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### Research Article

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## Glutathione Redox Cycle Protects Cultured Endothelial Cells against Lysis by Extracellularly Generated Hydrogen Peroxide

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**A**bstract. We have examined the role of the glutathione redox cycle as an antioxidant defense mechanism in cultured bovine and human endothelial cells by disrupting the glutathione redox cycle at several points. Endothelial glutathione reductase was selectively inhibited with 1,3-bis(chloroethyl)-1-nitrosourea (BCNU). Cellular stores of reduced glutathione were depleted by reaction with diethylmaleate (DEM) or 1-chloro-2,4-dinitrobenzene (CDNB) or by inhibition of glutathione synthesis with buthionine sulfoximine (BSO). Whereas several strains of untreated bovine and human endothelial cells were resistant to lysis by enzymatically generated hydrogen peroxide, BCNU-treated cells were readily lysed in a time- and dose-dependent manner. Glucose-glucose oxidase-mediated lysis of BCNU-treated bovine endothelial cells was catalase-inhibitable and directly related to BCNU concentration and endogenous glutathione reductase activity. Pretreatment of bovine endothelial cells with BCNU did not potentiate lysis by distilled water, calcium ionophore, lipopolysaccharide, or hypochlorous acid. Depletion of cellular reduced glutathione by reaction with

DEM or CDNB or by inhibition of glutathione synthesis by BSO also potentiated endothelial lysis by enzymatically generated hydrogen peroxide. Inhibition of endothelial glutathione reductase by BCNU or depletion of reduced glutathione by BSO increased endothelial susceptibility to lysis by hydrogen peroxide generated by phorbol myristate acetate-activated neutrophils. We conclude that the glutathione redox cycle plays an important role as an endogenous antioxidant defense mechanism in cultured endothelial cells.

### Introduction

Cells may detoxify hydrogen peroxide ( $H_2O_2$ ) by catalase or the glutathione peroxidase-glutathione reductase system. In tumor cells, susceptibility to lysis by either neutrophil- or macrophage-generated  $H_2O_2$  varies considerably and, in some, is dependent on the activity of the glutathione redox cycle but not catalase (1). The glutathione redox cycle is also important in protecting normal cells from oxidant injury (2-4).

In the glutathione redox cycle the oxidation of reduced glutathione by  $H_2O_2$  is catalyzed by glutathione peroxidase. The oxidized glutathione is then reconverted to reduced glutathione by glutathione reductase. The glutathione redox cycle can be disrupted at several points in vitro (4) (Fig. 1). Glutathione reductase may be selectively inhibited by 1,3-bis(chloroethyl)-1-nitrosourea (BCNU)<sup>1</sup> (1, 5, 6). Cellular stores of reduced glutathione can be depleted by formation of a thioether conjugate with electrophilic agents such as 1-chloro-2,4-dinitrobenzene

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1. Abbreviations used in this paper: BCNU, 1,3-bis(chloroethyl)-1-nitrosourea; BSO, buthionine sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; DEM, diethylmaleate;  $H_2O_2$ , hydrogen peroxide; LPS, lipopolysaccharide; NBCS, newborn calf serum; PMA, phorbol myristate acetate; <sup>51</sup>Cr, sodium chromate.

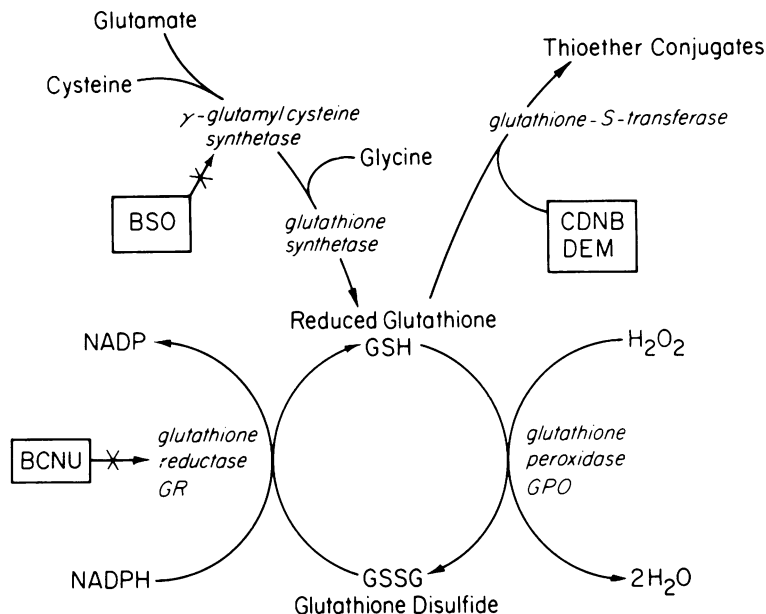


Figure 1. Pharmacologic modification of the glutathione redox cycle in vitro.

(CDNB) (7, 8) or diethylmaleate (DEM) (9–11) in a reaction catalyzed by endogenous glutathione-S-transferase. After cleavage of the  $\gamma$ -glutamyl and glycyl moieties of the glutathione and acetylation of the cysteine, the resulting mercapturic acid is excreted. Glutathione biosynthesis may be inhibited by buthionine sulfoximine (BSO), a selective inhibitor of  $\gamma$ -glutamylcysteine synthetase (4, 8, 11, 12).

We have examined the role of the glutathione redox cycle in modulating oxidant injury to endothelial cells in culture by disrupting the glutathione redox cycle at several points. Endothelial cell glutathione reductase was selectively inhibited with BCNU and cellular stores of reduced glutathione were depleted by reaction with CDNB or DEM or by inhibition of glutathione synthesis with BSO.

## Methods

**Preparation of endothelial cells.** Bovine aortic and pulmonary artery and human umbilical vein endothelial cells were obtained as previously described (13, 14) and cultured in Waymouth's medium MB-752/1 (Grand Island Biological Co. [Gibco], Grand Island, NY) supplemented with 10% newborn calf serum (NBCS) (Gibco). Bovine endothelial cells were used in 6th–15th passage. Because bovine endothelial cell susceptibility to lysis by the toxic agents varied somewhat between strains and passage number within the same strain, individual experiments were always performed with cells of the same strain and passage. Human umbilical vein endothelial cells were used in first or second passage only.

**Neutrophil preparation.** Purified human peripheral neutrophils were prepared by the standard technique of Ficoll-Hypaque gradient centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes (15).

**<sup>51</sup>Chromium-release assay.** Endothelial cells were plated on Falcon Microtest III plates (Falcon Labware, Div. of Becton-Dickinson & Co.,

Oxnard, CA) and labeled with sodium chromate (<sup>51</sup>Cr) (New England Nuclear, Boston, MA) as previously described (16). In some experiments endothelial cells were incubated with BSO during the overnight labeling. After overnight incubation, cells were washed three times with 1% NBCS in phosphate-buffered saline (PBS) (Gibco) and then incubated with either BCNU, DEM, CDNB, or ethanol control in 10% NBCS in Waymouth's medium. Endothelial cells were incubated with BCNU for 10 min, after which they were washed twice with 1% NBCS in PBS. Endothelial cells were incubated with DEM and CDNB for 30–60 min without further washing. Test or control medium was then added to a final volume of 100–200  $\mu$ l/well, and the cells were incubated at 37°C in a 95% air and 5% CO<sub>2</sub> atmosphere. 50–100  $\mu$ l of cell-free supernatant medium was removed at intervals for determination of specific <sup>51</sup>Cr-release as follows:  $(A - B/C - B) \times 100\%$ . A represents the mean test <sup>51</sup>Cr-cpm released, B represents the mean spontaneous <sup>51</sup>Cr-cpm released, and C represents the mean maximum <sup>51</sup>Cr-cpm released. Maximum <sup>51</sup>Cr-release was determined by incubation in 1% Triton X-100 (New England Nuclear). Spontaneous <sup>51</sup>Cr-release was determined in control monolayers incubated in 10% NBCS in Waymouth's medium only and was 10–15% of maximum <sup>51</sup>Cr-release after 6 h of incubation. Statistical significance was determined by comparing mean test and mean control <sup>51</sup>Cr-cpm released by two-tailed, unpaired, *t*-statistic.

For neutrophil experiments 50  $\mu$ l of neutrophils in 10% NBCS in Waymouth's medium was added and allowed to adhere to the endothelial monolayer for 10 min before addition of 50  $\mu$ l of medium with or without phorbol myristate acetate (PMA).

**Enzyme assays.** Duplicate 25-cm<sup>2</sup> flasks of BCNU-treated or ethanol (0.1%) control endothelial cells ( $2-3 \times 10^6$  cells) were solubilized by incubation with 0.2% Triton X-100 at room temperature for 1 h, frozen at  $-80^\circ\text{C}$ , and the supernatant medium was assayed for glutathione reductase activity and catalase by Dr. Ernest Beutler of the Scripps Clinic and Research Foundation, La Jolla, CA (17). Recovery of glutathione reductase activity after a 1-h incubation in 0.2% Triton X-100 at room temperature and subsequent storage at  $-80^\circ\text{C}$  for several weeks was 100%. Reduced soluble sulfhydryl content was measured in DEM-, CDNB-, BSO-treated, and control endothelial cells as follows (8, 18,

19). Duplicate 75-cm<sup>2</sup> flasks of endothelial cells (5–10 × 10<sup>6</sup> cells) of the same strain and passage were incubated with control medium, DEM (0.5 mM), CDNB (10 μM), or BSO (1 mM). After incubation the flasks were washed three times with 0.02% EDTA in PBS and the cells harvested with 0.05% trypsin and 0.02% EDTA in PBS buffer. An aliquot was then removed for determination of cell count by electronic counter (Particle Data, Inc., Elmhurst, IL). After centrifugation at 200 g for 10 min, the cell pellets were resuspended in 1.4 ml of 0.2% Triton X-100 and 2.5% sulfosalicylic acid in EDTA/PBS buffer. Solutions were cleared by centrifugation at 11,000 g for 5 min. A 1.0-ml aliquot of the acid-soluble supernatant medium was then added to 2.0 ml of 0.3 M Na<sub>2</sub>HPO<sub>4</sub> buffer. Spectrophotometric determinations were performed with a Gilford 300-N spectrophotometer (Gilford Instruments Laboratories, Inc., Oberlin, OH) at 412 nm immediately after the addition of 0.25 ml of 5,5-dithiobis-2-nitrobenzoic acid (40 mg/dl in 1% sodium citrate) (20). With each assay a standard curve was generated with known amounts of reduced glutathione (5–100 nmol). Recovery of reduced glutathione added at the time of cell lysis was >90%. Results are expressed as nanomoles of soluble reduced sulphydryls/10<sup>6</sup> endothelial cells.

**Reagents.** Catalase (11,800 U/mg protein), bovine serum albumin, reduced glutathione, glutathione reductase, 5,5-dithiobis-2-nitrobenzoic acid, superoxide dismutase (2,700 U/mg protein), glucose oxidase VII, xanthine oxidase gr. I, 5-sulfosalicylic acid, xanthine, hydrogen peroxide (30%), *Escherichia coli* 055:B5 lipopolysaccharide (LPS), and PMA were obtained from Sigma Chemical Co., St. Louis, MO. Trypan blue dye was obtained from Gibco. Sodium hypochlorite solution was obtained from Scientific Products, Redmond, WA and was standardized by reaction with KI and spectrophotometric measurement of I<sub>3</sub><sup>-</sup> formed using E<sub>353</sub> = 2.64 × 10<sup>4</sup> M<sup>-1</sup> cm<sup>-1</sup> (21). BCNU was obtained from Bristol Laboratories Div., Bristol-Meyers Co., Syracuse, NY and dissolved in ethanol at a 100-mg/ml stock just before each experiment. CDNB and DEM were obtained from Sigma Chemical Co., and dissolved in ethanol as 10<sup>-2</sup>- and 1-M stocks just before use. DL-buthionine-SR-sulfoximine was obtained from Chemalog, Div. of Chemical Dynamics Corporation, South Plainfield, NJ and dissolved in medium of 10<sup>-2</sup> M. The calcium ionophore, A23187, was obtained from Calbiochem-Behring Corp., La Jolla, CA and dissolved in dimethyl sulfoxide at 5 × 10<sup>-2</sup> M.

## Results

**Effect of BCNU on endothelial cell lysis by enzymatically generated H<sub>2</sub>O<sub>2</sub>.** Pretreatment of bovine pulmonary artery endothelial cells with BCNU (100 μg/ml) increased their susceptibility to lysis by both the H<sub>2</sub>O<sub>2</sub>-generating system, glucose-glucose oxidase, and the superoxide anion-generating system, xanthine-xanthine oxidase (Table I A). Pretreatment with BCNU alone (100 μg/ml for 10 min) did not significantly increase endothelial cell <sup>51</sup>Cr-release at 6 h compared with ethanol control. Although the susceptibility of untreated bovine pulmonary artery and aortic endothelial cells to glucose-glucose oxidase-mediated lysis varied somewhat between strains and passage number within the same strain, pretreatment with BCNU always potentiated H<sub>2</sub>O<sub>2</sub>-mediated lysis when assessed in a dose-response assay (Fig. 2). As with bovine endothelial cells, BCNU also significantly increased the susceptibility of human umbilical vein endothelial cells to lysis by enzymatically generated H<sub>2</sub>O<sub>2</sub> (Fig. 3), whereas BCNU alone did not significantly increase <sup>51</sup>Cr-release. Both glucose-glucose oxidase- and xanthine-xanthine oxidase-me-

Table I. Effect of BCNU on Bovine Endothelial Cell Lysis by Oxygen Radical-generating Systems

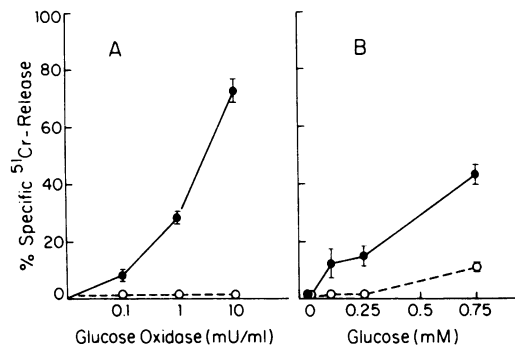
Addition to endothelial monolayer	% Specific <sup>51</sup> Cr-release	
	Pretreatment with	
	Ethanol control	BCNU
<b>A Bovine pulmonary artery</b>		
Glucose-glucose oxidase (4)	1.0±0.1	76.9±6.9
+ Catalase (4)	1.1±0.2	3.3±2.3
Xanthine-xanthine oxidase (4)	0.8±0.1	14.1±2.4
+ Catalase (4)	0.2±0.1	2.3±1.1
+ Superoxide dismutase (4)	0.2±0.1	12.8±1.5
<b>B Bovine aortic</b>		
Glucose-glucose oxidase (6)	16.2±0.9	61.2±1.7
+ Catalase (6)	0.4±0.1	1.0±0.1
+ Catalase boiled (6)	17.3±2.4	54.9±2.7

Glucose (27 mM) and glucose oxidase (10 mU/ml) or xanthine (10<sup>-5</sup> M) and xanthine oxidase (100 mU/ml) with or without catalase (3,000 U/ml), catalase boiled for 20 min before, or superoxide dismutase (270 U/ml) were added to <sup>51</sup>Cr-labeled bovine pulmonary artery (A) or aortic (B) endothelial cells that were preincubated for 10 min with BCNU (100 μg/ml) or ethanol control (0.1%). <sup>51</sup>Cr-release was determined at 4 h by aspirating and counting 50-μl aliquots of the cell-free supernatant medium. Values represent the means of (n) replicates±1 SE.

diated lysis of BCNU-treated cells were inhibited by catalase but not by superoxide dismutase (Table I A). Inhibition of endothelial lysis by catalase was due to its enzymatic activity, since boiled catalase was without protective effect (Table I B). Glucose-glucose oxidase-mediated bovine endothelial <sup>51</sup>Cr-release was associated with trypan blue dye uptake, indicating that endothelial cell lysis had occurred; in a 4-h incubation with glucose (27 mM)-glucose oxidase (10 mU/ml), the specific <sup>51</sup>Cr-release from BCNU-treated aortic endothelial cells was 58.2±1.4% and trypan blue dye uptake was 57.4±4.5% (means of four replicates ±1 SE).

**Time course of H<sub>2</sub>O<sub>2</sub>-mediated endothelial lysis.** Incubation with glucose-glucose oxidase did not induce significant <sup>51</sup>Cr-release until relatively late (3–4 h), even in BCNU-treated cells (Fig. 4). Despite the absence of specific <sup>51</sup>Cr-release in the first several hours of exposure to glucose-glucose oxidase, significant specific <sup>51</sup>Cr-release was observed at 6 h in endothelial cells exposed to the H<sub>2</sub>O<sub>2</sub>-generating system for only 1–2 h before addition of catalase (Fig. 5).

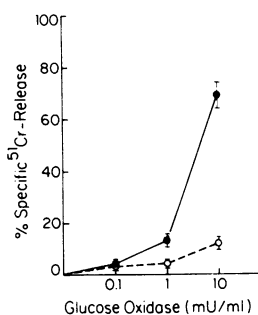
**Glutathione reductase activity in BCNU-treated endothelial cells.** Fig. 6 demonstrates that both bovine endothelial glutathione reductase activity and glucose-glucose oxidase-induced lysis were directly correlated with the concentration of BCNU. More importantly, bovine endothelial susceptibility to lysis by H<sub>2</sub>O<sub>2</sub> was inversely related to endogenous glutathione reductase



**Figure 2.** Dose-dependence of glucose-glucose oxidase-induced bovine endothelial <sup>51</sup>Cr-release. Bovine aortic endothelial cells of two different strains (*A* and *B*) were labeled overnight with <sup>51</sup>Cr, washed, and preincubated for 10 min with ethanol control (0.1%) (○) or BCNU (100 μg/ml) (●). The cells were washed again and then incubated with (*A*) 10% NBCS in Waymouth's medium or (*B*) 0.25% bovine serum albumin in PBS without glucose. In *A* the concentration of glucose was 27 mM and in *B* the concentration of glucose oxidase was 5 mU/ml. Aliquots of supernatant medium were removed after 4 h of incubation for determination of specific <sup>51</sup>Cr-release. Values represent means of six replicates ± 1 SE.

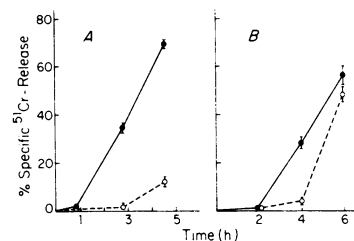
activity (Fig. 6). BCNU also markedly reduced human umbilical vein endothelial glutathione reductase activity (1.0 mIU/mg protein after a 10-min incubation with BCNU 100 μg/ml compared with 13.0 mIU/mg protein in 0.1% ethanol control). Endogenous catalase levels in the bovine endothelial cells were not affected by a 10-min incubation with 100 μg/ml BCNU (5.1 and 6.2 IU/mg protein in control cells compared with 5.1 and 14.6 IU/mg protein in BCNU-treated bovine aortic endothelial cells).

**Specificity of BCNU for H<sub>2</sub>O<sub>2</sub>-mediated injury in bovine endothelial cells.** Bovine aortic endothelial cell susceptibility to lysis by distilled water, calcium ionophore, and LPS was not affected by pretreatment with BCNU, despite BCNU potentiation of <sup>51</sup>Cr-release by enzymatically generated H<sub>2</sub>O<sub>2</sub> in the same cells (Table II). Moreover, BCNU did not potentiate en-



**Figure 3.** Dose-dependence of glucose-glucose oxidase-induced human endothelial cell <sup>51</sup>Cr-release. Human umbilical vein endothelial cells were labeled overnight with <sup>51</sup>Cr, washed, and preincubated for 10 min with ethanol control (0.1%) (○) or BCNU (100 μg/ml) (●). The cells were again washed and then incubated with glucose (27 mM) and glucose oxidase. Aliquots of supernatant medium were removed after 6 h of incubation for determination of specific <sup>51</sup>Cr-release. Values represent means of eight replicates ± 1 SE.

**Figure 3.** Dose-dependence of glucose-glucose oxidase-induced human endothelial cell <sup>51</sup>Cr-release. Human umbilical vein endothelial cells were labeled overnight with <sup>51</sup>Cr, washed, and preincubated for 10 min with ethanol control (0.1%) (○) or BCNU (100 μg/ml) (●). The cells were again washed and then incubated with glucose (27 mM) and glucose oxidase. Aliquots of supernatant medium were removed after 6 h of incubation for



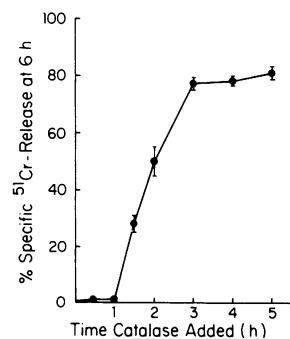
**Figure 4.** Time course of glucose-glucose oxidase-induced bovine endothelial <sup>51</sup>Cr-release. Bovine aortic endothelial cells of two different strains (*A* and *B*) were labeled overnight with <sup>51</sup>Cr, washed, and preincubated for 10 min with ethanol control (0.1%) (○) or BCNU (100 μg/ml) (●). The cells were washed again and then incubated with glucose (27 mM) and glucose oxidase (10 mU/ml in *A* and 25 mU/ml in *B*). Aliquots of supernatant medium were sampled for determination of specific <sup>51</sup>Cr-release at various intervals. Values represent means of eight replicates ± 1 SE.

dothelial lysis by the oxidizing agent, hypochlorous acid (Fig. 7 *B*), whereas it markedly increased bovine endothelial susceptibility to lysis by reagent H<sub>2</sub>O<sub>2</sub> (Fig. 7 *A*).

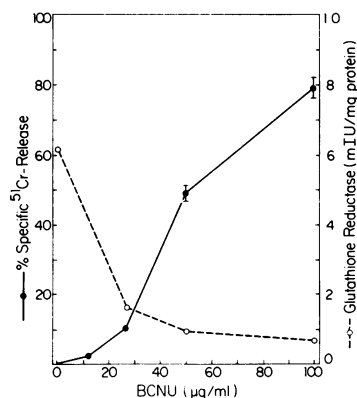
**Effect of glutathione depletion of H<sub>2</sub>O<sub>2</sub>-mediated endothelial cell lysis.** Since glutathione accounts for the majority of soluble-reduced sulfhydryls in cells (22), reduced glutathione levels in endothelial cells were determined by measuring total soluble-reduced sulfhydryl content without identifying the individual species of sulfhydryls.

Incubation of bovine aortic endothelial cells with CDNB (10 μM) produced a 68% reduction in soluble-reduced sulfhydryl content at 1 h (1.3 and 1.6 nmol/10<sup>6</sup> cells in CDNB-treated cells compared with 4.4 and 4.6 nmol/10<sup>6</sup> cells in ethanol control) without significantly increasing <sup>51</sup>Cr-release (0% specific <sup>51</sup>Cr-release in CDNB-treated cells at 6 h). Incubation with CDNB significantly increased bovine aortic endothelial cell susceptibility to glucose-glucose oxidase-mediated lysis (Fig. 8).

The soluble reduced sulfhydryl content of human umbilical vein endothelial cells incubated with DEM (0.5 mM) for 1 h was reduced 60% compared with ethanol control (4.1 and 3.4 nmol/10<sup>6</sup> cells with DEM compared with 9.0 and 9.6 nmol/10<sup>6</sup> cells in ethanol control). Although DEM (0.5 mM) alone did not produce significant <sup>51</sup>Cr-release (<5% specific <sup>51</sup>Cr-release at 6 h), incubation of human umbilical vein endothelial cells



**Figure 5.** Effect of the duration of initial exposure to glucose-glucose oxidase on subsequent <sup>51</sup>Cr-release. Bovine aortic endothelial cells were labeled overnight with <sup>51</sup>Cr, washed, and preincubated with BCNU (100 μg/ml). The cells were washed again and then incubated with glucose (27 mM) and glucose oxidase (25 mU/ml). Catalase (3,000 U/ml) was added at various time points and aliquots of supernatant medium were sampled at 6 h for determination of specific <sup>51</sup>Cr-release. Values represent means of eight replicates ± 1 SE.



**Figure 6.** Effect of BCNU concentration on glucose-glucose oxidase-induced bovine endothelial  $^{51}\text{Cr}$ -release and glutathione reductase activity. Glutathione reductase activity and glucose-glucose oxidase-induced  $^{51}\text{Cr}$ -release were determined in bovine pulmonary artery cells of the same strain and passage following a 10-min incubation with varying concentrations of BCNU. Specific  $^{51}\text{Cr}$ -re-

lease (●) was determined after incubation with glucose (27 mM) and glucose oxidase (10 mU/ml) for 4 h. Glutathione reductase activity (○) was determined in Triton-solubilized cells. Values for  $^{51}\text{Cr}$ -release represent means of eight replicates  $\pm 1$  SE. The glutathione reductase levels are the average of duplicate 25-cm<sup>2</sup> flasks.

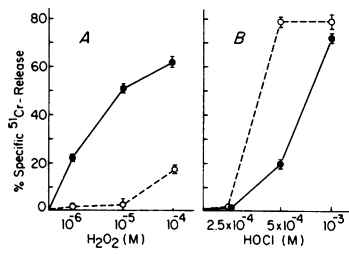
with DEM increased their susceptibility to glucose-glucose oxidase-mediated lysis (Fig. 9).

In concentrations up to 10 mM for 48 h, BSO was not toxic to human umbilical vein endothelial cells as assessed by phase-contrast microscopy and trypan blue dye exclusion.  $^{51}\text{Cr}$  uptake during overnight labeling and spontaneous release during a subsequent 6-h incubation were also unaffected by BSO (1 mM). In contrast to tumor cells where incubation with BSO produced >90% reduction in reduced glutathione levels (8, 11), incubation of human endothelial cells with BSO (1 mM) for 18 h produced only a 61% decrease in the soluble reduced sulfhydryl content ( $3.8 \pm 0.5$  nmol/ $10^6$  cells in BSO-treated cells compared to  $10.6 \pm 2.9$  nmol/ $10^6$  cells in control cells, means of three replicate flasks  $\pm 1$  SD). Increasing the concentration of BSO to 10 mM

**Table II.** Effect of BCNU on Bovine Endothelial Lysis by Nonoxidant Agents

Addition to endothelial monolayer	% Specific $^{51}\text{Cr}$ -release	
	Pretreatment with	
	Ethanol control	BCNU
Distilled water	28.3 $\pm$ 2.8	27.2 $\pm$ 1.1
A23187	54.4 $\pm$ 2.2	22.2 $\pm$ 2.2
LPS	5.1 $\pm$ 1.7	5.4 $\pm$ 1.1
Glucose-glucose oxidase	8.2 $\pm$ 1.0	31.2 $\pm$ 2.3

$^{51}\text{Cr}$ -labeled bovine aortic endothelial cell monolayers were preincubated for 10 min with BCNU (100  $\mu\text{g}/\text{ml}$ ) or ethanol control (0.1%). Specific  $^{51}\text{Cr}$ -release was determined after a 4-h incubation with distilled water, A23187 (50  $\mu\text{M}$ ), LPS (100  $\mu\text{g}/\text{ml}$ ), or glucose (27 mM)-glucose oxidase (4 mU/ml). Values represent the means of eight replicates  $\pm 1$  SE.

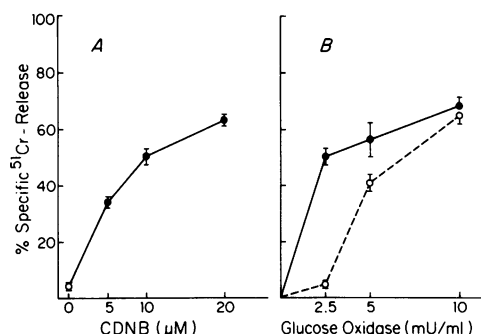


**Figure 7.** Specificity of BCNU for  $\text{H}_2\text{O}_2$ -mediated endothelial cell lysis. Bovine aortic endothelial cells of the same strain and passage were labeled overnight with  $^{51}\text{Cr}$ , washed, and preincubated for 10 min with 0.1% ethanol control (○) or BCNU (100  $\mu\text{g}/\text{ml}$ )

(●). The cells were washed again and then incubated with reagent  $\text{H}_2\text{O}_2$  (A) or HOCl (B). After a 4-h incubation, 50  $\mu\text{l}$  of supernatant medium were aspirated for determination of specific  $^{51}\text{Cr}$ -release. Values represent means of six replicates  $\pm 1$  SE.

did not cause further reduction in human endothelial reduced glutathione at 18 h. The failure of BSO to produce a greater depletion of human endothelial-reduced glutathione may be due to slower turnover of reduced glutathione in the confluent endothelial cells. Nevertheless, depletion of reduced glutathione by inhibition of its synthesis with BSO significantly increased human umbilical vein endothelial cell sensitivity to glucose-glucose oxidase-mediated lysis (Fig. 10).

**Role of glutathione redox cycle in the neutrophil-mediated lysis of endothelial cells.** PMA-activated neutrophils failed to induce significant  $^{51}\text{Cr}$ -release from untreated human or bovine endothelial monolayers. If the endothelial glutathione redox cycle was disrupted by pretreatment with BCNU, however, significant catalase-inhibitible lysis was observed (Table III). Depletion of endothelial reduced glutathione by inhibition of glutathione synthesis by BSO also significantly increased endothelial susceptibility to lysis by PMA-activated neutrophils (Fig. 11).



**Figure 8.** Effect of CDNB on glucose-glucose oxidase-induced bovine endothelial  $^{51}\text{Cr}$ -release. Bovine aortic endothelial cells of the same strain and passage were labeled overnight with  $^{51}\text{Cr}$ , washed, and preincubated for 30 min with ethanol control (0.1%) (○) or CDNB (●). Glucose (27 mM) and glucose oxidase were then added without further washing. In A the concentration of glucose oxidase was 2.5 mU/ml. In B the concentration of CDNB was 10  $\mu\text{M}$ . After 6 h, aliquots of supernatant medium were aspirated for determination of specific  $^{51}\text{Cr}$ -release. Values represent means of six replicates  $\pm 1$  SE.

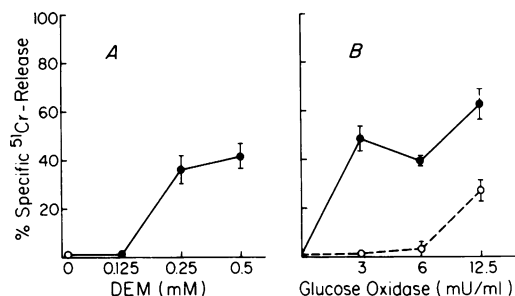


Figure 9. Effect of DEM on glucose-glucose oxidase-induced human endothelial  $^{51}\text{Cr}$ -release. Human umbilical vein endothelial cells from two different isolates (A and B) were labeled overnight with  $^{51}\text{Cr}$ , washed, and preincubated for 30 min with ethanol control (0.1%) (○) or DEM (●). Glucose (27 mM) and glucose oxidase were then added without further washing. In A the concentration of glucose oxidase was 25 mU/ml. In B the concentration of DEM was 0.5 mM. After 6 h, aliquots of supernatant medium were aspirated for determination of specific  $^{51}\text{Cr}$ -release. Values represent means of six replicates  $\pm$  1 SE.

## Discussion

In our previous studies of neutrophil-mediated endothelial cell injury we were unable to demonstrate significant human endothelial cell lysis by serum-treated zymosan-activated neutrophils (16). These results were in contrast to those of Sacks et al. (23) who observed small amounts of catalase-inhibitable  $^{51}\text{Cr}$ -release by complement- and zymosan-activated neutrophils, and Weiss et al. (24) who found significant catalase-inhibitable  $^{51}\text{Cr}$ -release with prolonged exposure to PMA-activated, but not zymosan-activated, neutrophils. These discrepancies in experimental results may relate in part to methodologic differences in the assays employed, but might also reflect differences in endogenous antioxidant defense mechanisms in cultured cells. Recently, Hoover et al. also failed to observe endothelial lysis by PMA-activated neutrophils and noted that neutrophil superoxide anion production was apparently reduced when neutrophils were in contact with endothelial monolayers, suggesting

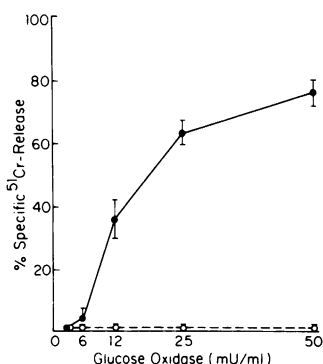


Figure 10. Effect of BSO on glucose-glucose oxidase-induced human endothelial cell  $^{51}\text{Cr}$ -release. Human umbilical vein endothelial cells were labeled overnight with  $^{51}\text{Cr}$  in 10% NBCS in Waymouth's medium with (●) or without (○) BSO (1 mM). The cells were then washed and 10% NBCS in Waymouth's medium with or without BSO (1 mM) was added, followed by glucose oxidase. After 4 h, aliquots of supernatant medium were removed for determination of specific  $^{51}\text{Cr}$ -release. Values represent means of six replicates  $\pm$  1 SE.

Table III. Effect of BCNU on Bovine and Human Endothelial Cell Lysis by PMA-activated Neutrophils

Addition to endothelial monolayer	% Specific $^{51}\text{Cr}$ -Release	
	Ethanol control	BCNU
<b>A Bovine pulmonary artery</b>		
PMN (8)	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1
PMN + PMA (8)	0.4 $\pm$ 0.1	15.1 $\pm$ 2.2
PMN + PMA + catalase (8)	0.1 $\pm$ 0.1	0.3 $\pm$ 0.2
<b>B Human umbilical vein</b>		
PMN (6)	4.6 $\pm$ 0.1	3.4 $\pm$ 0.1
PMN + PMA (6)	5.8 $\pm$ 0.1	19.6 $\pm$ 2.3
PMN + PMA + catalase (6)	0.9 $\pm$ 0.1	2.9 $\pm$ 0.2

$^{51}\text{Cr}$ -labeled bovine pulmonary artery (A), or human umbilical vein (B) endothelial cell monolayers were preincubated for 10 min with BCNU (100  $\mu\text{g}/\text{ml}$ ) or ethanol control (0.1%). 50  $\mu\text{l}$  of purified human neutrophils (PMN) in 10% NBCS in Waymouth's medium was then added, followed by 50  $\mu\text{l}$  of medium with or without PMA (10 ng/ml final). The neutrophil to endothelial cell ratio was  $\sim$ 25:1. Catalase (3,000 U/ml) was added to PMN before the addition of PMA. 50  $\mu\text{l}$  of cell-free supernatant medium was aspirated after 6 h of incubation for determination of specific  $^{51}\text{Cr}$ -release. Values represent the means of (n) replicates  $\pm$  1 SE.

some endogenous endothelial protective mechanism against neutrophil-mediated injury (25). The observations of Nathan et al. (1) and Arrick et al. (8) that tumor cell susceptibility to lysis by neutrophil-generated  $\text{H}_2\text{O}_2$  depended on endogenous glutathione reductase activity and glutathione levels suggested to us that the glutathione redox cycle might also be an important antioxidant defense mechanism in cultured endothelial cells and could account, in part, for our failure to detect endothelial cell lysis by activated neutrophils. Moreover, the glutathione

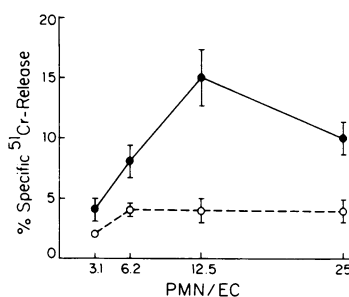


Figure 11. Effect of BSO on human endothelial  $^{51}\text{Cr}$ -release induced by PMA-activated neutrophils. Human umbilical vein endothelial cells were labeled overnight with  $^{51}\text{Cr}$  in 10% NBCS in Waymouth's medium with (●) or without (○) BSO (1 mM). The cells were then washed and 50  $\mu\text{l}$  of purified human peripheral blood neutrophils in 10% NBCS in Waymouth's medium was added at varying neutrophil (PMN) to endothelial cell (EC) ratios followed by 50  $\mu\text{l}$  of PMA (50 ng/ml final). After 6 h, aliquots of cell-free supernatant medium were aspirated for determination of specific  $^{51}\text{Cr}$ -release. Values represent means of six replicates  $\pm$  1 SE.

redox cycle may be important in preventing oxidant injury by other agents in addition to neutrophils.

The experiments presented in this report demonstrate that several strains of bovine aortic, bovine pulmonary artery, and human umbilical vein endothelial cells are relatively resistant to lysis by exogenously generated  $H_2O_2$ . If the glutathione redox cycle is disrupted by selective inhibition of endogenous glutathione reductase by BCNU, however, then enzymatically generated  $H_2O_2$  can induce endothelial cell lysis in a dose-dependent manner. Both glucose-glucose oxidase- and xanthine-xanthine oxidase-mediated lysis were catalase-inhibitable, indicating that  $H_2O_2$  or a  $H_2O_2$ -derived product was the actual mediator of cell lysis, as has been previously demonstrated in cultured endothelial cells (23, 24, 26).

The sensitivity of endothelial cell strains to  $H_2O_2$ -mediated lysis varied. Some human umbilical vein endothelial cell isolates were more readily lysed by glucose-glucose oxidase than others. Bovine endothelial cell strains also varied in their sensitivity to lysis by exogenously generated  $H_2O_2$ . For example, at the same concentration of glucose oxidase, significant lysis was observed in a strain of bovine aortic cells, whereas a strain of bovine pulmonary artery cells was unaffected (Table I). Although it is tempting to speculate that susceptibility to oxidant lysis might vary in endothelial cells derived from different vascular sites, it is important to consider that differences in sensitivity to  $H_2O_2$ -mediated injury in cultured endothelial cells might represent interanimal variation or result from repeated passage. These questions can be resolved only by comparing endothelial cells derived from different vascular sites in the same animal at similar passage.

The time course of  $H_2O_2$ -mediated endothelial lysis indicates that  $^{51}Cr$ -release is a relatively late event (5–6 h) in control cells and that lysis occurs earlier (3–4 h) when the glutathione redox cycle is disrupted by BCNU. Despite the fact that significant cell lysis was not observed after 2 h of incubation with glucose-glucose oxidase even in BCNU-treated cells, significant  $^{51}Cr$ -release was still noted at 6 h in cells exposed to the  $H_2O_2$ -generating system for only 2 h before the addition of catalase. This suggests that a lethal event may occur relatively early during exposure to enzymatically generated  $H_2O_2$ , but that it requires several hours to become manifest as  $^{51}Cr$ -release.

It seems unlikely that BCNU potentiation of  $H_2O_2$ -mediated endothelial cell lysis represents a nonspecific effect rather than selective inhibition of the glutathione redox cycle. BCNU pretreatment did not increase bovine endothelial cell susceptibility to lysis by nonoxidant toxic agents including distilled water, calcium ionophore, or LPS. Moreover, BCNU pretreatment did not potentiate lysis by reagent hypochlorous acid, a potent oxidizing agent generated by the myeloperoxidase system in neutrophils (27), whereas lysis by reagent  $H_2O_2$  was markedly enhanced in the same cells.

Further evidence that the glutathione redox cycle is specifically involved in protecting cultured endothelial cells against  $H_2O_2$ -mediated lysis is provided by the observation that depletion of cellular stores of reduced glutathione by reaction with the

electrophilic reagents DEM and CDNB and by inhibition of glutathione synthesis with BSO also increased endothelial cell susceptibility to lysis by extracellularly generated  $H_2O_2$ .

Even prolonged exposure to PMA-activated neutrophils did not produce significant lysis of control bovine and human endothelial cell monolayers at 6 h. Resistance to lysis by PMA-activated neutrophils in our assay appeared to be due, at least in part, to detoxification of  $H_2O_2$  by the glutathione redox cycle, since significant catalase-inhibitable  $^{51}Cr$ -release was observed when BSO- or BCNU-treated endothelial cells were exposed to PMA-activated neutrophils. Since  $H_2O_2$  production by neutrophils occurs primarily during an initial burst, the  $^{51}Cr$ -release observed at 6 h in BCNU- or BSO-treated cells following incubation with PMA-activated neutrophils may be analogous to the  $^{51}Cr$ -release noted at 6 h after addition of catalase to the glucose-glucose oxidase system at 1.5–2 h.

These studies demonstrate that interruption of one of the major endogenous antioxidant mechanisms, the glutathione redox cycle, significantly increases endothelial cell susceptibility to lysis by  $H_2O_2$  in vitro. The fact that disruption of the glutathione redox cycle potentiated  $H_2O_2$ -mediated lysis in primary passage human umbilical vein endothelial cells as well as the multiply passaged bovine aortic and pulmonary artery endothelial cells suggests that the glutathione redox cycle is a common mechanism for detoxifying  $H_2O_2$  in cultured endothelial cells. Further studies are required to determine the relative importance of endogenous catalase and whether simultaneous inhibition of catalase and the glutathione redox cycle would further increase endothelial susceptibility to lysis by enzymatically or neutrophil-generated  $H_2O_2$ .

Differences in levels of endogenous antioxidant enzymes in cultured endothelial cells may account for some of the discrepancies in experimental results observed in oxidant injury models in vitro (16, 23–26). More importantly, since the endothelium may frequently be exposed to oxygen products generated by inflammatory cells, toxic agents, or hyperoxia, endogenous antioxidant mechanisms such as the glutathione redox cycle may be critical in preventing or limiting vascular injury in vivo. (28)

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