ELSEVIER

European Journal of Pharmacology 351 (1998) 363–369

ejp

Protection of cultured gastric cells against *tert*-butyl hydroperoxide by glutathione isopropyl ester

Norihisa Yamaguchi, Noriaki Yajima, Motoo Ishida, Tadahito Shimada, Hideyuki Hiraishi *

Second Department of Internal Medicine, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan

Received 3 March 1998; revised 15 April 1998; accepted 17 April 1998

Abstract

Oxidants are involved in the pathogenesis of a variety of gastrointestinal disorders. Intracellular GSH protects rat gastric cells against oxidants. We examined whether GSH isopropyl ester (GSH ester) can protect against oxidants and whether a system of GSH ester uptake is present in these cells; we also investigated a possible role of GSH in inhibiting lipid peroxidation. Cytotoxicity was quantified by ^{51}Cr release. Lipid peroxidation was assessed by measuring malondialdehyde production. *tert*-Butyl hydroperoxide caused a dose-dependent increase in ^{51}Cr release. Treatment with GSH ester attenuated the toxicity of *tert*-butyl hydroperoxide. Incubation with GSH ester enhanced the cellular GSH content, which was prevented by DL-buthionine-[*S,R*]-sulfoximine, an inhibitor of γ-glutamylcysteine synthetase. GSH ester prevented *tert*-butyl hydroperoxide-induced lipid peroxidation, corresponding with the degree of protection. Therefore, we concluded that GSH isopropyl ester protects gastric cells against oxidants through the accumulation of intracellular GSH. This protection is mediated in part by the prevention of hydroperoxide-induced lipid peroxidation. However, the gastric epithelial system of GSH ester uptake appears distinctly different from those observed in hepatocytes, lymphoid cells and fibroblasts in terms of mediation of γ-glutamylcysteine synthetase activity.

Keywords: Gastric epithelial cell; *tert*-Butyl hydroperoxide; Glutathione; Lipid peroxidation; Cytolysis

1. Introduction

Reactive oxygen species are pathophysiologically involved in a variety of gastroduodenal disorders, such as those induced by ischemia/reoxygenation (Freeman and Crapo, 1982; Granger et al., 1986; McCord, 1986), ethanol (Mutoh et al., 1990; Szelenyi and Brune, 1988), or non-steroidal anti-inflammatory drugs (Vaananenn et al., 1991; Wallace et al., 1990). It is noteworthy that the gastric epithelium is continuously exposed to luminal oxidants generated from ingested food, catalase-negative bacteria, cast-off mucosal cells, and cigarette smoke and tar (Carlsson et al., 1983; Cross et al., 1984; Nakayama et al., 1984; Thomas et al., 1980). A recent report has also suggested a possible involvement of reactive oxygen species in the gastric mucosal injury and inflammation induced by *Helicobacter pylori* (*H. pylori*) infection (Suzuki et al., 1992), whose eradication reduces the recurrence of peptic ulcer diseases (Hentschel et al., 1993). However, healthy gastric epithelium appears unaffected despite the exposure to these reactive oxygen species, suggesting that gastric epithelial cells are endowed with effective anti-oxidant defenses, such as the glutathione redox cycle and superoxide dismutase (Hiraishi et al., 1991b, 1994b). Thus, an oxidation–reduction system in the epithelium of the stomach may represent the first line of defense against such oxidants.

It has been shown that GSH (γ-glutamylcysteinylglycine) is an important cellular protectant against reactive oxygen species in cultured gastric mucous (Hiraishi et al., 1987, 1991a,b) and chief cells (Olson, 1988), as well as in tumor cells (Nathan et al., 1980), endothelial cells (Harlan et al., 1984), or hepatocytes (Starke and Farber, 1985) by serving as a substrate for glutathione peroxide (Flohe, 1979; Wendel, 1980). Glutathione peroxidase utilizes GSH as a reductant to reduce toxic peroxide. While depletion of GSH by conjugation with electrophilic compounds such as diethyl maleate (Chasseaud, 1979) or by inhibition of γ-glutamylcysteine synthetase with DL-buthionine-[*S,R*]-sulfoximine (Griffith and Meister, 1979) potentiates injury from H_2O_2 (Hiraishi et al., 1987, 1991b; Olson, 1988), extracellular GSH provides protection against oxidant stress through the accumulation of intracellular GSH in cultured

* Corresponding author. Tel.: +81-282-87-2147; fax: +81-282-86-7761.

0014-2999/98/$19.00

gastric mucosal cells (Hiraishi et al., 1994a). Thus, there is much interest in procedures that increase the cellular levels of GSH. In this regard, it has been shown that the monoethyl, diethyl, and isopropyl esters of glutathione are more effective than native GSH in enhancing intracellular GSH in human lymphoid cells, fibroblasts (Wellner et al., 1984), endothelial cells (Tsan et al., 1989) and rat hepatocytes (Fernandez-Checa et al., 1991) in vitro. These glutathione esters, in which the carboxyl group of the glycine residue is esterified, are thought to be readily transported into these cells and hydrolyzed to GSH intracellularly.

Reactive oxygen species may cause tissue or cellular injury through a variety of processes including disruption of the interstitial matrix by the degradation of hyaluronic acid and collagen (Brown and Fridovich, 1980; McCord, 1974), lipid peroxidation of cellular components (Yamamoto et al., 1988), and DNA damage (Oleinick et al., 1984). In particular, we have previously demonstrated that oxidant stress causes lipid peroxidation, which is followed by cytolysis in cultured gastric cells (Yajima et al., 1995). However, the causal relationship between lipid peroxidation and GSH is not yet well established in gastric mucosal cells.

Accordingly, the present study was designed to examine (1) whether extracellular GSH isopropyl ester (GSH ester) can protect against oxidant stress, (2) whether an uptake system of GSH ester is present, and (3) whether GSH plays a role in preventing hydroperoxide-induced lipid peroxidation in cultured gastric mucosal cells.

2. Materials and methods

2.1. Animals and reagents

The experiments were carried out under approval of the institutional ethics committee. Sprague–Dawley rats, 7–10-days-old, (Dohken Laboratory, Ibaraki, Japan) of either sex were used. *tert*-Butyl hydroperoxide, GSH, and GSSG were purchased from Sigma. GSH isopropyl ester (YM737, GSH ester) was a kind gift from Yamanouchi Pharmaceutical (Tokyo, Japan). Malondialdehyde (98%) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Hanks' balanced salt solution (HBSS) and Earle's balanced salt solution (EBSS) supplemented with 15 mM Hepes were obtained from Sigma and adjusted to pH 7.4. ^{51}Cr (sodium chromate, 200–900 Ci/g chromium) was obtained from NEN (Boston, MA) Tissue culture plates and dishes were from Corning Glass Works (Corning, NY). Sources of other chemicals were described elsewhere (Hiraishi et al., 1987, 1991b, 1994a).

2.2. Cell culture

Primary culture of the gastric fundic mucosa from newborn rats was prepared, as described previously (Terano et al., 1982). Confluent monolayers were studied 3 days after seeding. The cells have been previously characterized as gastric mucus-producing epithelial cells.

2.3. ^{51}Cr release assay

Cytotoxicity was quantified by measuring ^{51}Cr release from prelabeled cells. Correlation between the result of specific ^{51}Cr release and of the dye exclusion test (the trypan blue test) has been reported to be strongly positive (Terano et al., 1987). After overnight prelabeling with ^{51}Cr, cells were washed three times with HBSS, then incubated with 1 ml EBSS only or EBSS containing test agents at 37°C in 95% room air–5% CO_2. In some experiments, cells were preincubated for 3 h with EBSS (control) or GSH ester (0.05–5 mM) before incubation with the test agents. Aliquots (200 μl) of cell-free supernatant buffer were removed at intervals for determination of specific ^{51}Cr release, as described previously (Terano et al., 1987; Hiraishi et al., 1987).

2.4. Assay for cellular glutathione content

Total glutathione (GSH + GSSG) was measured by a sensitive and specific assay to indicate the glutathione content of the cells. Monolayers in 35-mm culture dishes were incubated in EBSS (control) or GSH ester (0.5–5 mM) in the presence or absence of buthionine sulfoximine for 3 h. After incubation, the cells were washed three times with HBSS and solubilized in 0.2% Triton X-100. An aliquot of each sample was removed for protein assay. Then, cellular glutathione was assayed according to the method of Tietze (Akerboom and Sies, 1984; Tietze, 1969), as described previously (Hiraishi et al., 1994a). Data were expressed in 'GSH equivalents': GSH + 2GSSG nmol/mg protein.

2.5. Assay for malondialdehyde

Malondialdehyde was measured fluorometrically by adaptation of the method of Yagi (Yagi, 1976). In some experiments, monolayers in 60-mm dishes were pretreated with control buffer (EBSS without phenol red) or GSH ester (0.5–5 mM) before 3 h of exposure to *tert*-butyl hydroperoxide. The concentrations of *tert*-butyl hydroperoxide used in the cytotoxicity assay where cells on 24-well (2 cm^2/well) plates were exposed to 1 ml of *tert*-butyl hydroperoxide (0.025–0.5 mM), were equivalent to 0.0125–0.25 μmol of *tert*-butyl hydroperoxide/cm^2 of cells. Thus, these concentrations of *tert*-butyl hydroperoxide were used in malondialdehyde assay so that cells on 60-mm dishes were exposed to the same amounts of *tert*-butyl hydroperoxide used in the cytotoxicity assay. At the time indicated, the cells and incubation buffer were recovered and sonicated with a Sonifier model 250 (Branson Ultrasonics, Danbury, CT). Aliquots of the samples were removed for protein assay, then 50% trichloroacetic

acid was added to achieve a final concentration of 5%, and the mixture centrifuged at $3000 \times g$ for 30 min. Thereafter, malondialdehyde was assayed as described previously (Hiraishi et al., 1987; Yajima et al., 1995).

2.6. Calculation and statistics

Data are expressed as means ± S.E.M. Analysis of variance (ANOVA) was performed when more than two groups were compared. When significant ($P < 0.05$), Tukey's multiple comparison test was applied to test the differences between individual groups. The significance of the differences between two groups was evaluated by an unpaired Student's *t*-test (Woolson, 1987).

3. Results

3.1. Effect of pretreatment with GSH ester on tert-butyl hydroperoxide-induced ^{51}Cr release from culture cells

tert-Butyl hydroperoxide caused a dose-dependent increase in specific ^{51}Cr release during the 5-h incubation. In 5-h incubation with *tert*-butyl hydroperoxide (0.5 mM), the specific ^{51}Cr release from cultured gastric cells was 55.2 ± 6.2% (mean ± S.E.M. of four separate cultures) (Fig. 1). Preincubation with GSH ester (5 mM) significantly attenuated the toxicity of *tert*-butyl hydroperoxide, shifting the dose–response curve of *tert*-butyl hydroperoxide to the right (Fig. 1). Preincubation with increasing concentrations of GSH ester (0.05–5 mM) for 3 h caused dose-dependent inhibition of *tert*-butyl hydroperoxide (0.2 mM)-induced specific ^{51}Cr release (Fig. 2). Preincubation with GSH ester, at concentrations greater than 0.5 mM, significantly diminished specific ^{51}Cr release induced by *tert*-butyl hydroperoxide.

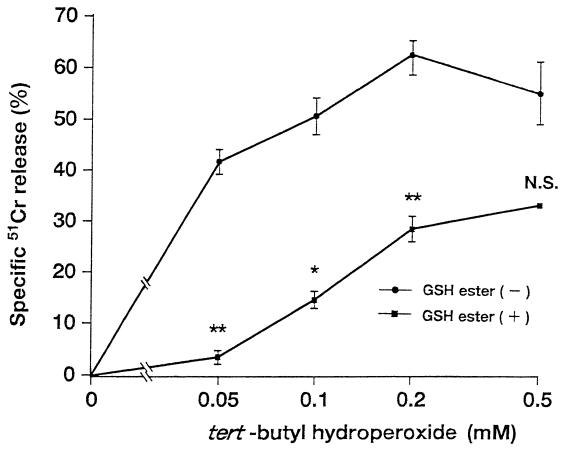

Fig. 1. Dose-dependent *tert*-butyl hydroperoxide-induced ^{51}Cr release and protection by GSH ester. Labeled cells were preincubated for 3 h with buffer or GSH ester (5 mM), then incubated for 5 h with increasing concentrations of *tert*-butyl hydroperoxide (0.05–0.5 mM). Values represent means of four separate cultures. Significant differences compared with control values without GSH ester pretreatment were determined by Student's *t*-test; $* P < 0.01$, $** P < 0.001$ and NS, not significant.

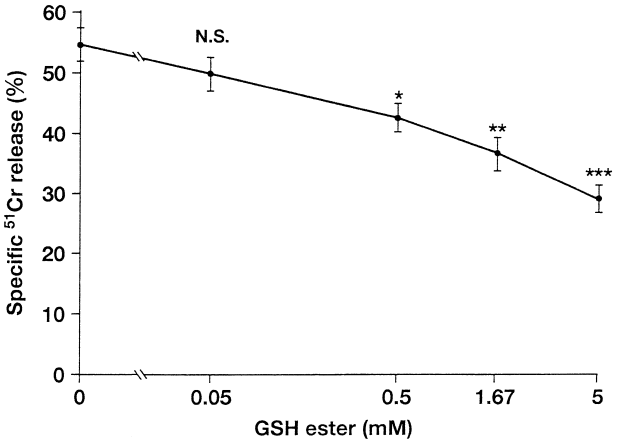

Fig. 2. Effect of preincubation with GSH ester on *tert*-butyl hydroperoxide-induced ^{51}Cr release. Labeled cells were preincubated for 3 h with buffer or GSH ester (0.05–5 mM), then incubated for 5 h with *tert*-butyl hydroperoxide (0.2 mM). ANOVA was performed with $P < 0.001$, and Tukey's multiple comparison test was applied to compare GSH ester-treated values with the control value (0 concentration); $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$ and NS, not significant.

3.2. Effect of buthionine sulfoximine on GSH ester-induced protection against tert-butyl hydroperoxide

Cells were preincubated in EBSS with or without GSH ester (5 mM) in the presence or absence of buthionine sulfoximine (0.1 mM) for 3 h, then exposed to *tert*-butyl hydroperoxide (0.1 mM) for 5 h. Buthionine sulfoximine inhibits GSH synthesis by irreversibly inhibiting γ-glutamylcysteine synthetase (Griffith and Meister, 1979). In control cultures without extracellular GSH ester in the absence of buthionine sulfoximine, *tert*-butyl hydroperoxide-induced ^{51}Cr release was 25.9 ± 3.8% (mean ±

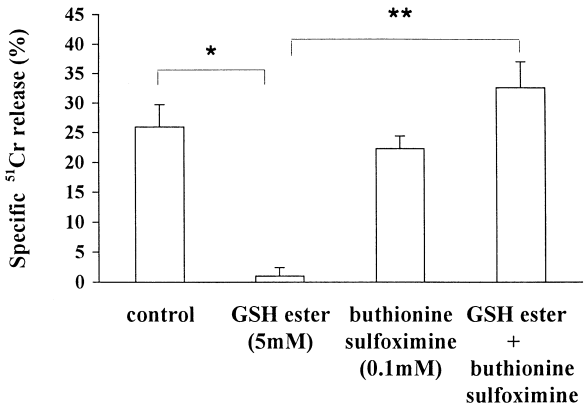

Fig. 3. Effect of preincubation with GSH ester/buthionine sulfoximine on *tert*-butyl hydroperoxide-induced ^{51}Cr release. Labeled cells were preincubated for 3 h with buffer or GSH ester (5 mM) in the presence or absence of buthionine sulfoximine (0.1 mM). After preincubation, cells were washed three times, then exposed to *tert*-butyl hydroperoxide (0.1 mM) for 5 h; $* P < 0.01$ and $** P < 0.001$.

S.E.M. of five separate cultures) (Fig. 3). Preincubation with 5 mM GSH ester in the absence of buthionine sulfoximine significantly decreased *tert*-butyl hydroperoxide-induced ^{51}Cr release to $1.0 \pm 1.4\%$ ($P < 0.01$). In cultures without GSH ester treatment in the presence of buthionine sulfoximine, *tert*-butyl hydroperoxide-induced ^{51}Cr release was $22.3 \pm 2.1\%$, which was not significantly different from the control values. The presence of buthionine sulfoximine during the preincubation period with GSH ester significantly increased *tert*-butyl hydroperoxide-induced ^{51}Cr release from 1.0 ± 1.4 to $32.6 \pm 4.4\%$ ($P < 0.001$), indicating that γ-glutamylcysteine synthetase activity is necessary for the protection afforded by extracellular GSH ester.

3.3. Effect of increasing concentrations of GSH ester on intracellular GSH content in the presence or absence of buthionine sulfoximine

Cells were incubated in EBSS with or without GSH ester (0.5–5 mM) in the presence or absence of buthionine sulfoximine (0.1 mM) for 3 h, washed three times, and the cellular GSH content was measured. In cultures without buthionine sulfoximine, incubation with extracellular GSH ester caused a dose-dependent increase in the intracellular GSH content; GSH ester (5 mM) increased the GSH content from 14.8 ± 2.1 (in control) to 61.3 ± 0.5 nmol/mg protein (means ± S.E.M. of three separate cultures; Fig. 4). The presence of buthionine sulfoximine during incubation with extracellular GSH ester inhibited the accumulation of intracellular GSH contents (Fig. 4), indicating that enhancement of intracellular GSH by extracellular GSH ester required the activity of γ-glutamylcysteine synthetase, and that extracellular GSH ester was not taken up by the cells in an intact form.

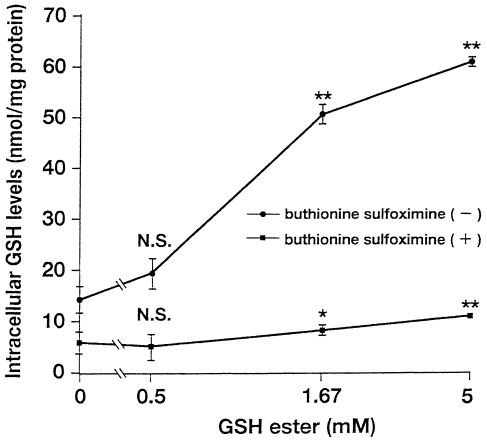

Fig. 4. Effects of GSH ester and buthionine sulfoximine on intracellular GSH levels. Cells were incubated for 3 h with buffer or GSH ester (0.5–5 mM) in the presence or absence of buthionine sulfoximine (0.1 mM) and washed three times with PBS; cellular GSH was extracted and measured. Values represent means of three separate cultures. In GSH ester-exposed cells in the absence of buthionine sulfoximine, ANOVA was performed and Tukey's multiple comparison test was applied to compare GSH ester-exposed values with the control values; $**P < 0.001$ and NS, not significant. Significant differences in buthionine sulfoximine-exposed values compared with corresponding values without buthionine sulfoximine were determined by Student's *t*-test; $*P < 0.01$, $**P < 0.001$ and NS, not significant.

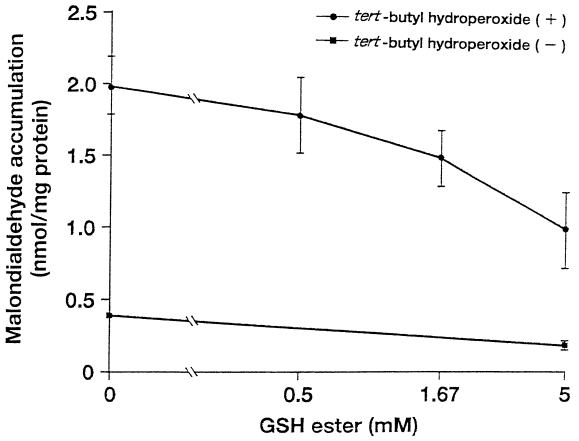

Fig. 5. Effect of GSH ester on *tert*-butyl hydroperoxide-induced lipid peroxidation. Cells were preincubated for 3 h with buffer or GSH ester (0.5–5 mM), then incubated for 5 h with or without *tert*-butyl hydroperoxide (0.1 μmol/cm^2, equivalent to 0.2 mM used in ^{51}Cr release assay). Values represent means of two separate cultures.

3.4. Effect of GSH ester on tert-butyl hydroperoxide-induced lipid peroxidation

Cultured cells were preincubated with or without GSH ester (0.5–5 mM) for 3 h, washed three times to remove

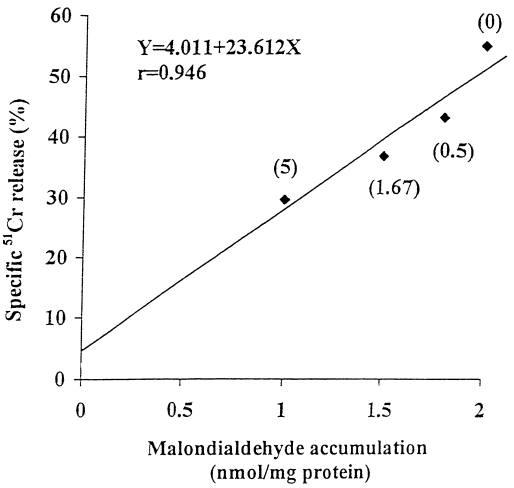

Fig. 6. Correlation between prevention of *tert*-butyl hydroperoxide-induced lipid peroxidation and inhibition of cytolysis by GSH ester. The effects of preincubation with GSH ester (0.5–5 mM) on *tert*-butyl hydroperoxide (0.2 mM)-induced specific ^{51}Cr release and on *tert*-butyl hydroperoxide (0.1 μmol/cm^2, equivalent to 0.2 mM used in ^{51}Cr release assay)-induced malondialdehyde accumulation were plotted. The numerals in parentheses indicate the concentrations of GSH ester administered in the preincubation period. The correlation between prevention of *tert*-butyl hydroperoxide-induced lipid peroxidation and inhibition of cytolysis by GSH ester was strongly positive ($r = 0.946$).

excess of GSH ester, then exposed to *tert*-butyl hydroperoxide for 5 h. Preincubation with GSH ester (0.5–5 mM) before *tert*-butyl hydroperoxide (0.1 μmol/cm^2, equivalent to 0.2 mM used in the cytotoxicity assay) exposure attenuated malondialdehyde production in a dose-dependent fashion at 5 h of incubation with *tert*-butyl hydroperoxide (Fig. 5). *tert*-Butyl hydroperoxide (0.1 μmol/cm^2) caused an increase in malondialdehyde production (2.0 ± 0.2 nmol/mg protein; mean $\pm$ S.E.M. of two separate cultures) compared with the control cultures (0.4 ± 0.1). Malondialdehyde accumulation in *tert*-butyl hydroperoxide-exposed cells after incubation with 5 mM GSH ester was 1.0 ± 0.2 nmol/mg protein, compared with 2.0 ± 0.2 in *tert*-butyl hydroperoxide-exposed, control cells (Fig. 5). Thus, enhanced lipid peroxidation, as assessed by the accumulation of malondialdehyde, was demonstrated in gastric cells exposed to *tert*-butyl hydroperoxide, but was prevented by preincubation with GSH ester. The degree of prevention of *tert*-butyl hydroperoxide-induced malondialdehyde production correlated well with that of decrease in specific ^{51}Cr release ($r = 0.946$, Fig. 6).

4. Discussion

The major findings of the current study were: (1) GSH ester protects cultured gastric mucosal cells against *tert*-butyl hydroperoxide through the accumulation of intracellular GSH; (2) exogenous oxidant stress induced by *tert*-butyl hydroperoxide accelerated lipid peroxidation, as assessed by enhanced malondialdehyde production; and (3) enhanced lipid peroxidation was significantly prevented by GSH ester. These findings suggest that GSH ester offers protection against oxidant stress in part by preventing *tert*-butyl hydroperoxide-induced lipid peroxidation in these cells.

Cultured gastric cells were loaded with *tert*-butyl hydroperoxide as oxidant stress in the present study. A range of toxic peroxides including *tert*-butyl hydroperoxide and H_2O_2 may be detoxified by glutathione peroxidase, which utilizes GSH as a reductant (Wendel, 1980). These cells are endowed with substantial levels of glutathione peroxidase and GSH (Hiraishi et al., 1987, 1991b). We have previously demonstrated that accumulation or depletion of intracellular GSH renders cells more or less resistant to oxidant stress in in vitro studies, respectively (Hiraishi et al., 1987, 1991b, 1994a). GSH esters may be more effective than GSH itself in enhancing the accumulation of intracellular GSH in a variety of cells (Fernandez-Checa et al., 1991; Maeno et al., 1989; Tsan et al., 1989; Wellner et al., 1984).

We have found that exogenous GSH ester provided significant protection of gastric cells against the toxicity of *tert*-butyl hydroperoxide when added in the preincubation period before *tert*-butyl hydroperoxide exposure (Figs. 1 and 2). Treatment with exogenous GSH ester was associated with a dose-dependent increase in cellular GSH (Fig. 4), corresponding with the degree of protection against subsequent exposure to *tert*-butyl hydroperoxide (Fig. 2). To determine whether this protection was caused by the uptake of GSH ester in an intact form and subsequent hydrolysis into GSH as observed in other cell types (Fernandez-Checa et al., 1991; Tsan et al., 1989; Wellner et al., 1984), we examined the effect of buthionine sulfoximine on protection afforded by GSH ester. Buthionine sulfoximine is known to inhibit GSH synthesis by irreversibly inhibiting γ-glutamylcysteine synthetase (Griffith and Meister, 1979). Buthionine sulfoximine, added with exogenous GSH ester during the incubation period, completely prevented protection by exogenous GSH ester against *tert*-butyl hydroperoxide (Fig. 3). Furthermore, intracellular GSH accumulation by incubation with GSH ester was also abolished by the presence of buthionine sulfoximine (Fig. 4).

Earlier studies on GSH esters, including monoethyl, diethyl, and isopropyl ester, in which the carboxyl group of the glycine residue is esterified, have shown that these esters protect animals against acetaminophen toxicity (Puri and Meister, 1983) and brain ischemia and hematoma (Maeno et al., 1989; Noguchi et al., 1989; Yamamoto et al., 1990) in vivo; they also protect lymphoid cells and fibroblasts against radiation injury (Wellner et al., 1984), and endothelial cells (Tsan et al., 1989) and hepatocytes against oxidants (Fernandez-Checa et al., 1991) in vitro. It has been hypothesized that these esters are readily transported into cells and hydrolyzed into GSH by substantial activities of cellular esterase, thus offering protection. Furthermore, Meister et al. demonstrated that, in buthionine sulfoximine-treated mice, both intraperitoneal and oral application of GSH ester leads to substantial increase in GSH levels and GSH ester is more effective than GSH in increasing cellular levels of GSH in the gastric mucosa in vivo (Martensson et al., 1990). However, the present study showed that this is not the case with cultured gastric mucosal cells from rats. Cellular GSH was increased by exogenously supplied GSH ester, but the increase was inhibited in the presence of buthionine sulfoximine (Fig. 4).

Several mechanisms may be involved in the utilization of extracellular GSH ester by these cells; (1) cleavage of GSH ester to its constituent amino acid(s) (which requires the activity of γ-glutamyltranspeptidase and dipeptidase) followed by their transport and intracellular GSH synthesis (which requires the activity of γ-glutamylcysteine synthetase); (2) formation of γ-glutamylcyst(e)ine by carboxypeptidase-type cleavage of GSH ester, or by transpeptidation between GSH and cyst(e)ine, followed by transport and conversion to GSH by GSH synthetase (which does not require the activity of γ-glutamylcysteine synthetase) (Anderson and Meister, 1983); and (3) transport of GSH ester and hydrolysis to GSH by esterase (which does not require the activity of γ-glutamylcysteine synthetase)

(Fernandez-Checa et al., 1991; Tsan et al., 1989; Wellner et al., 1984). Our results that accumulation of GSH by extracellular GSH ester is prevented by inhibition of γ-glutamylcysteine synthetase with buthionine sulfoximine (Fig. 4) suggest that the mechanisms of GSH ester metabolism by cells involve the breakdown of GSH ester, transport of the degradation product(s) (e.g., cyst(e)ine) and intracellular synthesis of GSH. The increase in intracellular GSH and protection by GSH ester may be sensitive to inhibition of γ-glutamyl transpeptidase by preventing breakdown of extracellular GSH ester. We have previously demonstrated that these cells have substantial level of γ-glutamyl transpeptidase activity and that treatment of cells with acivicin resulted in a significant inhibition of γ-glutamyl transpeptidase activity (Hiraishi et al., 1994a). However, since complete inhibition of γ-glutamyl transpeptidase cannot be achieved even with the highest concentration of acivicin, the effect of acivicin on the increase in intracellular GSH may be partial, as observed in our previous study on native GSH (Hiraishi et al., 1994a). Instead, we have shown that GSH accumulation as well as protection against *tert*-butyl hydroperoxide by GSH ester were completely prevented by buthionine sulfoximine (Figs. 3 and 4). Thus, gastric epithelial uptake system of GSH ester appears identical to that of native GSH by the cells (Hiraishi et al., 1994a) and distinctly different from that proposed in human lymphoid cells, fibroblasts (Wellner et al., 1984), endothelial cells (Tsan et al., 1989) and rat hepatocytes (Fernandez-Checa et al., 1991).

Lipid peroxidation is oxidant-mediated, chain reaction resulting in the oxidative deterioration of polyunsaturated fatty acids and reduced membrane fluidity of cytoplasm or subcellular organelles, which is essential for the proper functioning of biological membranes (Halliwell and Gutteridge, 1988; Noguchi et al., 1989; Yamamoto et al., 1988). It has been shown that lipid peroxidation is one of the mechanisms by which oxidant stress induces cytolysis. In the present study, we found that *tert*-butyl hydroperoxide-induced damage was associated with enhanced lipid peroxidation, as determined by the production of malondialdehyde (Figs. 1 and 5), consistent with the result of our previous study where lipid peroxidation was followed by cytolysis (Yajima et al., 1995). The current study also demonstrated that incubation with increasing concentrations of GSH ester (0.5–5 mM) caused a dose-dependent inhibition of *tert*-butyl hydroperoxide-induced malondialdehyde production (Fig. 5), corresponding with the degree of prevention of *tert*-butyl hydroperoxide-induced specific ^{51}Cr release (Fig. 2). The degree of inhibition of *tert*-butyl hydroperoxide-induced malondialdehyde accumulation correlated well with that of inhibition of cytolysis brought about by *tert*-butyl hydroperoxide (Fig. 6). Accordingly, these observations suggest that the mechanisms of protection by GSH ester involve the prevention of *tert*-butyl hydroperoxide-induced lipid peroxidation; this is the first report to indicate that cellular GSH is an essential protectant against oxidant-induced lipid peroxidation as well as cytolysis in gastric cells in vitro.

In summary, treatment of cultured gastric mucosal cells with exogenous GSH ester rendered cells more resistant to *tert*-butyl hydroperoxide. Exogenous oxidant stress induced by *tert*-butyl hydroperoxide accelerated lipid peroxidation, which was prevented by treatment with GSH ester. The degree of protection offered by graded doses of GSH ester against *tert*-butyl hydroperoxide correlated well with that of prevention of hydroperoxide-induced lipid peroxidation. These results lead to the conclusion that GSH offers protection against oxidant stress in part through the prevention of lipid peroxidation. However, gastric epithelial uptake of GSH ester seems distinctly different from that observed in other cell types, such as lymphoid and endothelial cells, fibroblasts and hepatocytes (Fernandez-Checa et al., 1991; Tsan et al., 1989; Wellner et al., 1984).

Since it is highly possible that reactive oxygen species are pathophysiologically involved in a variety of gastric mucosal injury and inflammation (Granger et al., 1986; Mutoh et al., 1990; Wallace et al., 1990), including *H. pylori* infection (Suzuki et al., 1992), a possible role of GSH in maintaining the integrity of the gastric mucosa against reactive oxygen species needs to be clarified in human gastric cells as well as in vivo.

Acknowledgements

This work was supported in part by a grant-in-aid for scientific research, and a grant-in-aid for encouragement of young scientists from the Ministry of Education, Science and Culture of Japan. We gratefully acknowledge the review of Professor Akira Terano, Dokkyo University School of Medicine, Tochigi, Japan.

References

Akerboom, T.P.M., Sies, H., 1984. Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological sample. Methods Enzymol. 77, 373–382.

Anderson, M.E., Meister, A., 1983. Transport and direct utilization of γ-glutamylcyst(e)ine for glutathione synthesis. Proc. Natl. Acad. Sci. USA 80, 707–711.

Brown, K., Fridovich, I., 1980. Superoxide radical and superoxide dismutase: threat and defense. Acta Physiol. Scand. 492, 9–18.

Carlsson, J., Iwami, Y., Yamada, T., 1983. Hydrogen peroxide excretion by oral streptococci and effect of lactoperoxidase–thiocyanate–hydrogen peroxide. Infect. Immun. 40, 70–80.

Chasseaud, L.F., 1979. The role of glutathione and glutathione-*S*-transferases in the metabolism of chemical carcinogens and other electrophilic agents. Adv. Cancer Res. 29, 175–274.

Cross, C.E., Halliwell, B., Allen, A., 1984. Antioxidant protection: a function of tracheobronchial and gastrointestinal mucus. Lancet 1, 1328–1330.

Fernandez-Checa, J.C., Garcia-Ruiz, C., Ookhtens, M., Kaplowitz, N.,

1991. Impaired uptake of glutathione by hepatic mitochondria from chronic ethanol-fed rats. J. Clin. Invest. 87, 397–405.

Flohe, L., 1979. Glutathione peroxidase: fact and fiction, In: Cohen, G., Greenwald, R.A. (Eds.), Oxygen Free Radicals and Tissue Damage. Ciba Foundation Symposium 65 (new series). Excerpta Medica, Amsterdam, pp. 95–122.

Freeman, B.A., Crapo, J.D., 1982. Biology of disease: free radical and tissue injury. Lab. Invest. 26, 412–426.

Granger, D.N., Hollwarth, M.E., Parks, D.A., 1986. Ischemia-reperfusion injury: role of oxygen-derived free radicals. Acta Physiol. Scand. (Suppl.) 548, 47–63.

Griffith, O.W., Meister, A., 1979. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (*S-n*-butyl homocysteine sulfoximine). J. Biol. Chem. 254, 7558–7560.

Halliwell, B., Gutteridge, J.M.C., 1988. Lipid peroxidation: a radical chain reaction. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine, 2nd edn. Oxford University Press, New York, NY, pp. 188–276.

Harlan, J.M., Levine, J.D., Callahan, K.S., Schwarz, B.R., 1984. Glutathione redox cycle protects cultured endothelial cells against lysis by extracellularly generated hydrogen peroxide. J. Clin. Invest. 73, 706–713.

Hentschel, E., Bandstatter, G., Dragosics, B., Hirschl, A.M., Nemec, H., Schutze, K., Traufer, M., Wurzer, H., 1993. Effect of ranitidine and amoxicillin plus metronidazole on the eradication of *Helicobacter pylori* and the recurrence of duodenal ulcer. New Engl. J. Med. 328, 308–312.

Hiraishi, H., Terano, A., Ota, S., Ivey, K.J., Sugimoto, T., 1987. Oxygen metabolite-induced cytotoxicity to cultured rat gastric mucosal cells. Am. J. Physiol. 253, G40–G48.

Hiraishi, H., Terano, A., Ota, S., Mutoh, H., Ivey, K.J., Sugimoto, T., 1991a. Role for iron in reactive oxygen species-mediated cytotoxicity to cultured rat gastric mucosal cells. Am. J. Physiol. 260, G556–G563.

Hiraishi, H., Terano, A., Ota, S., Mutoh, H., Sugimoto, T., Razandi, M., Ivey, K.J., 1991b. Antioxidant defenses of cultured gastric cells against oxygen metabolites: role of GSH redox cycle and endogenous catalase. Am. J. Physiol. 261, G921–G928.

Hiraishi, H., Terano, A., Ota, S., Mutoh, H., Sugimoto, T., Harada, T., Razandi, M., Ivey, K.J., 1994a. Protection of cultured rat gastric cells against oxidant-induced damage by exogenous glutathione. Gastroenterology 106, 1199–1207.

Hiraishi, H., Terano, A., Sugimoto, T., Harada, T., Razandi, M., Ivey, K.J., 1994b. Protective role of intracellular superoxide dismutase against extracellular oxidants in cultured rat gastric cells. J. Clin. Invest. 93, 331–338.

Maeno, H., Yamamoto, M., Iwai, A., Fujihara, A., Shikama, H., Noguchi, K., Watanabe, T., Nishiyama, M., Hori, T., Asano, T., 1989. Effects of glutathione and glutathione isopropyl ester on vasospasm in subarachnoid hemorrhage. In: Sakamoto, Y., Higashi, T., Taniguchi, N., Meister, A. (Eds.), Glutathione Centennial. Academic Press, New York, NY, pp. 317–331.

Martensson, J., Jain, A., Meister, A., 1990. Glutathione is required for intestinal function. Proc. Natl. Acad. Sci. USA 87, 1715–1719.

McCord, J.M., 1974. Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. Science Washington, DC 185, 529–531.

McCord, J.M., 1986. Oxygen-derived free radicals in postischemic tissue injury. New Engl. J. Med. 312, 159–163.

Mutoh, H., Hiraishi, H., Ota, S., Ivey, K.J., Terano, A., Sugimoto, T., 1990. Role of oxygen radicals in ethanol-induced damage to cultured gastric mucosal cells. Am. J. Physiol. 258, G603–G609.

Nakayama, T., Kodama, M., Nagata, C., 1984. Generation of hydrogen peroxide and superoxide anion radical from cigarette smoke. Jpn. J. Cancer Res. 75, 95–98.

Nathan, C.F., Arrick, B.A., Murray, H.W., Desatis, N.M., Cohn, Z.A., 1980. Tumor cell antioxidant defenses: inhibition of the glutathione redox cycle enhances macrophage-mediated cytolysis. J. Exp. Med. 153, 766–782.

Noguchi, K., Higuchi, S., Matsui, H., 1989. Effects of glutathione isopropyl ester on glutathione concentration in ischemic rat brain. Res. Commun. Chem. Pathol. Pharmacol. 64, 165–168.

Oleinick, N.L., Chiu, S., Friedman, L.R., 1984. Gamma radiation as a probe of chromatin structure: damage to and repair of active chromatin in the metaphase chromosome. Radiat. Res. 98, 629–641.

Olson, C.E., 1988. Glutathione modulates toxic oxygen metabolite injury of canine chief monolayers in primary culture. Am. J. Physiol. 254, G49–G56.

Puri, R., Meister, A., 1983. Transport of glutathione, as γ-glutamylcysteinylglycyl-ester, into the liver and kidney. Proc. Natl. Acad. Sci. USA 80, 5258–5260.

Starke, P.E., Farber, J.L., 1985. Endogenous defenses against the cytotoxicity of hydrogen peroxide in cultured rat hepatocytes. J. Biol. Chem. 260, 86–92.

Suzuki, M., Miura, S., Suematsu, M., Fukumura, D., Kurose, I., Suzuki, H., Kai, A., Kudoh, Y., Ohashi, M., Tsuchiya, M., 1992. *Helicobacter pylori*-associated ammonia production enhances neutrophil-dependent gastric mucosal cell injury. Am. J. Physiol. 263, G719–G725.

Szelenyi, I., Brune, K., 1988. Possible role of oxygen free radicals in ethanol-induced gastric mucosal damage in rats. Dig. Dis. Sci. 33, 865–871.

Terano, A., Ivey, K.J., Stachura, T., Sekhon, S., Hosojima, H., McKenzie, W.N., Krause, W.J., Wyche, J.H., 1982. Cell culture of rat gastric fundic mucosa. Gastroenterology 83, 1280–1291.

Terano, A., Ota, S., Mach, T., Hiraishi, H., Stachura, S., Tarnawski, A., Ivey, K.J., 1987. Prostaglandin protects against taurocholate-induced damage to rat gastric mucosal cell culture. Gastroenterology 92, 669–677.

Thomas, E.L., Bates, K.P., Jefferson, M.M., 1980. Hypothiocyanite ion: detection of the antimicrobial agent in human saliva. J. Dent. Res. 59, 1466–1472.

Tietze, F., 1969. Enzyme method for quantitative determination of nanogram amounts of total and oxidized glutathione. Anal. Biochem. 27, 502–522.

Tsan, M.F., White, J.E., Rosano, C.L., 1989. Modulation of endothelial GSH concentrations: effect of exogenous GSH and GSH monoethyl ester. J. Appl. Physiol. 66, 1029–1034.

Vaananenn, P.M., Meddings, J.B., Wallace, J.L., 1991. Role of oxygen-derived free radicals in indomethacin-induced gastric injury. Am. J. Physiol. 261, G470–G475.

Wallace, J.L., Keenan, C.M., Granger, D.N., 1990. Gastric ulceration induced by nonsteroidal antiinflammatory drugs is a neutrophil-dependent process. Am. J. Physiol. 259, G462–G467.

Wellner, V.P., Anderson, M.E., Puri, R.N., Jensen, G.L., Meister, A., 1984. Radioprotection by glutathione ester transport of glutathione ester into human lymphoid cells and fibroblasts. Proc. Natl. Acad. Sci. USA 81, 4732–4735.

Wendel, A., 1980. Glutathione peroxidase. In: Jakoby, W.B. (Ed.), Enzymatic Basis of Detoxification, Vol. 1. Academic Press, New York, NY, pp. 333–353.

Woolson, R.F., 1987. Statistical Methods for the Analysis of Biomedical Data. Wiley, New York, NY.

Yagi, K., 1976. A simple fluorometric assay for lipid peroxide in blood plasma. Biochem. Med. 15, 212–216.

Yajima, N., Hiraishi, H., Harada, T., 1995. Protection of cultured rat gastric cells against oxidant stress by iron chelation: role of lipid peroxidation. Dig. Dis. Sci. 40, 879–886.

Yamamoto, H., Nagano, T., Hirobe, M., 1988. Carbon tetrachloride toxicity on *Escherichia coli* exacerbated by superoxide. J. Biol. Chem. 263, 12224–12227.

Yamamoto, M., Sakamoto, N., Iwai, A., 1990. Pharmacological action of YM737, a new glutathione analogue, in rats with experimental hematoma. Arch. Int. Pharmacodyn. Ther. 308, 178–184.