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Role of glutathione in nitric oxide-mediated injury to rat gastric mucosal cells

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Abstract

Recent studies suggest that in some cell types, the activity of nitric oxide (NO) is influenced by the endogenous antioxidant, reduced glutathione (GSH). The present study has examined the role of GSH in NO-induced cytotoxicity in cells harvested from the rat gastric mucosa. Cell integrity was assessed by Trypan blue exclusion and alamar blue dye absorbance. Pretreatment of rats with bacterial endotoxin lipopolysaccharide increased Ca^{2+}-independent NO synthase (iNO synthase) activity (as detected by the radiolabeled conversion of [^{14}C]arginine to [^{14}C]citrulline, lowered GSH content and increased cell injury. Lipopolysaccharide treatment also resulted in a significant increase in the in vitro production of reactive oxygen metabolites as assessed by the fluorescent probe 2′,7′-dichlorofluorescein diacetate. Inhibition of iNO synthase activity by dexamethasone and N^G-nitro-L-arginine methyl ester prevented these effects. Similarly, the NO donor, *S*-nitroso acetyl-penicillamine depleted GSH stores and damaged cells in a dose-dependent manner. The effects of *S*-nitroso acetyl-penicillamine were diminished by the NO scavenger, 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide. In contrast, incubating cells with *N*-acetyl-L-cysteine to augment endogenous GSH synthesis, prevented the effects of *S*-nitroso acetyl-penicillamine. Reduction of GSH stores by pretreatment of rats with buthionine sulfoximine or incubating cells in vitro with diethyl maleate, increased oxidant production and exacerbated NO-induced cell injury. These results suggest that excessive levels of NO alter GSH homeostasis and increase the generation of oxidants leading to increased gastric cellular injury.

Keywords: Glutathione; Nitric oxide (NO); Oxidant; Cell damage; Gastric mucosa

1. Introduction

Nitric oxide (NO) is synthesized from L-arginine by either a Ca^{2+}-dependent constitutive or a Ca^{2+}-independent inducible isoform of the enzyme, nitric oxide synthase (Moncada et al., 1991). NO produced from both NO synthase isoforms has been detected in cells isolated from the rat gastric mucosa (Brown et al., 1992, 1994). Furthermore, NO formed by the Ca^{2+}-dependent enzyme is thought to maintain gastrointestinal mucosal integrity (Whittle, 1994), whereas high levels of NO released by the Ca^{2+}-independent isoform (iNO synthase) in response to bacterial endotoxin and/or cytokines injure gastrointestinal mucosal cells (Tepperman et al., 1993, 1994; Brown et al., 1994). An important mechanism for NO-induced cytotoxicity is through its action with the reactive oxygen metabolite, superoxide, to produce peroxynitrite (Crow and Beckman, 1995). This potent oxidizing agent can initiate lipid peroxidation and thus produce extreme cellular membrane damage (Beckman et al., 1990).

Mammalian cells possess many protective mechanisms which serve to minimize cellular damage from such reactive oxygen metabolites. A major protective mechanism is via the endogenous antioxidant defense system including the nonprotein thiol, reduced glutathione (GSH). GSH is present in most mammalian cells typically at concentrations of 0.5–10 mM and protects cells by functioning as a nucleophilic scavenger as well as acting as a cofactor in the GSH peroxidase-mediated reduction of hydrogen peroxide (Meister and Anderson, 1983). The gastric mucosa possesses a high basal concentration of GSH (approximately 8 mM) (Boyd et al., 1979) suggesting that it plays a role in maintaining the integrity of this tissue. Furthermore it has been demonstrated in vitro that endogenous GSH functions as an important antioxidant to protect cultured and isolated gastrointestinal epithelial cells from oxidative stress (Olson, 1988; Mårtensson et al., 1990) whereas

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depletion of intracellular GSH in gastric mucosa has been shown to increase the extent of ethanol-induced cellular damage in vitro (Mutoh et al., 1990) and hemorrhagic shock-reperfusion gastric mucosal damage in vivo (Stein et al., 1990).

A number of studies have suggested that in some mammalian cell types, GSH and the activity of NO are linked. In hamster fibroblasts, the cellular content of GSH was found to affect the sensitivity of the cells to NO-mediated cytotoxicity (Walker et al., 1995). Furthermore it has been proposed that NO may modulate GSH homeostasis in cytokine-induced hepatocyte injury (Kuo and Slivka, 1993). Therefore, in the present study we have examined the effects of high levels of NO in response to bacterial endotoxin or NO donors on the cellular levels of GSH and the viability of rat gastric mucosal cells. We have also investigated whether agents which alter the GSH content of gastric cells can influence the cytotoxic actions of the high levels of NO on these cells.

2. Materials and methods

2.1. Animals

Non-fasted Sprague-Dawley rats (250–350 g) purchased from Canada Breeding Labs. (St. Constant, Quebec, Canada) were used in these experiments. Animals were maintained in a temperature-controlled environment (22 ± 1°C) in a l2 h light and dark cycle with chow and water available ad libitum. All studies were approved by the University of Western Ontario Animal Care Committee and all animals were treated according to the guidelines set out by the Canadian Council on Animal Care.

2.2. Cell isolation technique

A crude preparation of gastric mucosal cells was harvested from everted sacs of the rat glandular stomach as described by Hatt and Hanson (1989) by 2.5 mg/ml pronase E (*Streptomyces griseus*; Sigma, St. Louis, MO, USA) digestion coupled with intermittent Ca^{2+} chelation by ethylenediamine-tetraacetic acid (EDTA, 2 mM; Sigma). Typically, the resultant cell yield per stomach was approximately 10^7 cells/ml.

Cells were suspended in 1 ml of medium containing: 0.5 mM NaH_2PO_4, 1.0 mM Na_2HPO_4, 20 mM $NaHCO_3$, 70 mM NaCl, 5 mM KCl, 1 mM glucose, 50 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1 mg/ml bovine serum albumin fraction V (BDH, Poole, UK) (pH 7.4; 37°C; 95% O_2/5% CO_2).

2.3. Treatments

Cells were harvested from control rats or rats treated 4 h previously with 3 mg/kg i.v. of bacterial endotoxin lipopolysaccharide (*Escherichia coli* serotype 0111:B4; Sigma). In some experiments rats were treated with the inhibitor of iNO synthase transcription, dexamethasone (1 mg/kg i.p.; Sigma) 1 h before endotoxin treatment, while other animals were injected with the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg s.c.; Sigma) l5 min after endotoxin administration. In in vitro studies, isolated cells harvested from control rats were treated with the spontaneous NO liberator, *S*-nitroso acetyl-penicillamine (0.1–2.0 mM; Sigma). Cells were treated with *S*-nitroso acetyl-penicillamine for 1 h at 37°C. Some cells exposed to the NO donor were co-incubated with 2-phenyl-4,4,5,5,-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO; 0.01–1.0 mM; Calbiochem). PTIO has been shown to oxidize NO to NO_2 followed by the generation of nitrate and nitrite in the presence of water to abolish the biological activity of NO without affecting synthase activity (Akaike et al., 1993; Amano and Noda, 1995).

In some groups of experiments, the cellular levels of reduced glutathione were decreased by treating isolated cells with diethyl maleate (0.75–14 mM; Eastman Kodak, Rochester, NY, USA), an agent which decreases endogenous GSH levels via irreversible conjugation (Meister and Anderson, 1983). Cells were incubated with diethyl maleate for 1 h prior to the determination of both cell viability and GSH levels. In similar studies, rats were treated in vivo with L-buthionine-*S*-*R*-sulfoximine (5 mmol/kg i.p., 3 h prior to killing; ICN) an irreversible inhibitor of γ-glutamylcysteine synthetase. L-Buthionine-*S*-*R*-sulfoximine treatment by this protocol has previously been shown to decrease cellular levels of reduced glutathione by inhibiting GSH biosynthesis (Griffith and Meister, 1979a,b).

Finally some cells were treated in vitro with *N*-acetyl-L-cysteine (10 mM; Sigma), an agent which has been shown to augment cellular levels of GSH (Kainz et al., 1993). Cells were incubated with *N*-acetyl-L-cysteine for 30 min prior to addition of *S*-nitroso acetyl-penicillamine (1 mM)

2.4. Assessment of cell integrity

Cell viability was assessed using Trypan blue. At the end of the incubation period in each experiment, Trypan blue (100 μl of a 1% (w/v) solution; Sigma) was added to the incubate and mixed. The percentage of stained cells was assessed by a naive observer. The number of stained cells is expressed as a percentage of the total number of cells counted.

Alamar blue which uses an indicator to assess cellular metabolic activity was also used in these studies. Alamar blue dye (Alamar, Sacramento, CA, USA) was added to a 250 μl aliquot of cell suspensions for 2 h at 37°C (95% O_2/5% CO_2 environment). The colorimetric determination was done at 570 nm and 600 nm on a plate reader (Spectra II, SLT Lab Instruments, Salzburg, Austria). The percent inhibition of metabolic activity in response to a

test agent as compared to untreated cells was calculated by the formula

$$100 - \frac{(OD_{570} - OD_{600}) \text{ of test agent dilutions}}{(OD_{570} - OD_{600}) \text{ of untreated control}} \times 100$$

2.5. Determination of cellular glutathione levels

GSH levels were determined by measuring the total reduced soluble thiol content using a previously described method (Mutoh et al., 1990). After the incubation period, cells were washed three times with the medium described above and centrifuged at $2000 \times g$ for 1 min. The pellet was resuspended in 0.7 ml of the solution containing 0.2% Triton X-100 (BDH) and 2.5 sulphosalicyclic acid (BDH) in 0.9% NaCl. The suspension was vortexed and centrifuged at $3000 \times g$ for 5 min. A 500 μl aliquot of the supernatant was added to 1.0 ml of 0.3 M Na_2HPO_4 buffer. Spectrophotometric determinations were performed at 412 nm immediately after the addition of 125 μl of 5,5′-dithiobis(-2-nitrobenzoic acid) (Ellman's reagent; Boehringer-Mannheim, Indianapolis, IN, USA; 40 mg/dl in 1% sodium citrate) to the supernatant-Na_2HPO_4 mixture.

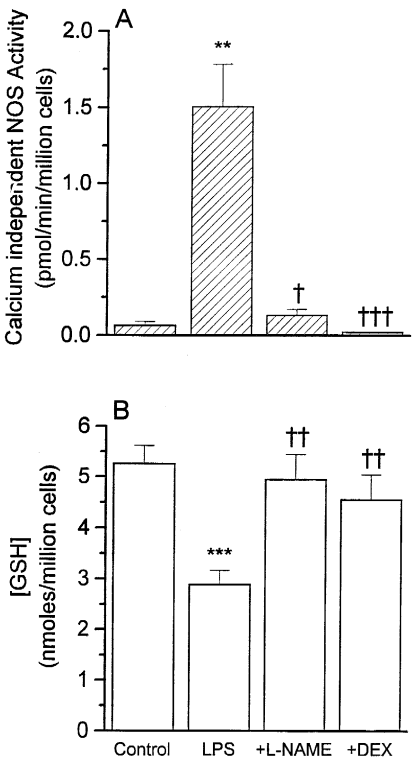

Fig. 1. Effect of bacterial endotoxin lipopolysaccharide (LPS; 3 mg/kg i.v.; 4 h prior to killing) administration on (A) Ca^{2+}-independent NO synthase (NOS) activity, and (B) cellular reduced glutathione (GSH). Some rats were also treated with either an inhibitor of inducible NO synthase transcription, dexamethasone (DEX; 1 mg/kg i.p.; 60 min prior to lipopolysaccharide), or L-NAME (10 mg/kg s.c.; 15 min after lipopolysaccharide). Results are means ± S.E. ($n = 9$–12), where ∗∗ ($P < 0.01$) and ∗∗∗ ($P < 0.001$) indicate significant difference from control, while † ($P < 0.05$), †† ($P < 0.01$) and ††† ($P < 0.001$) indicate significant difference from lipopolysaccharide treatment by nonparametric analysis of variance and either Dunn's or Student-Newman-Keuls multiple comparisons test.

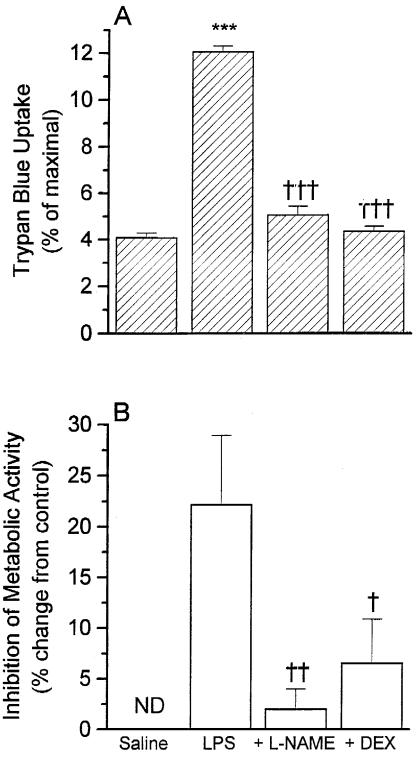

Fig. 2. Effect of lipopolysaccharide (LPS) administration (3 mg/kg i.v.; 4 h prior to killing) on cellular integrity in isolated gastric mucosal cells as assessed by (A) Trypan blue dye exclusion and (B) alamar blue dye absorbance. Some rats were treated with either dexamethasone (DEX; 1 mg/kg i.p.; 60 min prior to lipopolysaccharide), or L-NAME (10 mg/kg s.c.; 15 min post lipopolysaccharide). Results for Trypan blue are shown as the percentage of total cells failing to exclude the dye and are displayed as means ± S.E. ($n = 8$), where ∗∗∗ ($P < 0.001$) indicates significant difference from control and ††† ($P < 0.001$) indicates significant difference from lipopolysaccharide treatment by analysis of variance and Student-Newman-Keuls multiple comparisons test. Results for alamar blue dye absorbance are shown as inhibition of metabolic activity (percent change from control) and are means ± S.E. ($n = 9$), where † ($P < 0.05$) and †† ($P < 0.01$) indicate significant difference from lipopolysaccharide treatment by nonparametric analysis of variance and Dunn's multiple comparisons tests.

2.6. Estimation of NO synthase activity

The conversion of radiolabeled [^{14}C]L-arginine to radiolabeled [^{14}C]L-citrulline has been shown to provide an accurate and valid index of NO synthase activity (Moncada et al., 1991).

After the incubation period the cells were washed three times with assay buffer followed by centrifugation at $2000 \times g$ for 5 min. The supernatant was discarded and the pellet was resuspended in 200 μl of homogenization buffer (pH 7.4) which consisted of: 10 mM HEPES, 0.32 M sucrose (BDH), 0.1 mM EDTA, 1 mM dithiothreitol

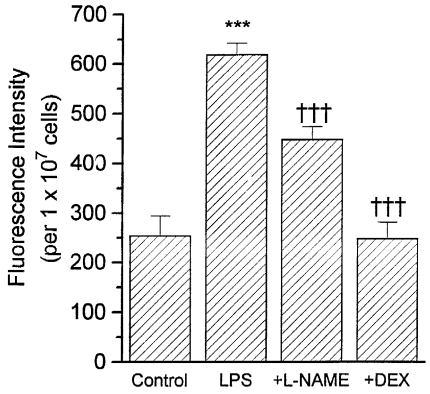

Fig. 3. Effect of lipopolysaccharide treatment (3 mg/kg i.v.; 4 h prior to killing) on in vitro production of intracellular reactive oxygen metabolite generation in rat isolated gastric mucosal cells. Cells were loaded with the probe 2′,7′-dichlorofluorescein diacetate (100 μM) and fluorescence of the probe was measured in the cell lysate. Some rats were treated with either dexamethasone (DEX; 1 mg/kg i.p.; 60 min prior to lipopolysaccharide), or L-NAME (10 mg/kg s.c.; 15 min after lipopolysaccharide). Results are expressed as the fluorescence intensity (per 1×10^7 cells) and are means ± S.E. ($n = 7$–9), where *** ($P < 0.001$) indicates significant difference from control and ††† ($P < 0.001$) indicates significant difference from lipopolysaccharide treatment by analysis of variance and Student-Newman-Keuls multiple comparisons test.

(Boehringer-Mannheim), 10 μg/ml soybean trypsin inhibitor (Sigma), 10 μg/ml leupeptin (Boehringer-Mannheim), and 2 μg/ml aprotinin (Sigma). Cells were lysed by three cycles of rapid freezing and thawing at 37°C. The lysates were centrifuged at $10\,000\times g$ for 20 min at 4°C. To determine total activity and Ca^{2+}-independent NO synthase activity, 20 μl of the supernatant was added to 50 μl of trace containing: 30 mM potassium phosphate, 150 μM $CaCl_2$, 0.7 mM $MgCl_2$, 15 μM [^{14}C]L-arginine (ICN), 0.7 mM β-NADPH, as well as, 7 mM L-valine to inhibit arginase activity. Incubations preceded at 37°C for 10 min and were terminated by addition of 500 μl of a 1:1 suspension of Dowex (AG 50W-8; Sigma) in distilled water to bind any excess radiolabeled L-arginine. Product formation that was inhibited by incubation with the NO synthase inhibitor N^G-mono-methyl-L-arginine (300 μM; Sigma) but not by EGTA (1 mM) was used as an index of Ca^{2+}-independent NO synthase activity. Results are expressed as pmoles of citrulline formed per min per 10^6 cells.

2.7. Intracellular oxidant production

Cells were suspended in a medium containing 100 μM 2′,7′-dichlorofluorescein diacetate (Molecular Probes) for 30 min at 37°C. Cells were washed twice with Hanks' balanced salt solution (Gibco) and sonicated in a buffer containing 50 mM K_2HPO_4, 0.1 mM EDTA, and 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (pH 7.0). The mixture was centrifuged at $2000\times g$ for 10 min at 4°C. The supernatant was used to determine intracellular oxidant production by monitoring its fluorescence on a Hitachi F-4010 fluorescence spectrophotomer at 502 nm excitation and 523 nm emission. Results are expressed as fluorescent intensity per 10^7 cells.

2.8. Statistical calculations

Statistical significance was estimated using analysis of variance and Dunn's multiple comparisons test or Student-Newman Keuls multiple comparisons test. $P < 0.05$ was the minimum accepted level of significance for all groups. Data are expressed as means ± S.E. with n

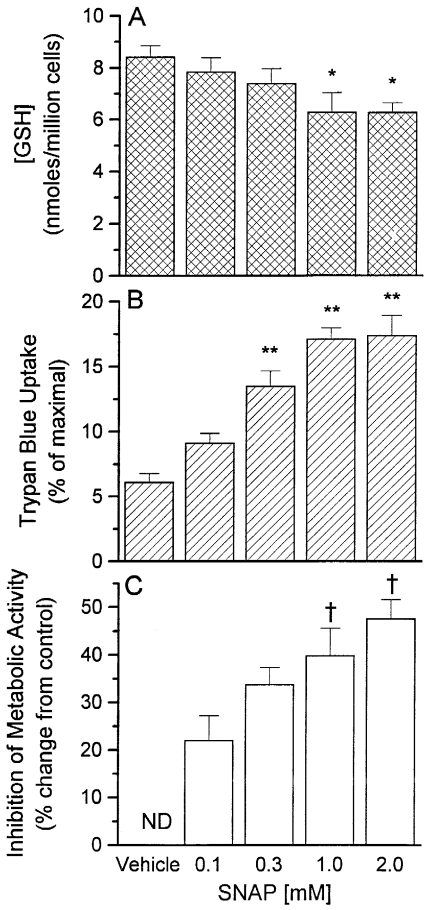

Fig. 4. Effect of in vitro treatment of isolated gastric cells with *S*-nitroso acetyl-penicillamine (SNAP; 0.1–2.0 mM; 60 min) on (A) cellular glutathione (GSH) levels and gastric cellular viability as assessed by (B) Trypan blue dye exclusion and (C) alamar blue dye absorbance. Results for glutathione content are expressed as nmoles of reduced glutathione per 10^6 cells and are means ± S.E. ($n = 7$–9), where * ($P < 0.05$) indicates significant difference from vehicle by analysis of variance and Student-Newman-Keuls multiple comparisons test. Results for Trypan blue are shown as the percentage of total cells failing to exclude the dye as means ± S.E. ($n = 7$–8), where ** ($P < 0.01$) indicates significant difference from vehicle by analysis of variance and Student-Newman-Keuls multiple comparisons test. Results for alamar blue absorbance are shown as percent inhibition activity in comparison to the vehicle and are means ± S.E. ($n = 5$–9), where † ($P < 0.05$) indicates significant difference from 0.1 mM *S*-nitroso acetyl-penicillamine by analysis of variance and Student-Newman-Keuls multiple comparisons test.

equaling the number of cell preparations, each from a different rat.

3. Results

Intravenous administration of the bacterial endotoxin *E. coli* lipopolysaccharide resulted in a significant ($P < 0.01$) increase in Ca^{2+}-independent NO synthase activity in cell suspensions isolated from the rat gastric mucosa when compared to iNO synthase activity of control rats (Fig. 1A). Lipopolysaccharide induction of iNO synthase activity was significantly reduced by pretreatment of rats with either dexamethasone or L-NAME (Fig. 1A). Furthermore the responses to dexamethasone or L-NAME alone were not significantly different from the iNO synthase activity observed in controls.

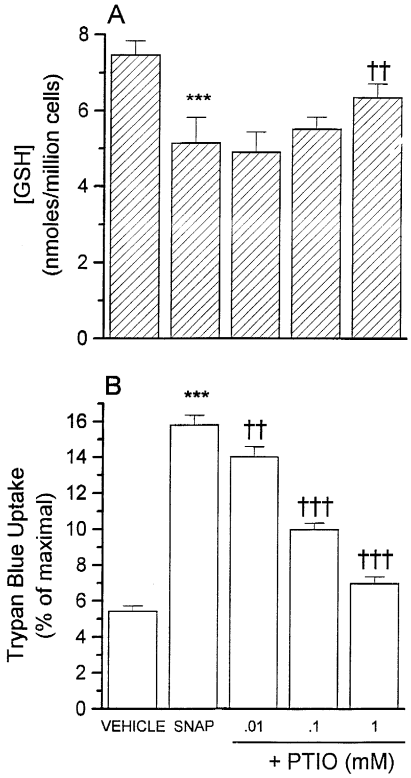

Fig. 5. Effect of treatment of isolated gastric cells with the NO scavenger α-phenyl-4,4,5,5,-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO; 0.01–1 mM) and *S*-nitroso acetyl-penicillamine (SNAP; 1 mM) on (A) intracellular glutathione (GSH) content and (B) viability of rat isolated gastric mucosal cells as assessed by Trypan blue dye uptake. Glutathione content is expressed as nmoles of reduced glutathione per 10^6 cells and are means ± S.E. ($n = 8$), where $***$ ($P < 0.001$) indicates significant difference from vehicle and †† ($P < 0.01$) indicates significant difference from *S*-nitroso acetyl-penicillamine treatment by analysis of variance and Student-Newman-Keuls multiple comparisons test. Results for Trypan blue are shown as the percentage of total cells failing to exclude the dye as means ± S.E. ($n = 8$), where $***$ ($P < 0.001$) indicates significant difference from vehicle, †† ($P < 0.01$), and ††† ($P < 0.001$) indicate significant difference from *S*-nitroso acetyl-penicillamine treatment by analysis of variance and Student-Newman-Keuls multiple comparisons test.

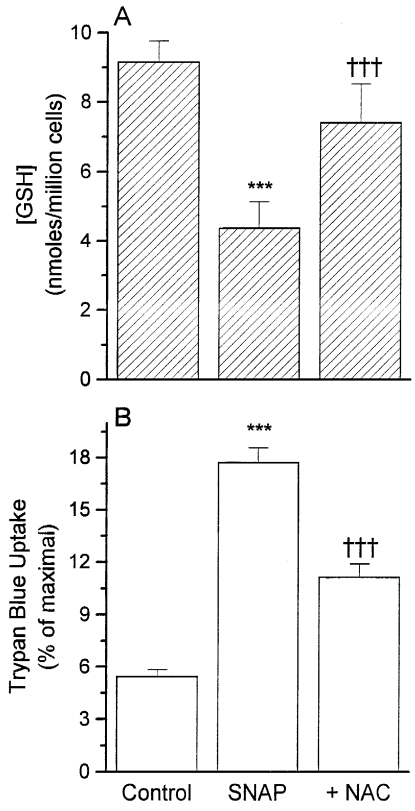

Fig. 6. Effect of pretreatment of gastric cells with *N*-acetyl-L-cysteine (NAC; 10 mM) on *S*-nitroso acetyl-penicillamine (SNAP; 1 mM)-induced reductions in cellular viability (A) glutathione levels and (B) Trypan blue dye uptake. Results for glutathione (GSH) are expressed as nmoles of reduced glutathione per 10^6 cells and are means ± S.E. ($n = 8$), where $***$ ($P < 0.001$) indicates significant difference from control, ††† ($P < 0.001$) indicates significant difference from *S*-nitroso acetyl-penicillamine treatment by analysis of variance and Student-Newman-Keuls multiple comparisons test. Results for Trypan blue are shown as the percentage of total cells failing to exclude the dye as means ± S.E. ($n = 8$), where $***$ ($P < 0.001$) indicates significant difference from control and ††† ($P < 0.001$) indicates significant difference from *S*-nitroso acetyl-penicillamine treatment by analysis of variance and Student-Newman-Keuls multiple comparisons test.

The induction of Ca^{2+}-independent NO synthase activity was also associated with a decrease in intracellular reduced glutathione (GSH) levels to 54.9% of control (Fig. 1B). The reduction in cellular GSH levels was ameliorated in response to treatment with dexamethasone or L-NAME (Fig. 1B). Cellular glutathione content in response to treatment of animals with L-NAME or dexamethasone alone was comparable to that observed in control cells. The viability of gastric mucosal cells isolated from rats treated 4 h prior with lipopolysaccharide was significantly reduced in comparison to cells harvested from control rats as assessed by Trypan blue dye exclusion (Fig. 2A). Furthermore metabolic activity of cells as assessed by alamar blue absorbance was inhibited when compared to control (Fig. 2B). However, the reduction in integrity of cells isolated from endotoxin-treated rats was significantly attenuated by dexamethasone and L-NAME treatments.

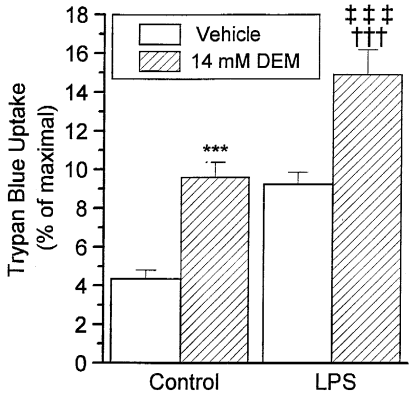

Fig. 7. Effect of glutathione (GSH) depletion with diethyl maleate (DEM; 14 mM; 60 min) on the cellular integrity of rat isolated gastric mucosal cells harvested from control and bacterial endotoxin (lipopolysaccharide (LPS); 3 mg/kg i.v.; 4 h prior to sacrifice) treated rats. Results are expressed as the number of cells (percentage of maximal) failing to exclude Trypan blue dye and are means ± S.E. ($n = 8$–9), where *** ($P < 0.001$) indicates significant difference from control, ††† ($P < 0.001$) indicates significant difference from lipopolysaccharide treatment and ††† ($P < 0.001$) indicates significant difference from diethyl maleate control by analysis of variance and Student-Newman-Keuls multiple comparisons test.

Lipopolysaccharide treatment also caused a significant increase in the in vitro production of intracellular reactive oxygen metabolites by isolated gastric mucosal cells as assessed by the fluorescent probe 2′,7′-dichlorofluorescein (Fig. 3). The inhibitors, dexamethasone and L-NAME, significantly ameliorated the increase in oxidant fluorescence in response to endotoxin pretreatment (Fig. 3).

Addition of *S*-nitroso acetyl-penicillamine (0.1–2 mM) to the incubation medium resulted in a decrease in GSH levels in isolated gastric cells to a maximum level of 74% of vehicle (Fig. 4A). Furthermore, incubation of cells in the presence of *S*-nitroso acetyl-penicillamine also resulted in a reduction in cellular viability (Fig. 4B and 4C).

The NO-mediated reduction in GSH was prevented by treating cells with the NO scavenger, PTIO (0.01–1 mM) (Fig. 5A). Similarly, the increase in Trypan blue dye uptake resulting from *S*-nitroso acetyl-penicillamine treatment was reduced by the addition of PTIO to the incubation medium (Fig. 5B). PTIO treatment alone did not significantly affect any of the parameters measured here. In contrast, incubating gastric cells with *N*-acetyl-L-cysteine (10 mM) reduced the damaging effect of *S*-nitroso acetyl-penicillamine (1 mM) and maintained the cellular levels of GSH (Fig. 6). *N*-Acetyl-L-cysteine treatment by itself significantly increased intracellular GSH content to approximately 134% of the value determined in cells from control animals.

Treatment of isolated gastric mucosal cells with diethyl maleate (14 mM for 1 h) significantly reduced cellular GSH from control levels of 6.2 ± 0.5 nmol/10^6 cells to 3 ± 0.4 nmol/10^6 cells. Diethyl maleate treatment increased Trypan blue uptake in control cells and exacerbated Trypan blue dye uptake in cells harvested from lipopolysaccharide-treated rats (Fig. 7). The extent of cell injury in diethyl maleate-treated cells from endotoxin-injected rats was significantly greater than that observed in diethyl maleate-treated cells from control rats (Fig. 7). Diethyl maleate treatment also resulted in an increase in oxidant production in gastric cells by $45 \pm 11\%$ ($n = 7$) from control levels.

Injection of L-buthionine-*S*-*R*-sulfoximine (5 mmol/kg) into rats resulted in a significant reduction in the GSH content of cells harvested from rat stomach (Fig. 8A). Furthermore L-buthionine-*S*-*R*-sulfoximine treatment resulted in a further reduction in the GSH content in response to *S*-nitroso acetyl-penicillamine treatment. Associated with the decrease in GSH content was a reduction in cell viability as assessed by Trypan blue dye uptake (Fig.

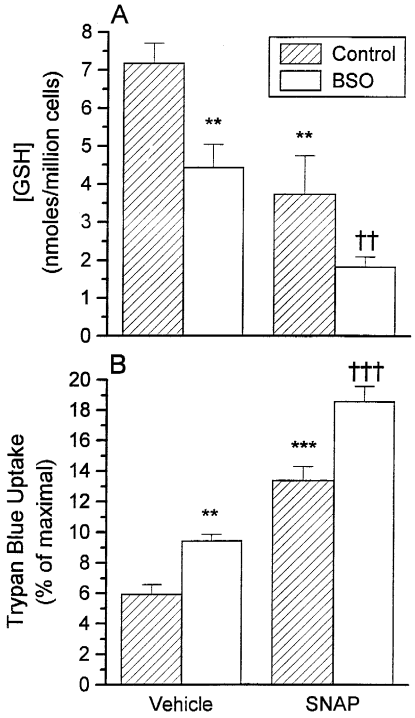

Fig. 8. Effect of pretreatment of rats with L-buthionine-*S*-*R*-sulfoximine (BSO; 5 mmol/kg i.p.) on (A) intracellular glutathione (GSH) levels and (B) cellular viability in rat isolated gastric mucosa cells as assessed by Trypan blue dye uptake. Cells harvested from these animals were then incubated in vitro in the presence of *S*-nitroso acetyl-penicillamine (SNAP; 1 mM). Results for glutathione are expressed as nmoles of reduced glutathione per 10^6 cells and are means ± S.E. ($n = 6$–9), where ** ($P < 0.01$) indicates significant difference from control and †† ($P < 0.01$) indicates significant difference from L-buthionine-*S*-*R*-sulfoximine treatment by analysis of variance and Student-Newman-Keuls multiple comparisons test. Results for Trypan blue are shown as the percentage of total cells failing to exclude the dye and are means ± S.E. ($n = 6$–9), where ** ($P < 0.01$) and *** ($P < 0.001$) indicate significant difference from control, and ††† ($P < 0.001$) indicates significant difference from L-buthionine-*S*-*R*-sulfoximine treatment by analysis of variance and Student-Newman-Keuls multiple comparisons test.

8B). Cell injury in response to *S*-nitroso acetyl-penicillamine treatment (1 mM) was significantly enhanced in cells harvested from L-buthionine-*S*-*R*-sulfoximine-treated rats. Furthermore, oxidant production in cells harvested from L-buthionine-*S*-*R*-sulfoximine-treated rats was increased by $30 \pm 14\%$ ($n = 5$) when compared to cells harvested from control rats.

4. Discussion

These data confirm the results of previous studies that have identified the presence of the inducible Ca^{2+}-independent isoform of NO synthase in cells isolated from the rat gastric mucosa of rats treated with bacterial lipopolysaccharide (Brown et al., 1994). While bacterial endotoxin treatment may also activate other enzymes systems associated with inflammation (e.g., cyclooxygenase-2) (Vane and Botting, 1995) the results of the present study also demonstrate that NO formation as well as cell injury were reduced by treatment with the NO synthase inhibitor, L-NAME. Therefore these data strongly suggest that the effects of lipopolysaccharide observed here are mediated to a large extent by NO release.

In the present study cell integrity is estimated by Trypan blue dye uptake and alamar blue dye absorbance. Trypan blue dye exclusion has previously been shown to be a reliable index of alterations in the permeability properties of gastric cell membranes (Wallace and Whittle, 1989; Tepperman et al., 1991; Brown et al., 1994; Dziki et al., 1995). However, Trypan blue is only a marker of plasma membrane permeability, therefore uptake of this dye may not necessarily reflect cellular toxicity. Yet, Trypan blue dye exclusion has been shown to directly correlate to other markers of cellular disruption such as acid phosphate release (Wong and Tepperman, 1994) and lactic dehydrogenase release (Nagy et al., 1994). Additionally, the alamar blue dye technique has been shown to detect changes in cellular metabolic activity (Pagé et al., 1993) and, thus, has been used to assess the cytotoxicity of test agents in this study. The alamar blue assay, which is a recently developed extension of the cytotoxicity assay based on the reduction of tetrazolium salts by the mitochondrial cytochromoxidase system (Mossman, 1983), has been shown to accurately assess mitochondrial integrity in isolated gastric mucosal cells from rat stomach (Nagy et al., 1994). In combination, the Trypan blue and alamar blue dye techniques provide reliable estimates of gastric cellular integrity.

In the present study gastric cell injury in response to bacterial endotoxin or high levels of NO ranged between 12–20% of the mixed cell population. This confirms previous results in rat gastric cells by Brown et al. (1994). In that study it was also found that NO induced a high degree of damage in a parietal cell-enriched fraction. It is possible that NO produced primarily by the mucous cells may affect viability of cells in close proximity, including parietal cells.

Lipopolysaccharide administration resulted in a significant decrease in cellular levels of GSH. It has been shown previously that excessive amounts of NO would reduce the intracellular levels of GSH in fibroblasts (Walker et al., 1995). As well, in the present study it has been demonstrated that intracellular stores of GSH can be depleted by treating cells with diethyl maleate, an electrophilic agent which forms thioester conjugates catalyzed by glutathione-*S*-transferase (Chasseaud, 1979). Depletion of endogenous GSH stores with diethyl maleate also enhanced cell damage as a result of in vivo endotoxin treatment. This finding confirms reports that a reduction in cellular GSH content by agents such as diethyl maleate (Chasseaud, 1979), calcium ionophore A23187 (Wong and Tepperman, 1994), and ethanol (Olson, 1988) result in an increase in the susceptibility to a cytotoxic challenge such as the induction of NO synthase in rat isolated gastric mucosal cells.

The NO donor *S*-nitroso acetyl-penicillamine was found to increase the extent of cell injury in these studies. Similarly, in other studies, high doses of NO donors have been shown to exert cytotoxic effects on gastrointestinal cells (Tepperman et al., 1994). Furthermore, the NO donor reduced cellular levels of GSH. The effects of *S*-nitroso acetyl-penicillamine on cell viability and GSH levels were attenuated by the NO scavenger, PTIO. This confirms previous studies in which PTIO was observed to effectively scavenge NO and as a result reduce NO-mediated tissue damage (Yoshida et al., 1994). These data suggest that NO, rather than an NO-independent mechanism, was responsible for the reduction in GSH levels observed in these studies.

In many of the studies described here, manipulations to reduced GSH levels did so proportionally to a lesser extent than reductions in cellular viability as assessed by Trypan blue dye exclusion and alamar blue dye absorbance. A possible explanation for this discrepancy is that perhaps the intracellular GSH pool of gastric epithelial cells needs only to be depleted below an undetermined threshold before NO-induced damage is augmented. It has been shown that cellular GSH levels must be depleted below a specific threshold value in order for lipid peroxidation and liver necrosis to occur in the rat (Casini et al., 1985). It is possible that a similar threshold exists in gastric epithelial cells and, therefore, it would not be expected that a 1:1 ratio exists between GSH depletion and NO-mediated cell injury. Furthermore, in the relatively crude cell preparation utilized in these studies, it cannot be guaranteed that the GSH pool of each cell is depleted to an equal extent. Instead it must be assumed that the GSH pool of each cell is depleted to varying degrees. Therefore, although the average GSH depletion observed was approximately 25%, only those cells whose GSH levels were reduced below the threshold value would be susceptible to NO-mediated injury. Similar results have been demonstrated using gastric

cell preparations from the rabbit in which a depletion in GSH levels by 35% in response to diethyl maleate treatment resulted in a disproportionate increase in Trypan blue dye uptake of 50% (Wong and Tepperman, 1994).

Similarly, *N*-acetyl-L-cysteine treatment increased cellular GSH levels. This confirms previous studies in which *N*-acetyl-L-cysteine treatment for over 30 min in vitro increased GSH pools in a number of cell types including ovarian cells and in cultured gastric cells (Issels et al., 1988; Mutoh et al., 1990). Furthermore, *N*-acetyl-L-cysteine maintained cell viability in cells challenged with high concentrations of *S*-nitroso acetyl-penicillamine. Similarly, *N*-acetyl-L-cysteine has been shown to protect hepatocytes from a cytotoxic challenge (Kainz et al., 1993). In addition GSH depletion by treating rats with L-buthionine-*S*-*R*-sulfoximine was associated with increased gastric cell injury in cells challenged with *S*-nitroso acetyl-penicillamine but this treatment was not cytotoxic in itself. These data confirm previous findings in which L-buthionine-*S*-*R*-sulfoximine treatment was shown to enhance cell injury in the liver and lungs of rats (Kainz et al., 1993; Thanislass et al., 1995). Similarly, it has been demonstrated that L-buthionine-*S*-*R*-sulfoximine treatment was associated with an increase in NO-mediated cytotoxicity in fibroblasts (Walker et al., 1995).

In the present study treatment of animals with the bacterial endotoxin, lipopolysaccharide, also resulted in an increase in the gastric cellular production of reactive oxidant species. Since these studies were done using relatively crude mucosal cells, the source of the free radicals determined here is uncertain. Similarly, the species of radical detected in these studies is also uncertain and may include superoxide, H_2O_2, $NO^{\cdot}$ or peroxynitrite. In additon, the present results demonstrate that the increase in oxidant production was ameliorated by agents that inhibited NO synthase activity. It has previously been shown that high levels of NO can generate reactive oxygen metabolites by causing mucosal iron release (Reif and Simmons, 1990) or by inactivation of the GSH-redox enzyme, glutathione peroxidase (Ashai et al., 1995). Since GSH peroxidase catalyses the reduction of oxidant species this may account for the increase in oxidant production in response to NO release. Thus a likely mechanism for the NO-mediated reduction in GSH levels which occurs via an increased conjugation with oxidants catalyzed via the glutathione-*S*-transferases (Chasseaud, 1979). The reduction in GSH levels therefore can enhance the extent of cellular injury in response to an NO challenge (Wink et al., 1994; Walker et al., 1995).

In conclusion, these data demonstrate that high levels of NO reduce gastric cell viability and also appear to alter intracellular GSH homeostasis and increase generation of intracellular oxidants. It is possible that the cellular content of the intracellular antioxidant, reduced glutathione, can affect the extent of damage observed in gastric mucosal cells in response to cytotoxic levels of NO.

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