Protective Role of Intracellular Superoxide Dismutase against Extracellular Oxidants in Cultured Rat Gastric Cells

Hideyuki Hiraishi,*§ Akira Terano,¶ Tsuneaki Sugimoto,¶ Takashi Harada,§ Mahnaz Razandi,* and Kevin J. Ivey*‡
**Department of Medicine, Veterans Affairs Medical Center, Long Beach, California 90822; ‡University of California, Irvine, California 92717; §Second Department of Internal Medicine, Dokkyo University School of Medicine, Tochigi 321-02 Japan; and ¶Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan*

Abstract

We examined the role of intracellular superoxide dismutase (SOD) as an antioxidant by studying the effect of diethyldithiocarbamate (DDC) on extracellular H_2O_2-induced damage in cultured rat gastric mucosal cells. ^{51}Cr-labeled monolayers from rat stomachs were exposed to glucose oxidase-generated H_2O_2 or reagent H_2O_2, which both caused a dose-dependent increase in ^{51}Cr release. DDC dose-dependently enhanced ^{51}Cr release by hydrogen peroxide, corresponding with inhibition of endogenous SOD activity. This inhibition was not associated either with modulation of other antioxidant defenses, or with potentiation of injury by nonoxidant toxic agents. Enhanced hydrogen peroxide damage by DDC was significantly prevented by chelating cellular iron with deferoxamine or phenanthroline. Inhibition of cellular xanthine oxidase (possible source of superoxide production) by oxypurinol neither prevented lysis by hydrogen peroxide nor diminished DDC-induced sensitization to H_2O_2. We conclude that (*a*) extracellular H_2O_2 induces dose dependent damage to cultured gastric mucosal cells; (*b*) intracellular SOD plays an important role in preventing H_2O_2 damage; (*c*) generation of superoxide seems to occur intracellularly after exposure to H_2O_2, but independent of cellular xanthine oxidase; and (*d*) cellular iron mediates the damage by catalyzing the production of more reactive species from superoxide and H_2O_2, the process which causes ultimate cell injury. (*J. Clin. Invest.* 1994. 93:331–338.) Key words: diethyldithiocarbamate • hydrogen peroxide • hydroxyl radical • iron • superoxide anion

Introduction

Gastric epithelium is constantly exposed to intraluminal generation of reactive oxygen metabolites (ROM)[1] (1–6). Gastric epithelial cells, together with the surface-covering mucus layer (7, 8), may represent an initial line of defense against such luminal oxidants. However, healthy gastric epithelium appears unaffected despite the constant exposure. Thus it is highly possible that gastric epithelial cells are endowed with effective antioxidant defenses such as the glutathione redox cycle, catalase and SOD. We have previously found that the glutathione redox cycle plays a principal role in detoxifying H_2O_2 in cultured gastric mucosal cells, whereas endogenous catalase plays a minor role (9). To date, however, the possible role of intracellular SOD against such injury remains undetermined in gastric cells. Moreover, there has been disagreement as to the central importance of SOD as an intracellular antioxidant defense in other cells or microorganisms (10–14).

$O_2^\bullet$, the one-electron reduction product of oxygen, is generated in aerobic organisms both spontaneously and as a result of pathological events such as neutrophil activation, hyperoxia, radiation, and ischemia/reperfusion (15–21). Thus, almost all aerobes elaborate forms of SOD, an enzyme which converts $O_2^\bullet$ to H_2O_2. In eukaryotes, SOD is characterized by the metal found at the active center and its cellular localization. Copper, zinc-containing SOD (Cu, Zn-SOD), the predominant form in mammalian cells, is located mainly in the cytosol; and the manganese-containing SOD (Mn-SOD) is primarily found in mitochondria (22). Diethyldithiocarbamate (DDC) has been shown to inhibit Cu, Zn-SOD activity by chelating copper ion, an active center of the enzyme (23, 24).

The present study attempted to clarify the role of intracellular SOD in maintaining the integrity of cultured gastric cells against ROM. We found that selective inhibition of intracellular SOD by DDC results in enhancement of H_2O_2 damage to cultured gastric mucosal cells. Our results also suggest that cellular iron mediates the damage by catalyzing production of more reactive species from $O_2^\bullet$ and H_2O_2 in these cells.

Methods

Animals and reagents. Rats, 7–10 d old, of either sex (Sprague-Dawley, Charles River Breeding Laboratories, Wilmington, MA) were used. Isolation medium consisted of Coon's modified Ham's F-12 medium (GIBCO, Grand Island, NY) containing 0.1% collagenase (0.52 U/mg, Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.05% hyaluronidase (type 1-S, 295 U/mg, Sigma Chemical Co., St. Louis, MO), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 25 μg/ml of gentamicin (Sigma). Culture medium consisted of F-12 medium supplemented with heat-inactivated (at 56°C for 30 min) 10% FBS (HyClone Laboratories, Inc., Logan, UT), 15 mM Hepes (Sigma), and the above antibiotics, b-D(+)glucose, glucose oxidase (GO, 192,100 U/g solid, lot no. 19F-3775), diethyldithiocarbamate (DDC), deferoxamine mesylate (DEF), 1,10-phenanthroline (PHE), GSH, oxidized glutathione (GSSG), glutathione reductase (200 U/mg protein), NADPH, H_2O_2 (30% solution), 1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol, SOD (from bovine liver, 2470 U/mg solid), xanthine, cytochrome *c*, sodium azide, BSA, EDTA, sulfosalicylic

Address correspondence to Dr. Kevin J. Ivey, 111-G, Department of Gastroenterology, Veterans Affairs Medical Center, 5901 East Seventh Street, Long Beach, CA 90822.

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1. *Abbreviations used in this paper:* CDNB, 1-chloro-2,4-dinitrobenzene; DDC, diethyldithiocarbamate; DEF, deferoxamine mesylate; EBSS, Earle's balanced salt solution; GO, glucose oxidase; GSSG, oxidized glutathione; GT, glutathione transferase; $O_2^\bullet$, superoxide anion radical; $OH^\bullet$, hydroxyl radical; OXY, oxypurinol; PHE, 1,10-phenanthroline; ROM, reactive oxygen metabolite(s); XD, xanthine dehydrogenase; XO, xanthine oxidase.

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acid, 5,5'-dithiobis (2-nitrobenzoic acid), TCA, ferrous ammonium sulfate, sodium thiocyanate, calcium ionophore A23187, DMSO, sodium taurocholate, oxypurinol (OXY), and Triton X-100 were purchased from Sigma. OXY was dissolved in 1 N NaOH as 100 mM stock. The stock solution was diluted in culture medium to obtain the final concentration (1 mM), and the pH of the solution was then brought to pH 7.4 by addition of 1N HCl. Calcium ionophore was dissolved in DMSO at 5×10^{-3} M. Perchloric acid ($HClO_4$) and 2-vinylpyridine were from Aldrich Chemical Co., Milwaukee, WI. Hanks' HBSS and Earle's balanced salt solution (EBSS) supplemented with 15 mM Hepes were obtained from GIBCO, and adjusted to pH 7.4. ^{51}Cr (sodium chromate, 200–900 Ci/g chromium) was obtained from ICN Biochemicals, Irvine, CA. Tissue culture plates and dishes were from Costar, Cambridge, MA.

Cell culture. Primary culture of the gastric fundic mucosa from 7–10-d old rats was prepared by the method of Terano et al. (25). In brief, the intact corpus (oxyntic glandular mucosa) area was excised from the antrum and forestomach, and the full wall thickness was minced into 2- to 3-mm^3 pieces. After incubation in isolation medium at 37°C for 60 min in an atmosphere of 5% CO_2-95% O_2, the tissues were pipetted several times and filtered through a nylon mesh and transferred to culture medium. The filtrate was washed by centrifuging at 50 *g* for 5 min in HBSS. Cells in culture medium were then inoculated into culture plates or dishes. The cultures were maintained at 37°C with 5% CO_2 in room air in a humidified atmosphere with the media changed daily. Confluent monolayers were studied 3 d after seeding.

^{51}Cr release assay. Cytotoxicity was quantified by measuring ^{51}Cr release from prelabeled cells. Culture medium containing 3 μCi/well per 0.5 ml ^{51}Cr was added to confluent monolayers on 24-well culture plates. After overnight prelabeling, cells were washed three times with HBSS and preincubated for 60 min with either EBSS or DDC, 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 prelabeled for 40 h with culture medium containing ^{51}Cr in the absence (vehicle control) or presence of OXY (1 mM). In GO-induced cytotoxicity assay, b-D(+)glucose was substituted for a-D(+)glucose in EBSS because GO uses b-D(+)glucose as substrate. 200 μl of cell-free supernatant buffer were removed at intervals for determination of specific ^{51}Cr release as follows: $(A - B/C - B) \times 100\%$, where A represents the mean test ^{51}Cr counts per minute (cpm) released, B represents the mean spontaneous ^{51}Cr cpm released, and C represents the mean maximum ^{51}Cr cpm released. Maximum ^{51}Cr release was determined by incubation in 0.2% Triton X-100. Spontaneous ^{51}Cr release was determined in control monolayers incubated in EBSS only and was 15–20% of maximum ^{51}Cr release after 5 h of incubation. ^{51}Cr radioactivity was counted with a Gamma 7000 Counting System (Beckman Instruments, Inc., Fullerton, CA).

H_2O_2 assay. Quantitation of H_2O_2 generated by GO was determined by the method of Thurman et al. (26). GO was incubated for 5 h with 10 mM b-D(+)glucose in a final volume of 1 ml in EBSS (without phenol red). The reaction was terminated by the addition of 0.1 ml TCA (50% wt/vol). The samples were centrifuged at 500 *g* for 10 min, and 0.2 ml of 10 mM ferrous ammonium sulfate and 0.1 ml of 2.5 M sodium thiocyanate added to the supernate. Absorption of the ferrithiocyanate complex was measured at 480 nm and compared to a standard curve generated from dilution of concentrated H_2O_2. The H_2O_2 concentration was calculated from the absorbance at 230 nm assuming an extinction coefficiency of 81 M^{-1} cm^{-1} (27).

Enzyme assay. 60-mm dishes of control buffer- or DDC-treated cells 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 glutathione peroxidase, glutathione reductase and catalase (28). Glutathione peroxidase and 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. For glutathione transferase (GT) assay, cells in 60 mm dishes were scraped into a solution of 50 mM Tris-HCl buffer containing 154 mM KCl, 4 mM EDTA, and 5 mM dithiothreitol, and sonicated. Solutions were cleared by centrifugation at 3,000 *g* for 5 min, and an aliquot of the supernatant was assayed for GT at 340 nm by measuring the formation of the conjugate of GSH and CDNB (29). A unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of S-2,4-dinitrophenylglutathione per minute at 30°C using 1 mM concentrations of GSH and CDNB. The specific activity of these enzymes was expressed as (m)U/mg protein as determined by the method of Bradford (30). Glutathione (GSH + GSSG) in 60 mm dishes of cells was measured by the method of Tietze (30, 31). After incubation, cells were washed three times with PBS, and solubilized in 0.2% Triton X-100. An aliquot of each sample was removed for protein assay. To another aliquot, an equal volume of 2 M $HClO_4$, 4 mM EDTA was added, and solutions were cleared by centrifugation at 3,000 *g* for 5 min to remove protein (32). An aliquot of the deproteinized, acid-soluble extract was neutralized with a solution containing 2 M KOH and 0.3 M *N*-morpholinopropane-sulfonic acid so that $HClO_4$ was removed by precipitation as the potassium salt (32), and the supernatant assayed for the sum of GSH and GSSG (31, 32). GSSG was assayed by masking GSH with 1% of 2-vinylpyridine (33). GSH and GSSG content was expressed as nmol/mg protein. The SOD activity was determined by lyzing cells in 0.2% Triton X-100 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.8. The lysate was centrifuged at 27,000 *g* for 30 min at 4°C. The supernatant material was dialyzed overnight at 4°C against 100 vol 50 mM potassium phosphate buffer with 0.1 mM EDTA (pH 7.8). The xanthine oxidase-cytochrome *c* method was used for SOD assay (34), and sodium azide (10 μM) was used to block peroxidases. The background activity was determined by adding the cell preparation to the cytochrome *c* mixture without xanthine oxidase. No reduction of cytochrome *c* was observed in the cell preparation. A unit of SOD activity was defined as the amount of enzyme which halved the rate of cytochrome *c* reduction. The enzyme activity was expressed as U/mg protein.

Statistical analysis. Data are expressed as means±SE. Analysis of variance and Student's *t* test were used to assess the significance of differences; $P < 0.05$ was considered significant.

Results

Cell culture. The cells reached confluency on day 3. Over 90% of the cells were identified as mucous-producing epithelial cells (surface epithelial cells and mucous neck cells) by PAS staining and ultrastructural studies (25, 35). Histochemical study with succinate dehydrogenase activity identified 5% of cells as parietal cells, whereas immunohistochemical study with pepsinogen failed to reveal any chief cells (25). These cells possessed the capability to synthesize DNA as well as cyclic nucleotides (25, 36), to produce and secrete mucous glycoprotein (25), and to generate prostaglandins (36).

Effect of pretreatment with DDC on cell lysis by enzymatically generated H_2O_2. The reaction of GO with the substrate b-D(+) glucose gives rise to the generation of H_2O_2 according to the following reaction:

$$\text{B-D(+)glucose} + O_2 + H_2O \xrightarrow{GO} \text{D-gluconic acid} + H_2O_2$$

The reaction mixture of 10 mM b-D(+)glucose and 20 mU/ml GO resulted in generation of H_2O_2 in amounts of 0.184±0.058 mM (mean±SE of six determinations) during 5-h incubation in a cell free system. GO (2.5–20 mU/ml) caused a dose-dependent increase in specific ^{51}Cr release in the presence of b-D-glucose (Fig. 1). GO (20 mU/ml) alone did not significantly increase ^{51}Cr release; specific ^{51}Cr release by GO (20 mU/ml) in the absence of glucose during 5-h incubation was 0.4±0.3% (mean±SE of quadruplicates on three separate preparations).

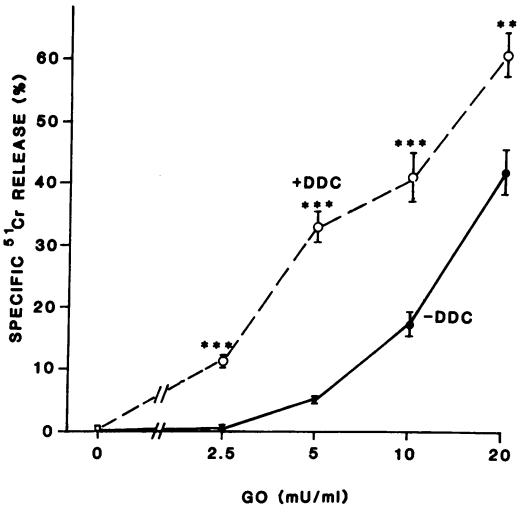

Figure 1. Effect of DDC on dose-dependent ^{51}Cr release induced by GO. Cells were labeled overnight with ^{51}Cr, washed, and preincubated for 60 min with buffer or DDC (10 μM). The cells were washed three times and then incubated with glucose (10 mM) and GO (2.5–20 mU/ml). Aliquots of supernatant buffer were removed after 5 h of incubation for determination of specific ^{51}Cr release. Values represent means±SE of quadruplicates on three separate preparations. Significant differences compared with control (−DDC) values were expressed as: $^{**}P < 0.01$; $^{***}P < 0.001$.

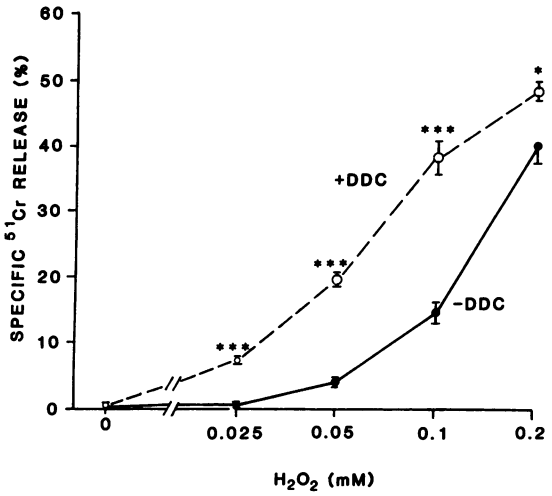

Figure 2. Effect of DDC on dose-dependent ^{51}Cr release induced by reagent H_2O_2. Cells were labeled overnight with ^{51}Cr, washed, and preincubated for 60 min with buffer or DDC (10 μM). The cells were washed three times and then incubated with reagent H_2O_2 (0.025–0.2 mM). Aliquots of supernatant buffer were removed after 5 h incubation for determination of specific ^{51}Cr release. Values represent means±SE of quadruplicates on three separate preparations. Significant differences compared with control (−DDC) values were expressed as: $^{*}P < 0.05$; $^{***}P < 0.001$.

GO-induced damage was inhibited specifically by extracellular catalase, and inhibition of damage was due to its enzymatic activity, since 3-amino-1,2,4-triazole (a specific inhibitor of catalase) (37) prevented the protective effect of catalase (38). Pretreatment with DDC (10 μM for 60 min) increased susceptibility to lysis by glucose-GO, i.e., it caused a left shift of the dose-response curve for GO (Fig. 1). Pretreatment with DDC alone (10 μM for 60 min) did not significantly increase ^{51}Cr release at 5 h compared with control; specific ^{51}Cr release after incubation with 10 μM DDC was 0.6±0.5%, compared to 0.0±0.4% (means±SE of quadruplicates on three separate preparations) in control cultures.

Effect of pretreatment with DDC on cell lysis by reagent H_2O_2. Reagent H_2O_2 (0.025–0.2 mM) caused a dose-dependent increase in specific ^{51}Cr release (Fig. 2). Pretreatment with DDC (10 μM for 60 min) also increased susceptibility to reagent H_2O_2 (0.025–0.2 mM) (Fig. 2).

Intracellular SOD activity in DDC-treated cells. Pretreatment with DDC (0.1–10 μM for 60 min) caused a dose-dependent increase of glucose/GO-induced ^{51}Cr release (Fig. 3). Treatment with DDC (10 μM), while inhibiting cellular SOD activity from 5.29±0.62 in control to 2.18±0.43 U/mg protein (means±SE of duplicates on three separate preparations), enhanced GO (5 mU/ml)-induced ^{51}Cr release from 5.3±0.4% in control to 33.1±2.5% (means±SE of sextuplicates on three separate preparations) (Fig. 3). Both cellular SOD activity and GO-induced damage were directly correlated with the concentration of DDC. Importantly, susceptibility to lysis by H_2O_2 was inversely related to endogenous SOD activity (Fig. 3). Similarly, pretreatment with DDC caused a dose-dependent increase in reagent H_2O_2-induced ^{51}Cr release (Fig. 4). DDC (10 μM) enhanced reagent H_2O_2 (0.05 mM)-induced ^{51}Cr release from 8.8±1.2% in control to 30.8±3.5% (means±SE of sextuplicates on 3 separate preparations) (Fig. 4).

Specificity of DDC for H_2O_2-mediated injury. Susceptibility to lysis by distilled water, calcium ionophore (5 μM), and taurocholate (5 mM) was not affected by pretreatment with DDC

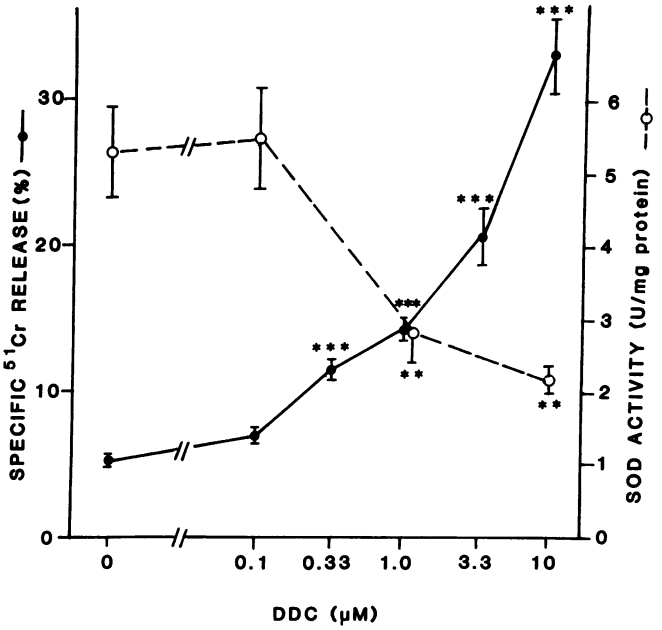

Figure 3. Effect of DDC concentration on SOD activity and GO-induced ^{51}Cr release. SOD activity and GO-induced ^{51}Cr release were determined in gastric cells after a 60-min incubation with increasing concentrations of DDC (0.1–10 μM). GO-induced ^{51}Cr release was determined after incubation with glucose (10 mM) and GO (5 mU/ml) for 5 h. Values for SOD activity and for ^{51}Cr release represent means±SE of duplicates and sextuplicates on three separate preparations, respectively. Significant differences compared with control (DDC 0) values were expressed as: $^{**}P < 0.01$; $^{***}P < 0.001$.

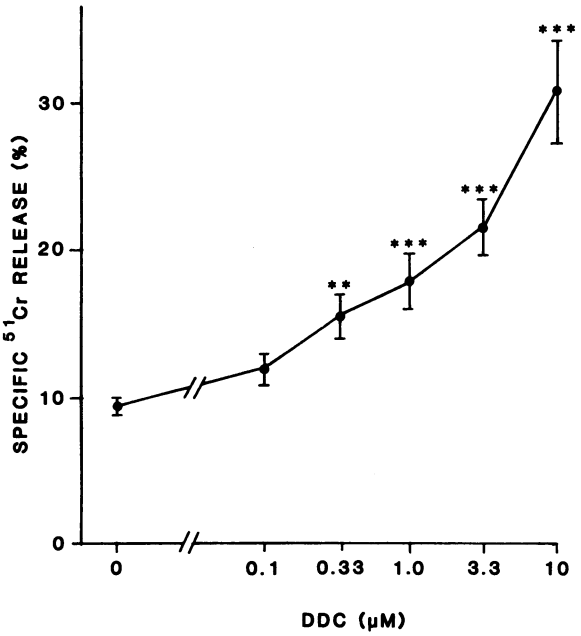

Figure 4. Effect of DDC concentration on reagent H_2O_2-induced ^{51}Cr release. Reagent H_2O_2-induced ^{51}Cr release was determined after a 60-min incubation with increasing concentrations of DDC (0.1–10 μM). Specific ^{51}Cr release was determined after incubation with reagent H_2O_2 (0.05 mM) for 5 h. Values for ^{51}Cr release represent means±SE of sextuplicates on three separate preparations. Significant differences compared with control (DDC 0) values were expressed as: $^{**}P < 0.01$; $^{***}P < 0.001$.

(10 μM for 60 min), despite DDC potentiation of ^{51}Cr release by reagent H_2O_2 (0.1 mM) in the same preparations (Table I).

Effect of iron chelation on DDC-induced enhancement of H_2O_2 injury. We have previously found that chelation of cellular iron inhibits H_2O_2-induced injury to these cells (38, 39). To further confirm the role of intracellular SOD in H_2O_2-induced injury, and to assess the possible importance of iron in mediating injury in cells with inhibited SOD, the effects of iron chelators, i.e., DEF (a chelator of Fe^{3+}) (40–42) and PHE (a chelator of divalent Fe^{2+}) (43, 44), were examined. In experiments with DEF, cells were preincubated for 60 min with or without DEF (20 mM), and then further incubated for 60 min with or without DDC (0.1–10 μM) before exposure to GO (5 mU/ml) (Fig. 5). In experiments with PHE, cells were preincubated with or without DDC (0.1–10 μM), and then incubated with reagent H_2O_2 (0.05 mM) in the presence or absence of PHE (10 μM) (Fig. 6). Strikingly, both DEF (20 mM) and PHE (10 μM) significantly and nearly completely prevented DDC-induced enhancement of susceptibility to lysis by H_2O_2 (Figs. 5 and 6).

Table I. Effect of DDC on ^{51}Cr Release Induced by Nonoxidant Agents

Addition	Percentage of specific ^{51}Cr release	
	−DDC	+DDC
Distilled water	19.5±0.9	18.9±1.5
A23187	20.9±1.4	20.4±1.5
Taurocholate	11.2±0.8	11.8±0.7
H_2O_2	14.6±1.6	38.4±2.5*

^{51}Cr-labeled cells were preincubated for 60 min with buffer or DDC (10 μM). The cells were washed three times and then incubated with distilled water for 30 min, A23187 (5 μM), taurocholate (5 mM), or H_2O_2 (0.1 mM) for 5 h. Aliquots of supernatant buffer were removed for determination of specific ^{51}Cr release. Values represent means±SE of quadruplicates on three separate preparations. Significant differences compared with control values were expressed as: * $P < 0.001$.

Effect of DDC on antioxidant defenses. We determined the effects of DDC on GSH and GSSG content, glutathione peroxidase, glutathione reductase, GT, and catalase activity. As summarized in Table II, inhibition of cellular SOD activity with DDC (10 μM for 60 min) was not associated with significant alteration of the glutathione redox cycle, GT or catalase. These results excluded the possibility that DDC potentiated H_2O_2-induced damage by impairing cellular antioxidant defenses (9) other than SOD.

Effect of OXY on DDC-induced sensitization of GO-induced injury. To determine whether cellular XO-derived $O_2^{\bullet}$ is involved in mediating H_2O_2-induced damage to cells with inhibited SOD, we examined the effect of OXY, an inhibitor of XO (38, 45, 46), on DDC-induced sensitization of gastric cell to GO damage. Cells were labeled with ^{51}Cr for 40 h in the absence or presence of OXY (1 mM), and preincubated for 60 min with buffer (control) or DDC (10 μM) before cytotoxicity assay by glucose/GO (7.5 mU/ml). Prelabeling with OXY (1 mM) did not influence the incorporation of ^{51}Cr applied into cells (data not shown). As shown in Table III, preincubation with OXY neither prevented lysis by GO nor diminished enhanced susceptibility to GO by DDC.

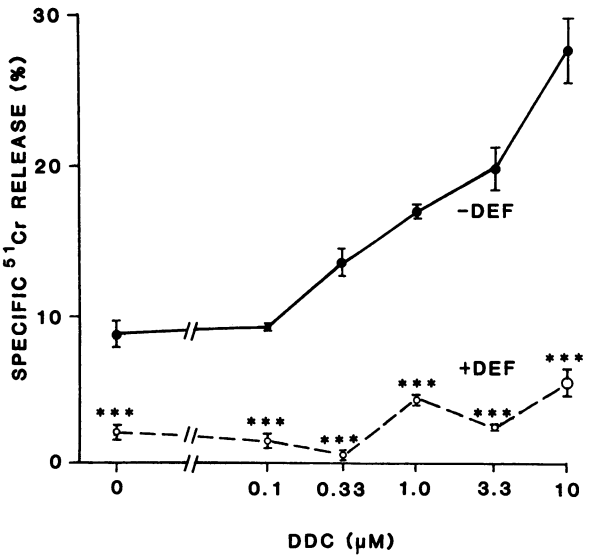

Figure 5. Effect of DEF on DDC-induced sensitization of lysis by GO. ^{51}Cr-labeled cells were incubated with buffer or 20 mM DEF for 60 min, washed, and then further incubated for 60 min with buffer or DDC (0.1–10 μM) before exposure to glucose (10 mM)/GO (5 mU/ml). Aliquots of supernatant buffer were removed after 5 h incubation for determination of specific ^{51}Cr release. Values represent means±SE of quadruplicates on three separate preparations. Significant differences compared with control (−DEF) values were expressed as: $^{***}P < 0.001$.

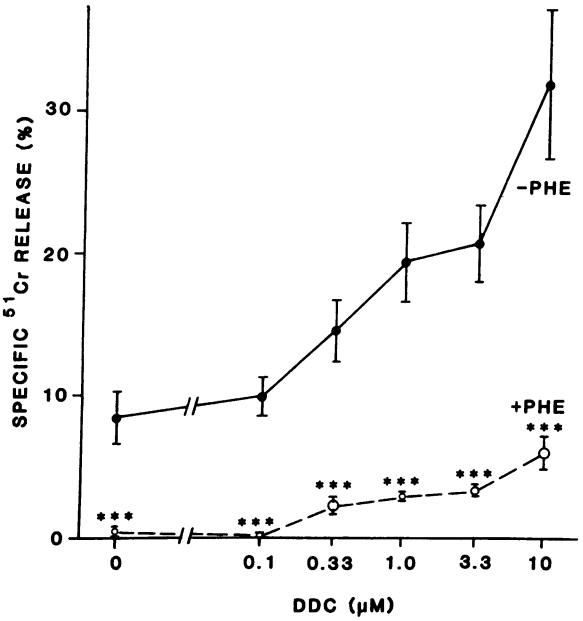

Figure 6. Effect of PHE on DDC-induced sensitization of lysis by H_2O_2. ^{51}Cr-labeled cells were preincubated for 60 min with buffer or DDC (0.1–10 μM). The cells were washed three times and then incubated with reagent H_2O_2 (0.05 mM) in the presence or absence of PHE (10 μM). Aliquots of supernatant buffer were removed after 5 h incubation for determination of specific ^{51}Cr release. Values represent means±SE of quadruplicates on three separate preparations. Significant differences compared with control (−PHE) values were expressed as: $***P < 0.001$.

Discussion

Although SOD is found intracellularly in almost all aerobes, its importance as an antioxidant is by no means certain. It has been reported that cultured endothelial cells with increased SOD activity (as much as 17-fold) after incubation with native SOD were more resistant to killing by H_2O_2 and activated neutrophils (10). In contrast, incubation of endothelial cells with polyethylene glycol conjugated SOD failed to protect cells from XO-induced ^{51}Cr release, although the manipulation increased cellular SOD by fourfold (11). Microinjection of SOD into endothelial cells was also shown not to be protective against hyperoxia-induced toxicity (12). In microorganisms, on the contrary, SOD-rich *E. coli* was less resistant to paraquat (an intracellular $O_2^{\bullet}$ generator), hyperoxia, or r-radiation (13, 14). Thus, it is possible that the protective role of cellular SOD 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 (14).

Therefore, the present study addressed the role of intracellular SOD in protection of cultured gastric cells against extracellular oxidants by inhibiting the enzyme to subnormal levels with DDC. DDC has been shown to inhibit Cu, Zn-SOD activity by chelating copper ion, an active center of the enzyme both in vivo and in vitro (23, 24). However, since there are many copper containing enzymes which may be affected by DDC, and since nonspecific effects of this agent other than through chelating copper ion have been described in other cell types (47–49), the effect of DDC on intracellular SOD activity as well as on oxidant-induced injury requires careful interpretation.

In cultured gastric mucosal cells, we found that treatment with DDC (10 μM for 60 min) significantly sensitized cells against extracellular H_2O_2, i.e., it caused a left shift of the dose response curve for enzymatically generated (continuous) and reagent (bolus) H_2O_2 (Figs. 1 and 2). Moreover, susceptibility to lysis by H_2O_2 was inversely related to inhibition of SOD activity, when cells were treated with increasing concentrations of DDC (1–10 μM) (Figs. 3 and 4). With regard to specificity of the effect of DDC on H_2O_2-induced gastric cell damage, susceptibility to lysis by nonoxidant toxic agents such as distilled water, calcium ionophore, or taurocholate was not affected by DDC, despite DDC potentiation of ^{51}Cr release by H_2O_2 in the same preparations (Table I).

The gastric mucosal cells used in the present study are endowed with the glutathione redox cycle and endogenous catalase against H_2O_2 (9), and glutathione transferase. As shown in Table II, inhibition of cellular SOD by DDC was not associated with significant alteration of the content or activity of these antioxidants, supporting the interpretation that potentiation of H_2O_2 damage by DDC was due to the inhibition of cellular SOD activity.

The use of DDC as an inhibitor of Cu, Zn-SOD has been criticized (47, 48). In erythrocytes with oxyhemoglobin, DDC may undergo a cyclic redox reaction to produce GSSG and hydrogen peroxide (47). This could increase cell death by a non-SOD inhibition pathway. We specifically tested whether GSSG was increased by DDC treatment in gastric cells, and found that the level of GSSG and the ratio of GSH to GSSG (GSH/GSSG) were not altered by DDC (Table II), consistent with results found in cultured cerebral astrocytes (49). Thus, it is unlikely that DDC undergoes such a cyclic redox reaction in gastric cells without oxyhemoglobin, which is in agreement with the findings in erythrocytes (47). The use of nonthiol copper chelators other than DDC was considered. However,

Table II. Effect of DDC on Antioxidant Defense

Addition	GSH + 2GSSG	2GSSG	GP	GR	GT	CAT
	nmol/mg protein		*mU/mg protein*			*U/mg*
Control	15.43±0.09	0.33±0.04	51.4±2.1	38.8±2.3	93.9±3.9	3.33±0.25
DDC	15.40±0.45	0.32±0.06	54.5±2.1	39.5±3.5	92.0±5.8	3.58±0.26

Cells were incubated for 60 min with buffer (control) or DDC (10 μM), and GSH and GSSG content and activity of glutathione peroxidase (GP), glutathione reductase (GR), glutathione transferase (GT), and catalase (CAT) were determined as described in the text. Values represent means±SE of duplicates on 3 separate preparations.

Table III. Effect of OXY on DDC-induced Sensitization of GO-induced Injury

Incubation	Percentage of specific ^{51}Cr release	
	−DDC	+DDC
Control	11.9±0.7	32.3±1.0
OXY 1.0 mM	12.0±0.7	33.8±1.2

Cells were labeled with ^{51}Cr for 40 h in the absence (vehicle control) or presence of OXY (1 mM), washed, and preincubated for 60 min with control buffer (−DDC) or DDC (10 μM). The cells were washed three times and then incubated with glucose (10 mM)/GO (7.5 mU/ml). Aliquots of supernatant buffer were removed after 5 h incubation for determination of specific ^{51}Cr release. Values represent means±SE of quadruplicates on three separate preparations.

other copper chelating agents have been shown to be less effective in inhibiting SOD activity, or not to inhibit SOD activity because of inability to remove copper from SOD (47). Therefore, DDC seems the most relevant copper chelator to examine the role of SOD in gastric cells.

Finally, the observation that chelation of cellular iron diminished DDC-enhanced sensitization of these cells against H_2O_2 provided other evidence that cellular SOD is specifically involved in protecting gastric cells against H_2O_2-induced lysis. Iron is stored in cells as low molecular weight iron chelates, heme-associated iron, and ferritin bound iron mostly in the form of ferric ion (Fe^{3+}) (41, 50). Fe^{3+} bound to ligands (L) may need to be reduced to Fe^{2+} so that H_2O_2 can react with Fe^{2+} to form a peroxo complex, yielding $OH^{\bullet}$ and/or being oxidized to higher oxidation states of iron as follows (51–54):

$$L\text{-}Fe^{3+} \xrightarrow{\text{reductant}} L\text{-}Fe^{2+}$$

$$L\text{-}Fe^{2+} + H_2O_2 \rightarrow L\text{-}Fe^{2+} - H_2O_2$$

$$L\text{-}Fe^{2+} - H_2O_2 \rightarrow L\text{-}Fe^{3+} + OH^- + OH^{\bullet}$$

$$L\text{-}Fe^{2+} - H_2O_2 \rightarrow L\text{-}Fe^{4+} + 2OH^-$$

$O_2^{\bullet}$ or other cellular reductants (e.g., ascorbate, thiols, etc.) (52, 55–58) are candidates to react with L-Fe^{3+} to form L-Fe^{2+}. We have previously found that DEF and PHE protect these cells against H_2O_2 by chelating cellular iron without affecting endogenous antioxidants, whereas chelation of iron was unable to protect against lysis by nonoxidant toxic agents (38). The current study demonstrated that, in cells where endogenous SOD was inhibited with DDC (0.1–10 μM), both iron chelators significantly and nearly completely prevented the DDC-induced enhancement of H_2O_2 damage (Figs. 5 and 6). These results suggest that DDC-induced sensitization against H_2O_2 is exclusively due to ROM-involved processes, and that the cellular iron is essential to mediate ROM-induced injury even in cells with inhibited SOD. Furthermore, these results also suggest that these two precursors, $O_2^{\bullet}$ and H_2O_2 alone without iron, are less toxic to gastric cells.

Thus among the candidates to reduce cellular Fe^{3+} to Fe^{2+} (52, 55–58), $O_2^{\bullet}$ seems most likely the one to react with Fe^{3+} to liberate Fe^{2+}. Thus we focused on the source of this $O_2^{\bullet}$ generation after exposure to H_2O_2. Exposure to H_2O_2 has been shown to cause a rapid and profound fall in cellular ATP levels with enhanced accumulation of its degradation products such as hypoxanthine in in vitro cells (59, 60); hypoxanthine can be a substrate of cellular XO to generate $O_2^{\bullet}$ (61, 62). Unexpectedly, however, 40 h of treatment with 1 mM OXY, which inhibits $O_2^{\bullet}$ generation from XO (45, 46), neither prevented lysis by H_2O_2 nor diminished increased susceptibility to H_2O_2 by DDC (Table III). Thus it seems unlikely that XO-derived $O_2^{\bullet}$ is involved in mediating H_2O_2-induced damage.

With respect to the source of $O_2^{\bullet}$, one possible explanation was suggested by Gutteridge et al. (63), who recently demonstrated that H_2O_2 produces hydroperoxyl radical acting on EDTA-Fe^{3+} through the formation of an intermediate iron–oxygen complex, which may in turn produce $O_2^{\bullet}$ as follows:

$$Fe^{3+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-}OOH^-\text{-EDTA} + H^+$$

$$Fe^{3+}\text{-}OOH^-\text{-EDTA} + H_2O_2 \rightarrow FeO^{2+}\text{-EDTA} + HO_2^{\bullet} + H_2O$$

$$FeO^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + HO_2^{\bullet} + OH^-$$

$$HO_2^{\bullet} \rightarrow H^+ + O_2^{\bullet}$$

Thus it is possible that H_2O_2 initiates this chain of reaction by interacting with Fe^{3+} bound to biomolecules (ATP or ADP) instead of EDTA (41). Alternatively, $O_2^{\bullet}$ spontaneously derived from mitochondria, chloroplast, or endoplasmic reticulum may act as a reductant of iron (58). Once $O_2^{\bullet}$ would be generated following exposure to excess H_2O_2, it would result in the formation of a peroxo complex which yields $OH^{\bullet}$ and/or is oxidized to higher oxidation states of iron by reducing Fe^{3+} (51–54).

In contrast to the protective role of intracellular SOD, we have previously demonstrated that extracellularly supplied SOD does not protect these cells from cytolysis induced by either XO or GO (38, 64). Thus, it is obvious that extracellular $O_2^{\bullet}$ is not involved in mediating H_2O_2 damage in gastric cells, consistent with earlier finding that $O_2^{\bullet}$ is unlikely to penetrate cell membranes because of its negative charge and high reactivity (65). Although extracellularly supplied SOD has been shown to prevent damage induced by GO in cultured hepatocytes (66), the discrepancy with respect to the role of extracellular SOD may depend on whether the cell type can take up the enzyme (67). Thus it is highly possible that gastric cells cannot take up the enzyme in its native form in a short incubation in vitro, in agreement with earlier observations in endothelial cells that SOD needs to be entrapped by liposomes or polyethylene glycol to get access to the interior of cells (11, 68).

In summary, we have demonstrated that (*a*) inhibition of cellular SOD activity with DDC dose dependently potentiated damage induced by H_2O_2, but not by non-oxidant toxic agents; (*b*) potentiation of H_2O_2 damage by DDC correlated with the degree of inhibition of the enzyme activity; (*c*) treatment with DDC was not associated with alteration of other antioxidant defenses such as the glutathione redox cycle, glutathione transferase, or catalase; (*d*) enhancement of H_2O_2-mediated damage by DDC was significantly prevented by chelation of cellular iron with DEF or PHE; however, (*e*) inhibition of cellular XO by oxypurinol neither prevented lysis by H_2O_2 nor diminished DDC-induced sensitization to H_2O_2.

Based on these observations, together with our previous findings (9, 38, 39, 64), we postulate the following mechanisms of H_2O_2-induced damage to gastric cells and enhance-

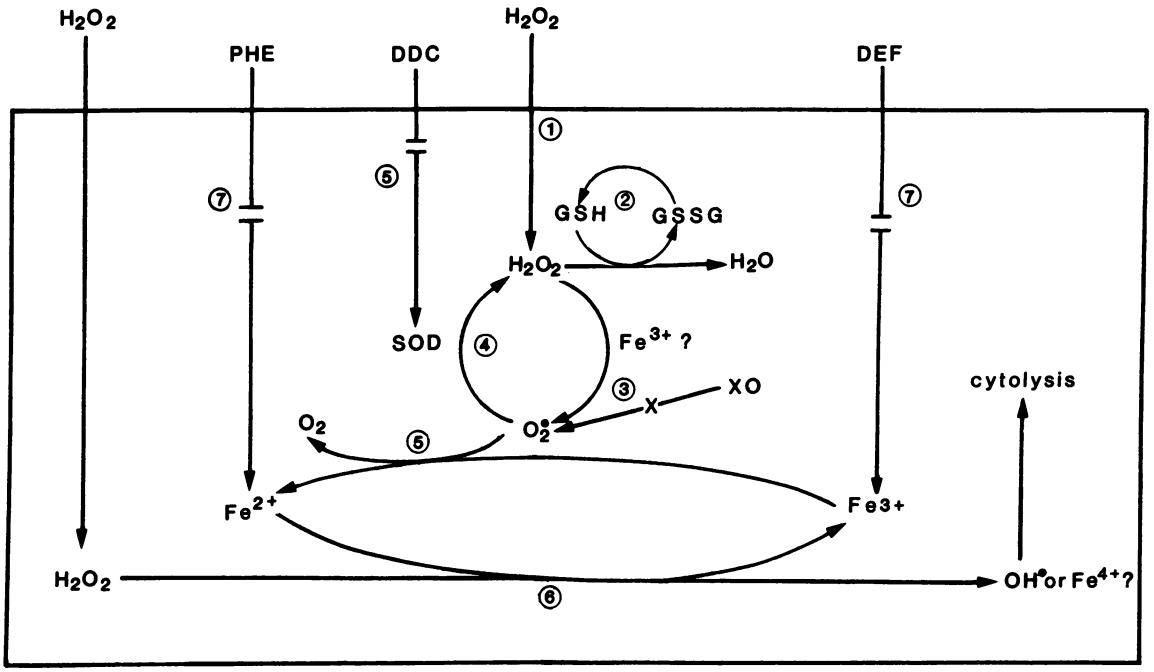

Figure 7. Postulated mechanisms of augmentation of H_2O_2-induced injury by inhibition of cellular SOD. See details in the final part of the Discussion.

ment of damage by inhibition of intracellular SOD (Fig. 7): (*1*) extracellular H_2O_2 penetrates cell membranes because it is electroneutral (65); (*2*) H_2O_2, which enters the interior of cells, is detoxified principally by the glutathione redox cycle (9): (*3*) excess H_2O_2 causes $O_2^{\bullet}$ generation intracellularly (possibly through reacting with Fe^{3+}), which process is independent of the cellular XO/XD system; (*4*) cellular SOD prevents reduction of Fe^{3+} to Fe^{2+} by dismutating $O_2^{\bullet}$ into H_2O_2; (*5*) excess H_2O_2 may also inhibit SOD activity simultaneously (69). When SOD is disrupted by DDC or overwhelmed, accumulated $O_2^{\bullet}$ initiates reduction of stored Fe^{3+} to Fe^{2+}; (*6*) reduced Fe^{2+} reacts with H_2O_2 to form more toxic species, which ultimately causes cell damage; and (*7*) DEF and PHE protect cells by chelating stored iron (Fe^{3+}) and reduced iron (Fe^{2+}), respectively, presumably through inhibition of generation of such species.

Since the gastric epithelium is constantly exposed to intraluminal oxidants, we may speculate that cellular SOD may also be critical in preventing gastric epithelial injury in vivo.

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