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Endothelial Cell Heterogeneity: Antioxidant Profiles Determine Vulnerability to Oxidant Injury[1] (42652)

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Abstract. Human umbilical vein endothelial cells were more sensitive to hydrogen peroxide lysis than cow pulmonary artery endothelial cells. Conversely, activated neutrophils which utilize hydrogen peroxide-mediated cell cytotoxicity cell mechanisms were more toxic to the cow pulmonary artery cells. This discordance was not related to neutrophil adhesion to either cell type or cell passage number. The antioxidant profiles of the endothelial cells revealed that cow pulmonary artery cells were rich in catalase to consume bolus hydrogen peroxide presented to them, while human umbilical vein endothelial cells utilize glutathione peroxidase-linked mechanisms to detoxify a slower more sustained release of hydrogen peroxide generated by neutrophils. Endothelial cells from different species and sites may utilize diversified antioxidant protective mechanisms.

Neutrophil-mediated endothelial damage may occur in the clinical syndromes associated with pulmonary dysfunction during hemodialysis or as seen in the adult respiratory distress syndrome (1–3). Neutrophil-derived oxidants such as hydrogen peroxide have been implicated as the primary effector of endothelial cell lysis (4–6). Our laboratory has demonstrated that neutrophil-mediated endothelial injury was inhibited by superoxide dismutase and catalase and depends upon tight adhesion of the neutrophil to the endothelial cell (4). Other investigators have confirmed this basic observation and suggested additional injury may result from other neutrophil products such as hydroxyl radical species and neutrophil-derived proteases (7–9). Endothelial cells are not passive participants. Rather, they contain endogenous antioxidants which protect them from neutrophil-mediated damage. Specifically, Harlan *et al.* (10) have suggested that an intact glutathione redox cycle is critical for the maintenance of endothelial integrity in the face of a hydrogen peroxide oxidant challenge. Shingu *et al.* (11) demonstrated that human umbilical vein endothelial cells contain negligible amounts of catalase which correlates with their enhanced sensitivity to high concentrations of hydrogen peroxide. Hydrogen peroxide, in addition to inducing irreversible lytic damage, can also cause induction of endothelial intracellular calcium flux (12) and the alterations in the production of prostaglandins (13–15). Therefore the endothelial cell defense against hydrogen peroxide is fundamental in maintaining its responsiveness and may be critical for this cell's interaction with PMNs and platelets. There are variations in the reported susceptibility of endothelial cells to oxidants generated by PMNs or enzymatic systems. While it is generally agreed that prolonged exposure of endothelial cells to hydrogen peroxide induces lethal injury, discrepant results have been reported by various investigators which suggest that a complex balance between exogenous oxidants and endogenous antioxidants underlies these differences. Our serendipitous observation utilizing two endothelial cell types suggests that endothelial cell types are variable with respect to their utilization of various antioxidant defense systems. These results indicate that a cow pulmonary artery cell line relies heavily upon catalase to inactivate hydrogen peroxide while human umbilical vein endothelial cells rely on an intact glutathione redox cycle. The data also imply that neutrophil-mediated endothelial injury is best retarded by reduced glutathione-linked mechanisms.

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Materials and Methods. *Reagents.* Phorbol myristate acetate (PMA), glucose oxidase (Type V *Aspergillus niger*), superoxide dismutase (SOD, Type 1 bovine erythrocytes, 27–50 u/mg protein), catalase (bovine liver, 17,000 u/mg protein thymol free), dithionitrobenzoic acid (DTNB), 3-amino 1,2,4-triazole, dimethylsulfoxide, and hydrogen peroxide were obtained from Sigma Chemical Co. (St. Louis, MO). 1,3-Bis(chloroethyl)-1-nitrosourea (BCNU) was obtained from Bristol Myers (Evansville, IN). Sodium ^{51}Cr and ^{111}In oxine were obtained from Amersham Corp. (Arlington Heights, IL). Hanks' balanced salt solution (HBSS) was obtained from GIBCO Laboratories (Grand Island, NY).

Endothelial cell cultures. Human umbilical vein endothelial cells (HUEC) harvested from umbilical cords by collagenase digestion as previously described (16, 17), exhibiting von Willebrand factor antigen (vWF Ag) and Weibel–Palade bodies, were grown on 2-cm^2 24-well plates (Costar, Cambridge, MA), 100-mm culture dishes, or microtiter wells (Falcon 3070, Becton–Dickinson; Oxnard, CA) to confluence, and used as primary cultures. In some experiments HUEC were passaged three times before subculturing to the microtiter wells. Cow pulmonary artery cells (CPA), a generous gift of Dr. Una Ryan of the University of Miami, were maintained in the laboratory in M199 with 20% Ryan red media growth supplement. Both human and bovine cells exhibit von Willebrand factor antigen by immunofluorescence and Weibel–Palade bodies by transmission electron microscopy (7, 16, 17).

Preparation of PMNs. After receiving informed consent (following guidelines of the Committee on the Use of Human Subjects in Research of the University of Minnesota) 40-ml human volunteer blood samples were drawn into a plastic syringe containing 20 ml of hydroxyethylstarch (Hespan, American Hospital Supply Corp., Irvine, CA) and 200 units of preservative-free heparin (Upjohn, Kalamazoo, MI). The mixture was allowed to sediment at room temperature and the supernatant was collected and centrifuged at 400*g* for 5 min at 4°C. The pellet was resuspended in 0.2 ml ice-cold Hanks' balanced salt solution containing 100 mg% glucose. Residual erythrocytes were lysed in 15 ml of ice-cold water and after 25 sec, isotonicity was reconstituted by addition of 5 ml of 3.6% sodium chloride. This suspension was centrifuged in the cold at 400*g* for 5 min, and the pellet was resuspended in 5 ml of HBSS, carefully layered on top of Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) at 1.075 specific gravity, and then centrifuged at 20,000*g* for 30 min at 4°C. The resulting PMNs (greater than 95%) were washed once again, counted, and resuspended in 10 ml of HBSS. Viability was assessed by trypan blue exclusion and exceeded 95%.

Endothelial cytotoxicity assay. Confluent endothelial cells grown in microtiter wells were radiolabeled with 1 μCi sodium ^{51}Cr in serum-free Medium 199 (GIBCO) for 6 hr, washed four times with HBSS containing 25 m*M* Hepes, and then exposed to various concentrations of reagent hydrogen peroxide or glucose/glucose oxidase. In each experiment, HBSS alone or 10% H_2O_2 was added to assess spontaneous and maximal chromium release, respectively. The endothelial cells were incubated with various dilutions of reagent hydrogen peroxide for 90 min or with glucose (27 m*M* in HBSS)/glucose oxidase (10^{-6} to 10 u/ml) for 6 hr. Cells exposed to similar concentrations of boiled glucose oxidase served as controls. In similar studies, PMNs (5.0×10^6 in HBSS) were added to labeled endothelial cell monolayers in 2-cm^2 tissue culture wells. The PMNs were centrifuged onto the endothelial monolayers at 50*g* for 3 min to obtain maximum cell adhesion and then stimulated with PMA (100 ng/ml). In some experiments 10 μg/ml superoxide dismutase and/or 50 μg/ml catalase were added to the PMNs. The PMN-treated endothelial cell cultures were then incubated for 18 hr at 37° in a humidified atmosphere of 95% air, 5% CO_2. Following incubation in each cytotoxicity assay, the cells were pelleted (175*g* for 3 min) and 500-μl supernatant samples were removed for determination of the percentage of soluble ^{51}Cr release from the endothelial cells (Beckman Gamma 5500; Beckman Instruments, Irvine, CA). The percentage ^{51}Cr specific release from HUEC and cow pulmonary artery cells after

exposure to reagent H_2O_2, glucose/glucose oxidase, or activated PMNs was calculated using the following formula: (experimental cpm − HBSS (spontaneous cpm)) ÷ 10% H_2O_2 cpm (maximal release) − HBSS (spontaneous cpm)] × 100%. Spontaneous ^{51}Cr for the reagent H_2O_2 experiments averaged 6%, the glucose/glucose oxidase experiments 10%, and the PMN experiments 18%.

PMN adhesion to endothelial cell monolayers. ^{111}In-radiolabeled PMNs were added to human HUEC or CPA monolayers for 30 min and washed three times, and residual counts were quantified. Adherence is expressed as a percentage of total PMNs ^{111}In recoverable from each well (9). [A 30-min incubation time was chosen since adhesion was maximal. Longer incubation periods (>4 hr) resulted in diminished adherence due to cellular alterations *in vitro.*]

Catalase and glutathione levels. Endothelial cells were grown in 100-mm dishes to confluence. Cells were removed with a rubber policeman and sonicated. Catalase was quantified spectrophotometrically by assessing the capacity of endothelial cell lysates to reduce hydrogen peroxide compared with a standard curve using reagent catalase (18). Glutathione (GSH) levels were determined by titrating total sulfhydryl groups with dithionitrobenzoic acid (10). Levels of catalase and GSH are expressed per milligram endothelial cell protein as measured by the method of Lowry. Inhibition of glutathione reductase was accomplished by the addition of 10 μg of BCNU dissolved in 0.1% ethanol for 10 min prior to the cytotoxicity assay. In control experiments the ethanol vehicle in the same concentrations was added to the endothelial cells.

Inhibition of catalase was accomplished by 12-hr incubation of the endothelial cells with aminotriazole (50 m*M*) prior to the cytotoxicity assay. Such treatment did not in itself significantly reduce viability, as measured by trypan blue exclusion and spontaneous ^{51}Cr release.

H_2O_2 consumption by endothelial cells. Hydrogen peroxide consumption by HUEC or CPA by adding various concentrations of hydrogen peroxide was assayed spectrophotometrically as the timed disappearance of H_2O_2 (230 nm). In some experiments, endothelial cells were preincubated with aminotriazole as described above before performance of the H_2O_2 consumption assay.

Statistics. The data are presented as the means ± SE. Statistical analysis was performed by utilizing the Student *t* test.

Results. Bovine pulmonary artery endothelial cells exposed to reagent hydrogen peroxide were more resistant to lysis as measured by the specific ^{51}Cr release after 90 min incubation compared to similarly treated human umbilical vein endothelial cells. The addition of 0.18 nmol of hydrogen peroxide induced reproducibly measurable lysis of human umbilical vein endothelial cells, while the same degree of lysis was not seen until 180 nmol H_2O_2 for the cow pulmonary artery cells (Fig. 1A). Human umbilical vein endothelial cells demonstrated a 10-fold increase in sensitivity to hydrogen peroxide, compared to cow pulmonary artery cells, at every concentration studied until more than 50% of the cells were lysed. Similarly, enzymatically (glucose/glucose oxidase) generated hydrogen peroxide was 10- to 100-fold more toxic to human umbilical vein endothelial cells than similarly treated cow pulmonary artery cells (Fig. 1B). Such enzyme systems generate hydrogen peroxide continuously and are more efficient killers of all endothelial cell types as determined by ^{51}Cr release; glucose (27 m*M*) and glucose oxidase (1 unit/ml) produced 8.8 nmol of hydrogen peroxide in 6 hr. The apparent enhanced sensitivity of both cell types to continuously generated hydrogen peroxide may reflect an accumulative damaging effect on the endothelial cell metabolism over the 6-hr incubation period used in the enzymatic generation of H_2O_2. In both instances, catalase completely abolished ^{51}Cr leak from the endothelial cells (not shown).

Since we had previously shown that neutrophil-derived oxidants can damage endothelial cells, the susceptibility of human and bovine pulmonary endothelial cells in a neutrophil-mediated endothelial injury assay was then examined. As seen in Fig. 2A, neutrophils activated by phorbol myristate acetate injured both human umbilical vein and cow pulmonary artery endothelial cells but

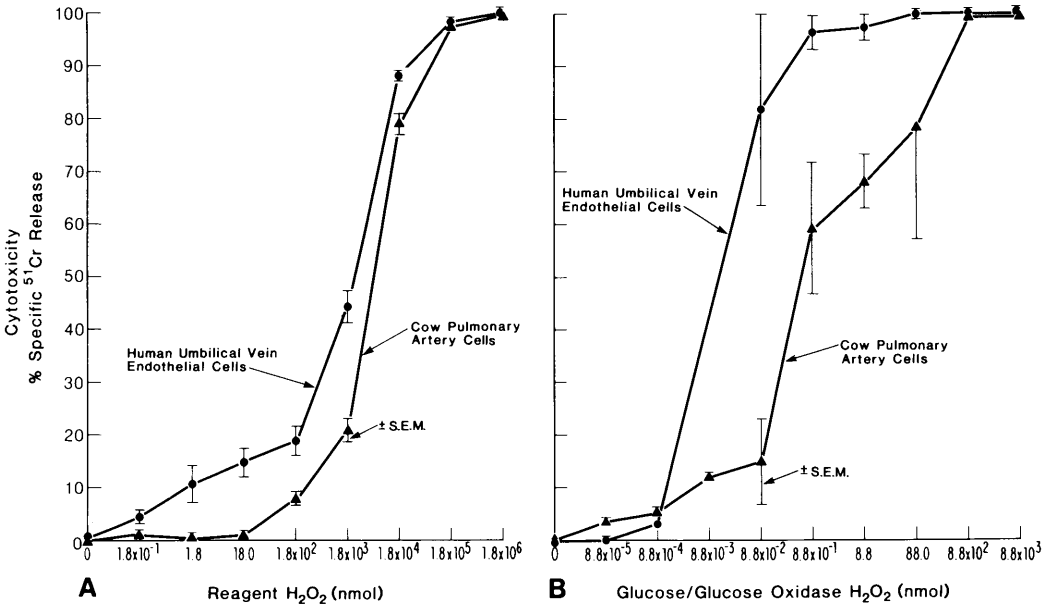

FIG. 1. Hydrogen peroxide-induced endothelial cell toxicity. Specific ^{51}Cr release from endothelial cells was determined as described under Materials and Methods after exposure of cells grown in microtiter wells to reagent H_2O_2 (A) or glucose and glucose oxidase (B). The results represent the means and SEM of 10 experiments done in sextuplicate (A) or 4 experiments done in triplicate (B).

in contrast to the bolus H_2O_2-induced damage, the cow pulmonary artery cells were significantly ($P < 0.05$) more sensitive to activated neutrophil killing than the human umbilical vein endothelial cells. This neutrophil-mediated endothelial damage was indeed due to neutrophil-derived oxidants since 10 μg/ml superoxide dismutase plus 50 μg/ml catalase completely abolished the ^{51}Cr leak from both cell types (not shown).

Since neutrophil-mediated endothelial injury is dependent on tight adhesion of the neutrophils to the endothelium, we measured whether the differential susceptibility of cytotoxicity of each endothelial cell type was related to alterations of PMN adhesion. As seen in Fig. 2B, neutrophils adhered equally well to both endothelial cell types, suggesting that differences between injury due to neutrophil-mediated mechanisms and that due to direct H_2O_2 exposure might be related to other mechanisms such as differential endogenous antioxidant mechanisms. It was demonstrated that vulnerability to H_2O_2 was not altered by passage in cell culture, since the second and third passaged human cells were identical to the primary human vein endothelial cells in their enhanced sensitivity to reagent H_2O_2 (not shown).

Intracellular concentrations of glutathione and catalase were measured in the human umbilical vein endothelial cells and the cow pulmonary artery endothelial cells. As seen in Table I, human umbilical vein endothelial cells contain threefold more glutathione than cow pulmonary artery cells, while cow pulmonary artery cells contain 2.5 times as much catalase as the human umbilical vein endothelial cells. This suggested that glutathione may be critical for the defense of human umbilical vein endothelial cells against oxidant damage while catalase was the primary defense utilized by the cow pulmonary artery cells. To further explore the concept that the differential cytotoxicity reflected the catalase-mediated consumption of hydrogen peroxide by cow pulmonary artery cells, H_2O_2 consumption was compared to that of the human umbilical vein endothe-

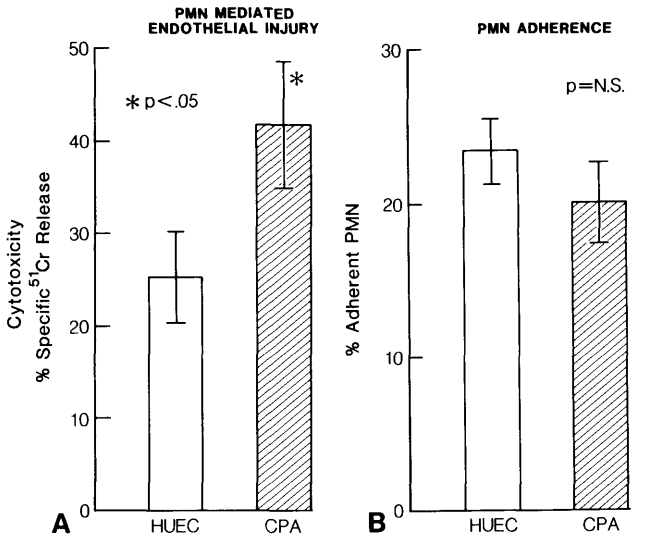

FIG. 2. Neutrophil (PMN)-induced specific endothelial cell injury and adherence. (A) Specific release of soluble ^{51}Cr from endothelial cells exposed to 5×10^6 PMN as described under Materials and Methods; $N = 4$ experiments run in triplicate. (B) Adhesion of ^{111}In-labeled PMN to endothelial cells described under Materials and Methods; $N = 4$ experiments in triplicate. Results depict the means and SEM.

lial cells. Cow pulmonary artery cells consumed hydrogen peroxide at a significantly greater rate than the human umbilical vein endothelial cells (Fig. 3), consistent with the greater catalase level in these. This increased H_2O_2 consumption was indeed due to catalase since aminotriazole, a noncompetitive inhibitor of catalase, inhibited the hydrogen peroxide consumption by both cell types.

Not only did aminotriazole inhibition of catalase reduce H_2O_2 consumption, but, as expected, it enhanced endothelial susceptibility to H_2O_2. Amino-1,2,4-triazole treatment increased the sensitivity of both cell types to hydrogen peroxide but the degree of left shift of the cytotoxicity curve was much greater for the cow pulmonary artery cells (Fig. 4A) than the human umbilical vein endothelial cells (Fig. 4B), supporting the enhanced dependence of the cow pulmonary artery cells on catalase as its primary protection against bolus H_2O_2.

TABLE I. ANTIOXIDANT LEVELS OF HUEC AND CPA

	GSH	Catalase
	(nmol/mg EC protein)	
CPA	15.7 ± 3.8	12.3 ± 3.6
HUEC	40.4 ± 9.0	5.2 ± 1.3
	$P < .05$	$P < .05$

Note. Glutathione (GSH) and catalase levels in endothelial cells were measured as described under Materials and Methods. Results reflect the means ± SEM of four separate experiments.

BCNU, an inhibitor of glutathione reductase, was added to both endothelial cell types to reduce the activity of the glutathione redox cycle. As predicted, BCNU-treated human endothelial cells became slightly more sensitive to hydrogen peroxide, but BCNU had virtually no effect on sensitivity of the cow pulmonary artery cells to peroxide (Fig. 5).

Discussion. Endothelial cells have variable responses and sensitivity to biologic oxidants whether generated by activated neutrophils or enzymatic systems or presented as a chemical reagent. One apparent explanation may relate to the differing cellular concentration of antioxidants. Heterogeneity of the endothelial response is reflected by the enhanced sensitivity of both human umbilical vein endothelial cells to either reagent hydrogen peroxide or enzymatically derived

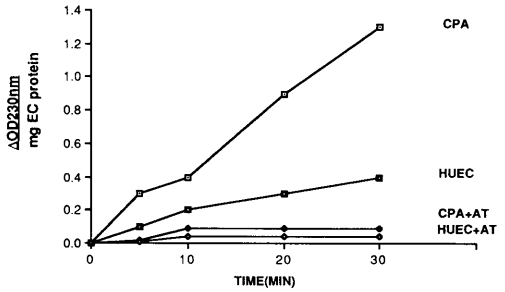

FIG. 3. Consumption of hydrogen peroxide by endothelial cells before and after treatment with 3-amino 1,2,4 triazole (AT) for 12 hr as described under Materials and Methods. Results are expressed as the changes in absorption at 230 nm/mg of endothelial cell protein.

hydrogen peroxide compared to cow pulmonary artery cells. In contrast, human umbilical vein endothelial cells were more resistant to neutrophil-mediated injury when compared with the bovine cells. This apparent discrepancy seems to correlate with the different antioxidant content of each cell type. Catalase-rich, glutathione-poor cow pulmonary artery cells are resistant to hydrogen peroxide and to neutrophil injury. Conversely, glutathione-rich, catalase-poor human umbilical vein endothelium are more easily injured by reagent (bolus) and enzymatically generated (continuous) peroxide lysis, while resisting neutrophil damage. Inhibitors of catalase rendered the bovine cells signifcantly more vulnerable to H_2O_2, while the glutathione reductase inhibitor, BCNU, made human endothelial cells more sensitive to neutrophil-mediated lysis (10, 19, 20).

Many laboratories have reported that neutrophil-derived oxidant species including H_2O_2, $OH^{\cdot}$, and iron radicals, can damage endothelial cells *in vitro* (5–7); however, hydrogen peroxide is essential for generation of these alternate chemical mediators and cellular cytotoxicity. Hydrogen peroxide exposure induces endothelial cell retraction (12), a change in intracellular calcium (12), and stimulation of prostaglandin production and release (13, 14), and alters pyrimidine nu-

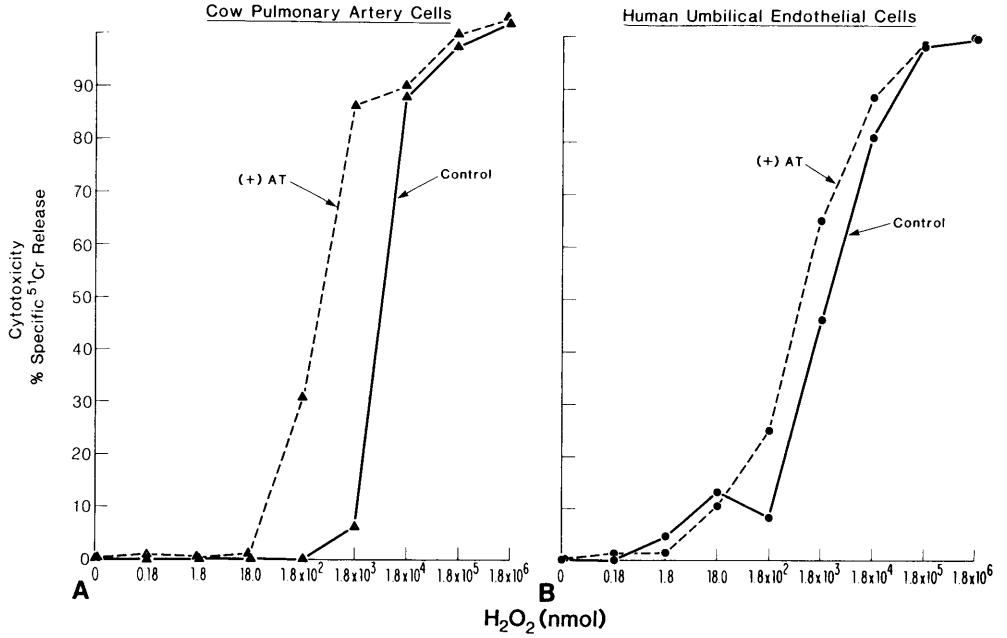

FIG. 4. 3-Amino 1,2,4 triazole (AT) inhibition of catalase increased vulnerability of cow pulmonary artery endothelial cells (A) more than human umbilical endothelial cells (B) to reagent H_2O_2. Cytotoxicity measured as described under Materials and Methods. The results are from one of those separate experiments performed in triplicate with simultaneous controls.

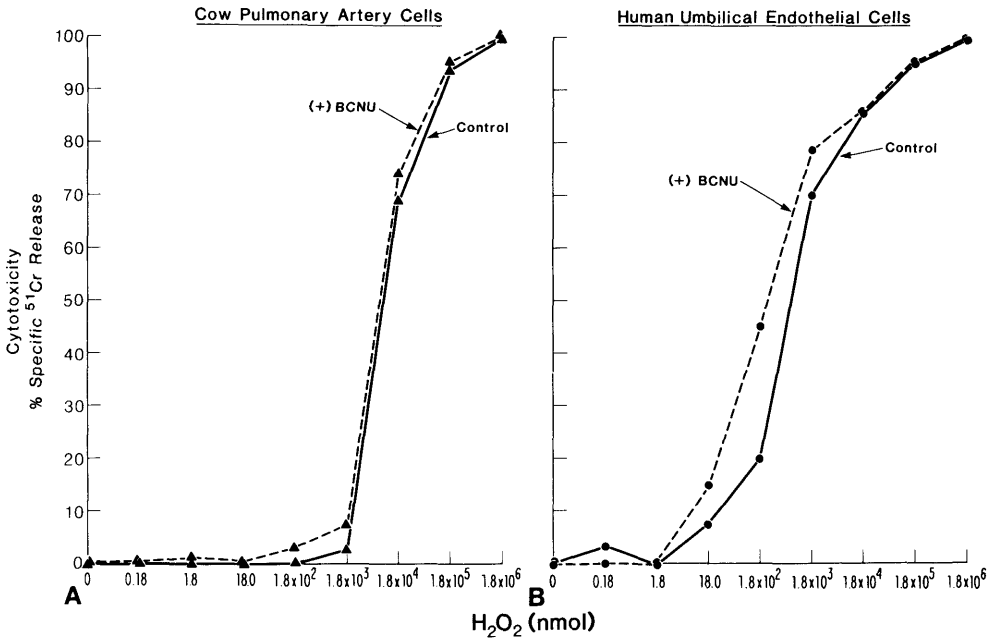

FIG. 5. BCNU-mediated inhibition of glutathione reductase increases sensitivity of HUEC (B) but not CPA (A) to H_2O_2. Cytotoxicity measured as described under Materials and Methods. The results are representative experimental data for one of three separate studies performed in triplicate with simultaneous controls.

cleotide levels (21). Finally, prolonged H_2O_2 treatment causes a decrease in cellular ATP with eventual nuclear fragmentation and cell death (22). Several investigators have stressed the importance of cellular antioxidants, primarily the glutathione redox system, in protecting endothelium from neutrophil-derived hydrogen peroxide (10, 19, 20). Depletion of glutathione stores in endothelium markedly enhances endothelial cell susceptibility to hydrogen peroxide, suggesting that endothelial damage occurs when the ability of the endothelium to degrade hydrogen peroxide is overwhelmed.

Endothelial antioxidant levels may also be affected by the composition of the tissue culture media used to grow the cells *in vitro*. For instance, high concentrations of reduced glutathione are present in RPMI (23). Since intracellular glutathione concentrations may be modified by the extracellular medium, we considered the possbility that our results were due to differences in growth conditions, but several control studies mitigate against this: Human umbilical vein endothelial cells grown in the same media as the cow pulmonary artery cells maintained increased sensitivity to bolus hydrogen peroxide. In addition, early and late passage cow pulmonary artery cells displayed equivalent sensitivity to hydrogen peroxide, suggesting that endothelial cell adaptation to culture conditions did not change oxidant sensitivity. Finally, all cells were labeled in the same medium for 6 hr before study to minimize culture-related differences.

Induction of antioxidant has been demonstrated *in vitro* upon exposure to oxidants in several cell types. For example, hyperoxia or lipopolysaccharide increases antioxidant levels in lung homogenates (24, 25). It is possible that the multiply-passaged cow pulmonary artery cells responded to this stress by evolving a new antioxidant profile. In our studies human umbilical vein endothelial cells passaged three times did not display an enhanced sensitivity to hydrogen peroxide when compared with primary cultures. The

cow pulmonary artery cells maintained endothelial morphology and von Willebrand factor antigen (by immunofluorescence). We also do not feel that the differences in pulmonary artery endothelial cells and human umbilical vein endothelial cells are simply due to species variation. Bovine *aortic* endothelium, like the pulmonary artery cells, had abundant catalase relative to human endothelial cells (13.5 u/mg protein), but unlike the cow pulmonary artery cells these aortic cells had a very high GSH level (23.2 nmol/mg protein). Of interest, these bovine *aortic* endothelial cells were resistant to low concentrations of hydrogen peroxide, consistent with their higher catalase levels.

Several factors must be considered when comparing results from endothelial cell physiology experiments. In the studies in which the neutrophil is the effector, Cronstein *et al.* (23) have emphasized the modulating role of adenosine and other media nutrients. In addition, modest amounts of lipopolysaccharide contaminating the tissue culture media or cultures could prime neutrophils (26), augmenting their ability to injure endothelium. Neutrophil preparation techniques, especially those permitting contamination with platelets or endotoxin, might also augment neutrophil-mediated endothelial cell cytotoxicity (27).

Our data support an important role for glutathione-linked pathways in protection of human endothelial cells against neutrophil-derived oxidants. Why neutrophil-derived oxidants need to be detoxified by glutathione more than by catalase is unclear. One possible explanation was suggested by Dobrina and Patriarca (28). Data in bovine microvascular cells indicated that the contribution of the glutathione cycle or catalase depends upon the rate of hydrogen peroxide generation. For example, at low rate $\times$ concentration products for hydrogen peroxide, the glutathione cycle accounted for greater than 75% of the hydrogen peroxide degradation, while at higher hydrogen peroxide rate concentration products the contribution of catalase became prominent. This seems in agreement with the lower affinity of catalase for hydrogen peroxide compared to glutathione peroxidase (28).

Our studies demonstrating enhanced degradation of bolus hydrogen peroxide by endothelial cells richer in catalase (Fig. 3) supports these observations. Furthermore, the differences between reagent- or enzyme-derived hydrogen peroxide and neutrophil-derived oxidants may also reflect contributions of other neutrophil prooxidative constituents (myeloperoxidase, lactoferrin, or hydroxyl radical) which might focus oxidant effects at the membrane. This would require altered antioxidant defense strategy in addition to the elimination of hydrogen peroxide.

In conclusion, these results clearly indicate that there is heterogeneity in antioxidant defense strategems utilized by different endothelial cells in culture. Studies *in vivo* comparing endothelial cytotoxicity should account for the endothelial cell's proactive contribution to these events.

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