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Tumor Necrosis Factor-α-mediated Decrease in Glutathione Increases the Sensitivity of Pulmonary Vascular Endothelial Cells to H_2O_2

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Abstract

We examined the effects of tumor necrosis factor-α (TNFα) stimulation of endothelial cells on the increase in endothelial permeability induced by H_2O_2. Bovine pulmonary microvascular endothelial cells (BPMVEC) were grown to confluence on a microporous filter and the ^{125}I-albumin clearance rate across the monolayer was determined. Pretreatment with TNFα (100 U/ml) for 6 h had no direct effect on transendothelial ^{125}I-albumin permeability. However, TNFα pretreatment enhanced the susceptibility of BPMVEC to H_2O_2; that is, H_2O_2 (10 μM) alone had no direct effect, whereas H_2O_2 increased ^{125}I-albumin permeability more than threefold when added to monolayers pretreated for 6 h with TNFα. Determination of lactate dehydrogenase release indicated that increased permeability was not due to cytolysis. We measured the intracellular contents of GSH and catalase to determine their possible role in mediating the increased susceptibility to H_2O_2. TNFα treatment (100 U/ml for 6 h) decreased total GSH content and concomitantly increased the oxidized GSH content, but did not alter the cellular catalase activity. The role of GSH was examined by pretreating endothelial cells with 2 mM GSH for 3 h, which produced an 80% increase in intracellular GSH content. GSH repletion inhibited the increased sensitivity of the TNFα-treated endothelial cells to H_2O_2. We tested the effects of xanthine oxidase (XO) inhibition since XO activation may be a source of oxidants responsible for the decrease in cellular GSH content. Pretreatment with 0.5 mM oxypurinol attenuated the synergistic effect of TNFα and H_2O_2 on endothelial permeability. The results indicate that decreased oxidant buffering capacity secondary to TNFα-induced reduction in intracellular GSH content mediates the increased susceptibility of endothelial cells to H_2O_2. This mechanism may contribute to oxidant-dependent vascular endothelial injury in septicemia associated with TNFα release. (*J. Clin. Invest.* 1992. 89:794–802.) Key words: endothelial permeability • oxygen free radicals • anti-oxidants • cytokines • vascular injury

Introduction

Tumor necrosis factor-α (TNFα)[1] is an important mediator of endotoxic shock and the associated high permeability pulmonary edema (1, 2). Infusion of human recombinant TNFα has been shown to induce a vascular "leak" syndrome in animal models (3, 4). There are two described pathways involved in TNFα-induced vascular endothelial injury: (*a*) direct effects of TNFα and of secondary mediators released by TNFα on endothelial cells, and (*b*) a neutrophil (PMN)-dependent pathway. In support of the first pathway, reports indicate that TNFα can directly increase endothelial permeability in vitro (3, 5–7) and in vivo (3). The TNFα-induced release of inflammatory mediators such as platelet activating factor (8), interleukin 1 (9), granulocyte-macrophage colony-stimulating factor (10), and possibly reactive oxygen species (11, 12) may contribute to the permeability-increasing effect of TNFα. The second pathway involving PMN (13–16) may be the result of TNFα-induced augmentation of PMN activation, resulting in the release of oxygen free radicals (17) and arachidonic acid metabolites (18). The released oxidants, in particular H_2O_2, can directly increase vascular endothelial permeability (19). TNFα can also mediate PMN adhesion to endothelial cells by increasing the expression of adhesion molecules (20, 21) and thereby promote cell–cell contact and enhance PMN activation (22).

Recent studies have suggested a third potentially important mechanism involving TNFα-mediated increase in the susceptibility of vascular endothelial cells to oxidants (23, 24). The possibility has been raised that TNFα can interfere with the intracellular oxidant buffering capacity such that cells become more sensitive to oxidant-mediated injury (25, 26). Moreover, we have recently shown that TNF$_\alpha$ augmented PMN-mediated endothelial injury (26a), which could be ascribed to an effect of TNF$_\alpha$ on endothelial anti-oxidants. In this study we examined whether such a mechanism contributed to the increase in endothelial permeability in response to oxidant exposure. We measured the alterations in permeability of TNFα-treated bovine pulmonary microvascular endothelial cells in response to H_2O_2 and the roles of GSH and catalase, the primary intracellular antioxidant defenses against H_2O_2.

Methods

Reagents. DME, HBSS, and fetal bovine serum (FBS) were purchased from Gibco Laboratories, Grand Island, NY. BSA (Fraction V), Hepes, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), NADPH, glutathione reductase, 4-vinylpyridine, hydrogen peroxide (30% solution), GSH, and

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[1] *Abbreviations used in this paper:* BPMVEC, bovine pulmonary microvascular endothelial cells; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); FBS, fetal bovine serum; GSSG, oxidized glutathione; LDH, lactate dehydrogenase; TNFα, tumor necrosis factor-α; XO, xanthine oxidase.

oxypurinol were purchased from Sigma Chemical Co., St. Louis, MO. ^{125}I was obtained from New England Nuclear, Boston, MA.

Pulmonary microvascular endothelial cells. Bovine pulmonary microvascular endothelial cells (BPMVEC) were isolated using the technique described previously (27). Briefly, tissue from the periphery of bovine lung was minced, exposed to collagenase, filtered, centrifuged, and resuspended in DME containing 20% FBS. After several days of incubation, colonies were selected based on uniform morphology and isolated with a cloning ring. The cells were confirmed to be endothelial in origin by the presence of Factor VIII–related antigen and incorporation of acylated LDL. The endothelial cells were harvested at 18–24 population doublings using 0.025% trypsin and centrifuged at 100 *g* for 5 min. The cells were resuspended in culture media at 2×10^5 cells/ml and seeded as described below.

Preparation of monolayers on filters and permeability assay. Polycarbonate microporous membrane filters (13 mm diam, 0.8-μm pore size; Nucleopore Corp., Pleasanton, CA) were coated with gelatin (type III calf skin gelatin; Sigma Chemical Co.) as previously described (28) and mounted on the bottom of plastic cylinders (9 mm i.d.; Adaps, Dedham, MA). These cylinders were suspended in 12-well culture plates, sterilized by ultraviolet light for 24 h, and coated with 30 μg/ml of ovine fibronectin. Endothelial cells were seeded with 0.5 ml of cell suspension at a density of 2.0×10^5 cells/ml and cultured for 4 d in 5% CO_2 at 37°C to allow the cells to grow to confluency.

The system for determining transendothelial ^{125}I-albumin flux has been described by us (29). Culture medium in the upper chamber (monolayer mounted cylinder) was replaced with 600 μl of HBSS containing 0.5% BSA and 20 mM Hepes (medium A) containing tracer ^{125}I-labeled albumin. The upper chamber was floated by means of a styrofoam collar in a larger lower chamber filled with 25 ml of medium A. The lower chamber was stirred continuously for complete mixing and the whole system was kept in a water bath at a constant temperature of 37°C. After the addition of different concentration of H_2O_2 solution in 50 μl of medium A to the upper compartment, samples were taken from the lower chamber every 5 min for 60 min. The radioactivity of the samples was measured in a gamma counter and transendothelial clearance rates of ^{125}I-albumin were calculated by weighted least-squares nonlinear regression (BMDP Statistical Software, Berkeley, CA) (29). The clearance rates were corrected for differences in free-to-bound ^{125}I ratios by determination of free ^{125}I concentrations using trichloroacetic acid precipitation.

Treatment of endothelial monolayers with TNFα. Recombinant human TNFα (Cetus Corp., Emeryville, CA) with a specific bioactivity of 25×10^6 U/mg protein was used. Endotoxin contamination was 0.05 ng/ml by a limulus amebocyte lysate assay. This level had no effect on the cellular parameters measured in this study. Confluent monolayers in DME containing 20% FBS were treated with TNFα in 50 μl DME to give a final concentration of 0, 10^2, 10^3, or 10^4 U/ml, and then incubated at 37°C for periods of 1, 3, or 6 h.

In some studies heat-inactivated (90°C, 20 min) TNFα was used to exclude the possible effects of contaminating endotoxin on endothelial permeability. Furthermore, neutralizing polyclonal rabbit anti-human TNFα antibody (gift of Dr. Mary E. Gerritsen, Miles Laboratories, New Haven, CT) or an equivalent concentration of nonrelevant control rabbit IgG (Calbiochem Corp., La Jolla, CA) was used to confirm that the observed effects were due to TNFα.

In some experiments, 2 mM reduced GSH was added to endothelial cells as described (30) at 0, 3, or 6 h before the permeability assay in order to increase the intracellular GSH concentration. In other experiments, the xanthine oxidase (XO) inhibitor, oxypurinol (0.5 mM), was added to monolayers 30 min before the application of TNFα. Oxypurinol at 0.5 mM inhibited the XO activity in BPMVEC (control XO activity was 1.40±0.16 nmol/min per 2×10^6 cells and the 6-h post-oxypurinol value was undetectable) as measured using the assay of Terada et al. (31).

Glutathione and catalase assays. We measured intracellular contents of GSH and catalase in BPMVEC after treatment with TNFα to determine possible alterations in these antioxidants. Endothelial cells were seeded onto six-well plastic tissue culture plates coated with fibronectin. Confluent monolayers were incubated for 1, 3, or 6 h with different concentrations of TNFα diluted into the culture medium. After incubation, monolayers were washed twice with PBS and lysed with 1% Triton X-100.

To assay total GSH (i.e., the sum of reduced GSH and oxidized GSH [GSSG]), 100 μl cell lysate was incubated at 30°C with 800 μl of 0.3 mM NADPH, 125 mM sodium phosphate buffer with 6.3 mM EDTA, pH 7.5, and 100 μl of 6 mM DTNB (32). After addition of 20 μl of 25 U/ml GSH reductase, the change in optical density at 412 nm was measured. To measure GSSG, GSH in samples was derivatized by adding 2 μl of 20 mM 4-vinylpyridine per 125 μl solution and mixing vigorously for 1 min. GSSG was measured in the same manner as GSH (32). The concentration of GSH was calculated as the difference between total GSH and GSSG. In parallel experiments, the number of endothelial cells was determined in order to express the results as the amount of GSH (nanomoles) per 10^6 cells. In some experiments, the GSH and GSSH assays were carried out after exposure of endothelial cells to 2 mM GSH in culture medium for 1, 2, 3, or 6 h.

Catalase activity was determined using the assay described by Beers and Sizer (33). In a spectrophotometer cuvette, 1.9 ml reagent grade water and 1 ml of substrate solution consisting of 59 mM H_2O_2 in 0.05 M potassium phosphate (pH 7.0) were added. The cuvette was incubated in spectrophotometer for 5 min, and 0.1 ml of the cell lysate was added. The decrease in absorbance at 240 nm was recorded for 2–3 min.

Lactate dehydrogenase. Lactate dehydrogenase (LDH) release from BPMVEC was determined to assess whether the permeability-increasing effects of TNFα were due to cytolysis. Endothelial monolayers were plated on six-well culture plates as above. The culture medium was removed after incubation with TNFα in DME containing 20% FBS for 6 h. Some monolayers were washed twice with PBS and reincubated for 1 h in HBSS with or without 10 μM H_2O_2. LDH activity was assayed in the culture media after 6 h incubation and after further 1 h incubation using an LDH assay kit (LD-L20; Sigma Diagnostics, St. Louis, MO). Released LDH was expressed as a percentage of total cellular LDH, which was determined after cell lysis with 1% Triton X-100. Values of LDH released into medium were corrected by subtracting the baseline LDH activity.

Morphologic analysis. Changes in the actin microfilament cytoskeleton of endothelial monolayers grown on filters were assessed using the rhodamine phalloidin stain (Molecular Probes, Inc., Eugene, OR) as described (34). Monolayers were examined and photographed using a fluorescence microscope equipped with epi-illumination (Nikon Optiphot; Nikon, Garden City, NY).

Statistics. Differences between two group means were compared by *t* test. Multigroup comparisons were made by one-way analysis of variance.

Results

Effects of TNFα on endothelial permeability. Treatment of BPMVEC monolayers with TNFα (10^2–10^4 U/ml) for 6 h increased the ^{125}I-albumin transendothelial clearance rates (the measure of transendothelial albumin permeability) in a concentration-dependent manner (Fig. 1). The lowest concentration of TNFα (10^2 U/ml), which we subsequently used for all studies reported below, had no independent effect on permeability. Monolayers exposed to 10^3 or 10^4 U/ml of TNFα, however, showed significant increases in permeability within 1 h of challenge and in a time-dependent manner until 6 h (data not shown).

Addition of H_2O_2 (30–1,000 μM) to endothelial monolayers resulted in concentration-dependent increases in ^{125}I-albumin permeability (Fig. 2). However, pretreatment with 100 U/ml of TNFα for 6 h (which had no direct effect on permeabil-

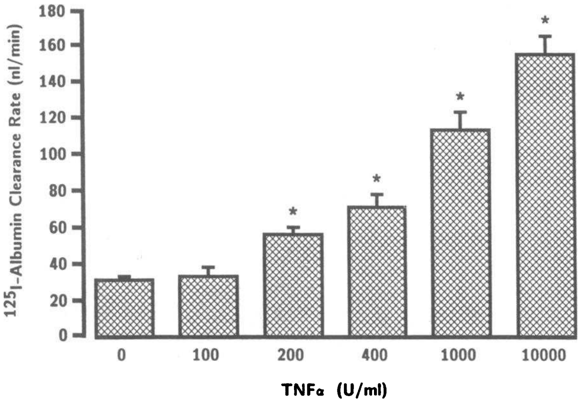


Figure 1. Effect of TNFα on transendothelial ^{125}I-albumin clearance rate across the bovine pulmonary microvascular endothelial monolayers. Albumin clearance rates were measured for 1 h after a 6-h incubation with various concentrations of TNFα. Values are means±SE; $n = 8$ in each group. $^*P < 0.05$ compared with control.

ity itself, as shown in Fig. 1) significantly augmented the sensitivity of BPMVEC monolayers to H_2O_2. 10 μM H_2O_2, a concentration with no direct permeability-increasing effect, increased ^{125}I-albumin transendothelial clearance rate more than threefold (Fig. 2). The increased sensitivity to H_2O_2 was not observed in monolayers treated with buffer (Fig. 2). The permeability-enhancing effect of TNFα pretreatment was not evident after 1 h treatment with 100 U/ml TNFα; the effect became apparent only after 3 h and was augmented further after a 6-h TNFα treatment period (Fig. 3). Heat-inactivated TNFα had no permeability-enhancing effect (Fig. 4). Anti-TNFα antibody abolished the permeability-enhancing effect of TNFα, but this did not occur with a control IgG (Fig. 4).

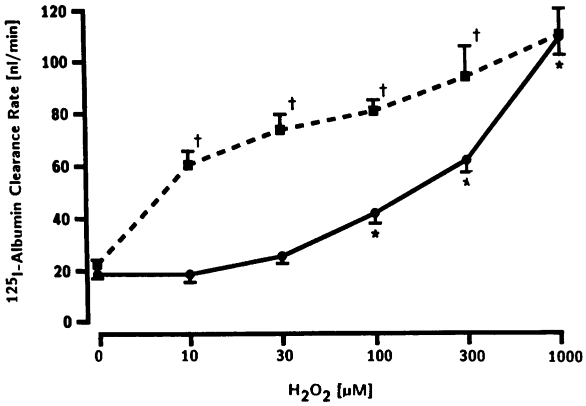


Figure 2. Effect of TNFα on endothelial susceptibility to H_2O_2-induced increase in permeability. Bovine pulmonary microvascular endothelial monolayers were preincubated with (■) or without (●) 100 U/ml TNFα for 6 h and the transendothelial ^{125}I-albumin clearance rates were measured for 1 h after addition of varying concentrations of H_2O_2. H_2O_2 increased ^{125}I-albumin permeability in control monolayers without TNFα pretreatment in a dose-dependent manner (*solid line*). Pretreatment with TNFα significantly enhanced the response to H_2O_2 (*dashed line*). Values are means±SE; $n = 8$ in each group. $^*P < 0.05$ compared with 0 μM of H_2O_2. $^\dagger P < 0.05$ compared with control monolayers without TNFα.

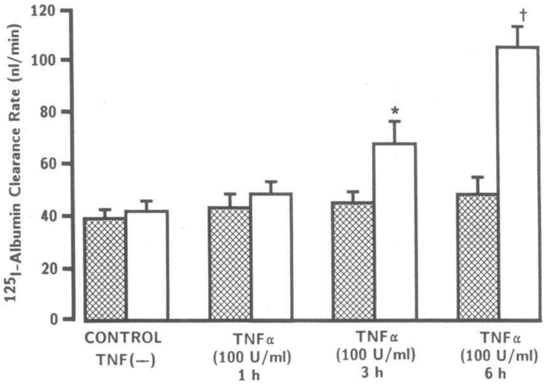


Figure 3. Time course of the "priming" effect of TNFα on endothelial sensitivity to H_2O_2-induced increase in permeability. Bovine pulmonary microvascular endothelial monolayers were preincubated with (*open bars*) or without (*cross-hatched bars*) 100 U/ml of TNFα for 1, 3, or 6 h. The transendothelial ^{125}I-albumin clearance rates were measured after addition or no addition of 10 μM H_2O_2. Values are mean±SE; $n = 8$ in each group. $^*P < 0.05$ and $^\dagger P < 0.01$ compared with monolayers without H_2O_2.

Cytotoxicity assay of TNFα and H_2O_2. Treatment of BPMVEC with TNFα (10^2, 10^3, 10^4 U/ml) for 6 h and treatment with 10 μM H_2O_2 for 1 h after the 100 U/ml TNFα for 6 h did not increase LDH release (Table I). This finding indicates that the increase in endothelial permeability was not due to a cytolytic effect of TNFα when combined with H_2O_2. Monolayer cell numbers were also not altered by these interventions.

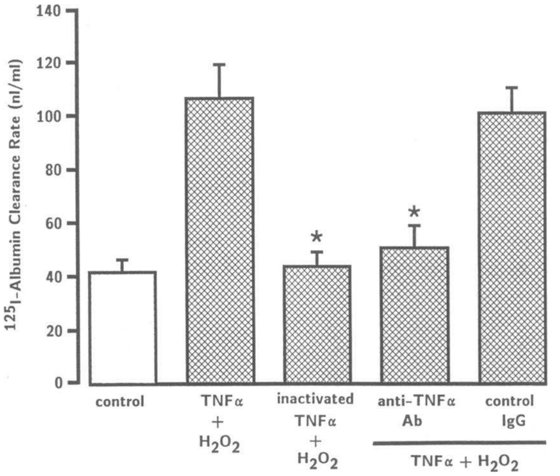


Figure 4. Effect of anti-TNFα antibody and heat-inactivated TNFα on the permeability-enhancing effect. Polyclonal anti-TNFα antibody was preincubated with TNFα for 30 min and then applied to endothelial monolayers. An equivalent concentration of nonrelevant rabbit IgG was used as a control protein. Heat-inactivated TNFα (100°C, 20 min) was also used instead of TNFα. Endothelial monolayers were incubated for 6 h with TNFα (100 U/ml), a combination of TNFα and anti-TNFα antibody, or heat-inactivated TNFα. The medium was then replaced with buffer. The transendothelial ^{125}I-albumin clearance rates were measured after addition of 10 μM H_2O_2. Values are means±SE; $n = 8$ in each group. $^*P < 0.01$ decreased when compared with the TNFα + H_2O_2 group.

Table I. LDH Release from Pulmonary Microvascular Endothelial Cells

Treatment	%LDH*
TNFα (U/ml) for 6 h‡	
0	6.8±1.1
100	6.5±0.7
1,000	5.9±0.8
10,000	6.5±1.6
H_2O_2 treatment for 1 h§	
Control cells + H_2O_2, 0 μM	1.0±0.5
Control cells + H_2O_2, 10 μM	0.8±0.3
TNFα pretreated cells + H_2O_2, 0 μM	0.7±0.4
TNFα pretreated cells + H_2O_2, 10 μM	1.2±0.4

Values are means±SE; $n = 4$.

* %LDH = (LDH in medium/[LDH in medium + LDH in cell lysate]) × 100.

‡ BPMVEC were incubated with various concentrations of TNFα in DME with 20% FBS. Values were corrected by subtraction of baseline LDH activity in the culture medium.

§ BPMVEC were washed with PBS after incubation with or without TNFα (100 U/ml) for 6 h, and then further incubated with or without H_2O_2 (10 μM) in HBSS for 1 h.

Changes in GSH and catalase. Treatment with TNFα (10^2–10^4 U/ml) for 6 h significantly decreased intracellular GSH content in a concentration-dependent manner (Table II). The decrease in GSH was associated with an increase in GSSG such that the GSSG/GSH ratio increased significantly after TNFα treatment. The effect of TNFα was time dependent; that is, treatment with 100 U/ml TNFα for 1 h showed no change in either GSH or GSSH content, 3 h TNFα treatment showed a small decrease ($P < 0.05$) in GSH, which became more pronounced after 6 h TNFα treatment and was accompanied by an increase in GSSH (Table II). GSSG concentration in the culture medium was also increased from 0.12±0.02 (control) to 0.17±0.02 nmol/ml after TNFα treatment (100 U/ml, 6 h) (mean±SE; $P < 0.05$). In contrast, the BPMVEC catalase activity was not significantly altered at any time point within 6 h after TNFα treatment (Table III).

Table II. Changes in Intracellular Glutathione Content after Exposure to TNFα

TNFα		GSH	GSSG	GSSG/GSH ratio
U/ml		*nmol/10⁶ cells*		
Control		6.20±0.16	0.25±0.03	0.040±0.004
100	1 h	6.22±0.20	0.26±0.03	0.041±0.005
100	3 h	5.58±0.16*	0.27±0.02	0.050±0.005*
100	6 h	5.25±0.26*	0.30±0.03*	0.059±0.007*
1,000	6 h	4.71±0.38*	0.34±0.02*	0.078±0.009*
10,000	6 h	4.28±0.25*	0.36±0.03*	0.086±0.008*

Values are means±SE; n = 8–12 per group. BPMVEC were incubated with various concentrations of TNFα in culture medium.

* Increased or decreased from control; $P < 0.05$.

Table III. Catalase Activity in BPMVEC after 6-h Treatment with TNFα

TNFα *U/ml*	0	100	1,000	10,000
Catalase (U/10⁶ cells)	6.09±0.15	6.37±0.20	6.08±0.18	5.85±0.12

Values are means±SE; n = 6.

Effect of exogenous GSH on intracellular GSH and GSSG. Intracellular total GSH content increased in a time-dependent manner after addition of 2 mM reduced GSH to the culture medium, and the GSH value reached a plateau at twice the control value by 3 h (Fig. 5). The increase in intracellular GSH content was not influenced by the presence of 100 U/ml TNFα in the culture medium (Fig. 5). Changes in GSH and GSSG contents and GSSG/GSH ratios are shown in Table IV. The increase in cellular GSH content was accompanied by an increase in the GSSG content after treatment with exogenous GSH (Table IV).

Effect of exogenous GSH on endothelial permeability. We examined the effects of GSH supplementation on the H_2O_2-induced increase in permeability of TNFα-pretreated endothelial monolayers. GSH was added to BPMVEC culture media at the beginning, at the halfway point (i.e., at 3 h), or at the end of 6 h TNFα treatment, and was coincubated for 6, 3, or 0 h, respectively. The 3-h treatment with GSH (which doubled the intracellular GSH content [as shown in Fig. 5]) significantly reduced the rise in ^{125}I-albumin permeability mediated by the combination of TNFα and H_2O_2 regimen (Fig. 6). A 6-h period of GSH incubation was as protective as the 3-h GSH incubation period (Fig. 6), which is consistent with 3- and 6-h GSH treatment periods producing the same increases in intracellular GSH content (Fig. 5). Treatment with GSH alone for 6 h had no effect on baseline permeability. In the control group (0 h), GSH was added and immediately removed when the 6 h TNFα treatment period ended, and the cells were then challenged with

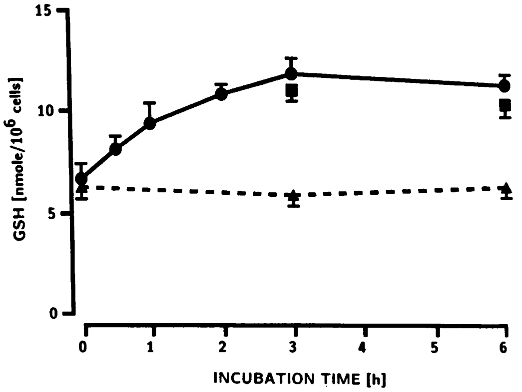


Figure 5. Effect of exogenous GSH on intracellular total GSH content in pulmonary microvascular endothelial cells. Monolayers were incubated with 2 mM GSH in the presence (*squares*) or absence (*circles*) of TNFα (100 U/ml) for 0, 1, 2, 3, or 6 h. Baseline values of GSH in untreated cells showed no change (*triangles*). Values are mean±SE; n = 4–6 in each group.

Table IV. Changes in GSH and GSSG Content after Treatment with Exogenous GSH and TNFα

	GSH	GSSG	GSSG/GSH ratio
	nmol/10⁶ cells		
Control	6.09±0.25	0.15±0.06	0.024±0.004
GSH 3 h	10.7±0.75*	0.38±0.08*	0.034±0.008
GSH 6 h	10.3±0.58*	0.33±0.10*	0.032±0.012
TNFα 6 h	5.43±0.20‡	0.28±0.08*	0.049±0.010*
TNFα 6 h§ + GSH 3 h	10.0±0.94*	0.35±0.11*	0.035±0.011
TNFα 6 h§ + GSH 6 h	10.8±1.15*	0.40±0.16*	0.037±0.013

Values are mean±SE; $n = 6$ per group. Exogenous GSH concentration was 2 mM. TNFα concentration was 100 U/ml.

* Increased from control; $P < 0.05$.

‡ Decreased from control; $P < 0.05$.

§ Endothelial cells were treated with TNFα and GSH at the same time.

Table V. Effect of Oxypurinol on TNFα-induced Changes of GSH and GSSG in Endothelial Cells

	GSH	GSSG	GSSG/GSH ratio
	nmol/10⁶ cells		
Control	5.96±0.13	0.15±0.03	0.025±0.006
TNFα	4.80±0.31*	0.26±0.02*	0.054±0.006*
Oxypurinol	6.21±0.27	0.25±0.05*	0.040±0.010*
Oxypurinol + TNFα	6.18±0.26	0.24±0.04*	0.039±0.008*

Endothelial cells were incubated with or without TNFα (100 U/ml) for 6 h. 0.5 mM oxypurinol was added 30 min before the 6-h incubation. Values are means±SE; $n = 6$.

* $P < 0.05$ compared with control.

H_2O_2. This short-term GSH incubation period had no protective effect (Fig. 6), excluding the possibility that residual extracellular contamination with GSH was responsible for the protective effect of GSH repletion.

Effect of oxypurinol on intracellular GSH and GSSG. Oxypurinol treatment (0.5 mM; 6 h) slightly increased intracellular GSH and GSSG as compared with the control value (Table V). The GSSG/GSH ratio also increased. Addition of oxypurinol 30 min before TNFα treatment prevented the decrease in GSH content induced by the 6-h TNFα treatment period (Table V).

Effect of oxypurinol on permeability. Treatment of BPMVEC with 0.5 mM oxypurinol 30 min before addition of 100 U/ml TNFα prevented the synergistic effect of the TNFα and H_2O_2 regimen in increasing transendothelial ^{125}I-albumin permeability (Fig. 7). Treatment with oxypurinol alone had no effect on baseline permeability values. Oxypurinol added at the end of the TNFα treatment period had no protective effect, excluding the possibility that residual oxypurinol directly interfered with the H_2O_2 effect.

Morphological changes. Control cells showed characteristic peripheral bands and close cell–cell contact (Fig. 8 *A*). Neither TNFα (100 U/ml, 6 h) nor H_2O_2 (10 μM, 1 h) treatments caused significant change in this pattern. TNFα treatment and subsequent H_2O_2 challenge resulted in the development of randomly oriented stress fibers, disappearance of peripheral bands, cell retraction, and intercellular gaps (Fig. 8 *B*). Addition of oxypurinol 30 min before TNFα treatment prevented the changes caused by the combination of TNFα and H_2O_2 (Fig. 8 *C*).

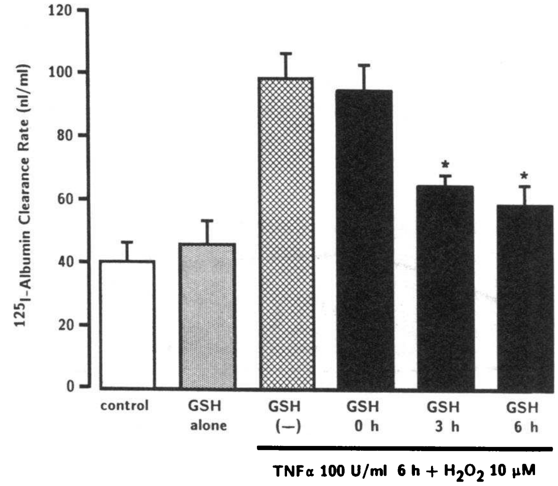


Figure 6. Effect of exogenous GSH on increased endothelial permeability induced by a combination of TNFα (100 U/ml for 6 h) and subsequent H_2O_2 (10 μM). 2 mM GSH was added to the medium at the beginning, halfway point, or end of 6-h TNFα treatment and coincubated for 6, 3, or 0 h, respectively. The medium was then replaced with buffer. The transendothelial ^{125}I-albumin clearance rates were measured after addition of H_2O_2. Values are means±SE; $n = 8$ in each group. *$P < 0.05$ decreased when compared with TNFα and H_2O_2 treated group without GSH.

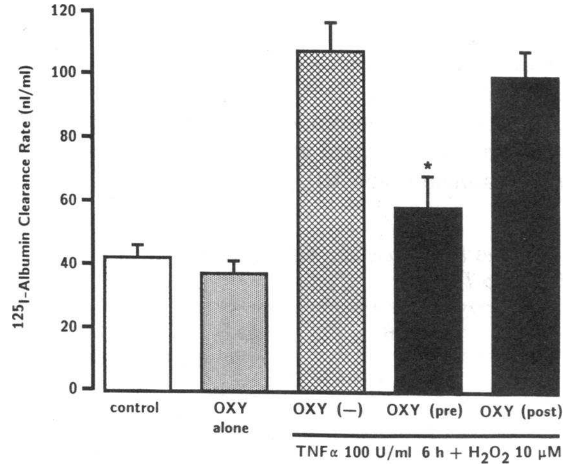
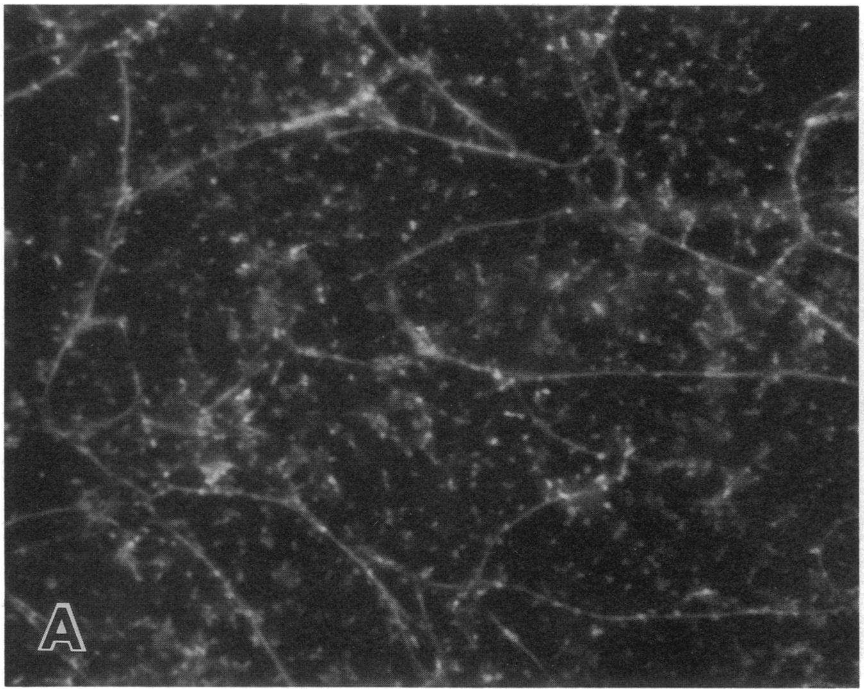


Figure 7. Effect of oxypurinol on increased endothelial permeability induced by a combination of TNFα and subsequent H_2O_2. Oxypurinol was added to the medium 30 min before (*pre*) or at the end (*post*) of TNFα treatment (100 U/ml for 6 h). The transendothelial ^{125}I-albumin clearance rates were measured after addition of 10 μM H_2O_2. Values are means±SE; $n = 8$ in each group. *$P < 0.05$ significantly decreased when compared with the TNFα- and H_2O_2-treated groups without oxypurinol.



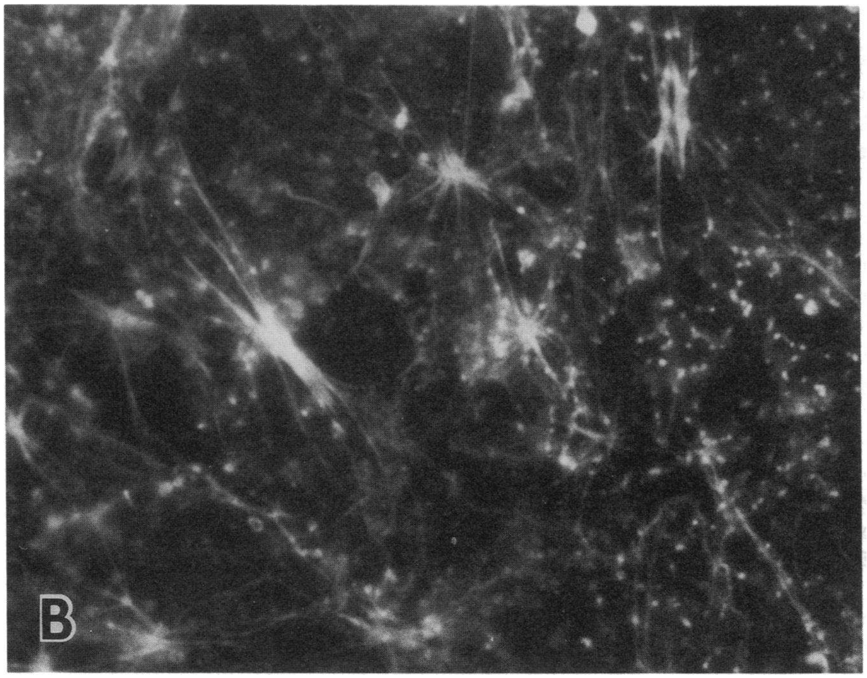


Figure 8. Morphological changes in endothelial cells. Monolayers were stained for actin microfilaments using rhodamine phalloidin (see Methods). (*A*) Control monolayer, (*B*) monolayers treated with TNFα (100 U/ml, 6 h) and subsequent H_2O_2 (10 μM, 1 h), and (*C*) as in (*B*) except with 30-min pretreatment with 0.5 mM oxypurinol (×500).

Discussion

TNFα is an important mediator of endotoxic shock and the associated increase in vascular endothelial permeability and tissue edema as observed in the adult respiratory distress syndrome (1, 2). Several studies have suggested a critical role for TNFα in the pathogenesis of lung vascular injury in adult respiratory distress syndrome (3, 4, 35, 36). In previous studies, concentrations of TNFα ranging from 0.5 to 22 ng/ml were shown to increase endothelial permeability and produce actin cytoskeletal redistribution (7) in the absence of cytolysis (5, 6). However, it is doubtful that these concentrations of TNFα alone can explain the high permeability pulmonary edema associated with endotoxemia. Serum TNFα concentrations in septic patients are relatively low (median concentration in nonsurvivors of 0.33 ng/ml) and almost never exceed 4 ng/ml (corresponding to 100 U/ml of TNFα used in this study) (37, 38). We showed in this study that endothelial permeability did not increase in BPMVEC in response to 4 ng/ml (100 U/ml) TNFα. However, even a low TNFα concentration (100 U/ml), which had no direct permeability-increasing effect, was capable of "priming" endothelial cells and rendering them susceptible

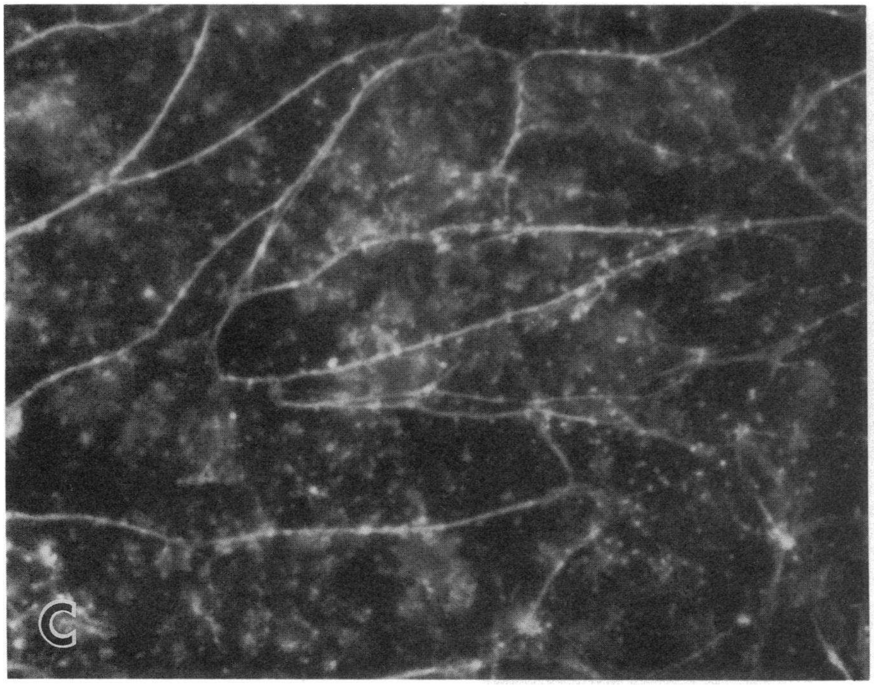


Figure 8 (Continued)

to injury by H_2O_2, an oxidant produced by activated PMN (39). Therefore, H_2O_2 resulted in an increase in endothelial permeability in TNFα "primed" cells even at an H_2O_2 concentration of 10 μM that in control endothelial cells had no effect on permeability.

The permeability-increasing effect of subthreshold concentrations of TNFα combined with H_2O_2 was associated with changes in the shape of endothelial cells and redistribution of cytoskeletal actin filaments, but was not the result of cytolysis. The observed effects were also not due to contamination of the TNFα preparation, since heat-inactivation of the cytokine had no effect and neutralizing TNFα with an antibody prevented the TNFα-mediated augmentation of the permeability increase.

In light of observations that sensitivity of cultured tumor cells to TNFα can be regulated by the capacity of these cells to scavenge free radicals (25), we examined a possible basis of the TNFα-mediated effect on permeability by determining alterations in intracellular antioxidants of TNFα-exposed BPMVEC to H_2O_2. There is some precedence for invoking a role of TNFα in modulating intracellular antioxidants. Intravenous administration of TNFα in rats increased plasma GSSG concentration (40), an index of oxidation of the antioxidant GSH (41). TNFα has also been shown to decrease intracellular thiols, with the most abundant being GSH (42). Since the GSH redox cycle as well as intracellular catalase are the primary antioxidant defense mechanisms against H_2O_2 (26), we determined whether TNFα-mediated alterations in intracellular GSH and catalase contributed to the increased susceptibility of TNFα-exposed endothelial cells to H_2O_2. The results indicated that the GSH content was reduced in a concentration-dependent manner after TNFα challenges. TNFα appeared to be responsible for GSH oxidation since the decrease in GSH was associated with a concomitant increase in GSSG content. A likely mechanism of reduction in GSH content may be generation of oxidants during TNFα exposure and the conversion of GSH to GSSG (43). GSSG formed by oxidative stress is subsequently reduced by glutathione reductase and reconverted to GSH; however, intracellular GSSG may accumulate when the rate of GSSG formation exceeds that of its reduction or when the glutathione reductase system is impaired (43). In such a case, GSSG can be extruded into the extracellular space or may form mixed disulfides with intracellular or extracellular proteins resulting in a net loss of GSH (44). In this study, only a small increase in GSSG in the medium was detected in conjunction with intracellular accumulation of GSSG after treatment with TNFα; therefore, a major part of loss of GSH induced by TNFα may be the result of formation of mixed disulfides.

The decrease in intracellular GSH content was seen in parallel with the increase in permeability; that is, GSH content decreased slightly after the 3-h TNFα treatment and this was associated with increased susceptibility to H_2O_2, but the greater decrease in GSH occurring within 6 h after TNFα treatment augmented the sensitivity to H_2O_2. Tsan et al. (26) have reported that depletion of cellular GSH by the GSH synthesis inhibitor, buthionine sulphoxamine, increased the susceptibility of endothelial cells to lysis by H_2O_2. The role of decreased cellular GSH observed in the present study in enhancing endothelial sensitivity to H_2O_2 is consistent with this observation.

In contrast to the TNFα-induced decrease in intracellular GSH content, even high concentrations of TNFα had no significant effect on the endothelial catalase activity. Shiki et al. (45) also showed that high concentration of endotoxin did not alter Cu/Zn SOD and catalase contents in cultured bovine endothelial cells, although Mn SOD content was significantly increased. Similarly, Shaffer et al. (46) have shown that a high concentration of TNFα did not affect either catalase or CuZn

SOD mRNA signals. Therefore, endothelial cell catalase activity appears to be resistant to TNFα, and it is therefore unlikely that it is an important determinant of the increased susceptibility to H_2O_2 observed in TNFα-treated endothelial cells.

Since the decrease in cellular GSH content may be a determinant of the observed increase in sensitivity of endothelial cells to H_2O_2, we tested whether supplementation of GSH might prevent the "priming" effect of TNFα on endothelial cells. Exogenous GSH increased the intracellular GSH concentration by 180% as has been demonstrated previously using endothelial cells (30). This increase in GSH significantly reduced the H_2O_2-mediated increase in endothelial permeability in TNFα-treated endothelial cells.

The question of why TNFα caused the reduction in the endothelial GSH content is unresolved. Generation of oxygen free radicals by endothelial cells in response to TNFα (11) may cause oxidation of GSH, and thus may contribute to the decrease in GSH content. Possible sources of the oxygen free radical induced by TNFα include activation of (*a*) XO (12) and (*b*) arachidonic acid metabolism (11). The source of oxidants may depend on species and organs from which endothelial cells were derived. Schuger et al. (36) showed that TNFα-induced cytotoxicity in human umbilical vein endothelial cells was prevented with cyclooxygenase inhibitors, but not the XO inhibitor, allopurinol. In contrast, endotoxin-induced injury of bovine pulmonary endothelial cells was prevented with allopurinol (47). In this study oxypurinol (used in a concentration that inhibited XO activity in BPMVEC) prevented both the decrease in cellular GSH content and the increase in permeability that occurred in the combination TNFα and H_2O_2 regimen. This finding suggests that XO activation may be the source of oxygen free radical after TNFα challenge of BPMVEC and that this is responsible for oxidation of GSH.

In summary, we have shown that TNFα pretreatment of endothelial cells for 3–6 h significantly augmented the increase in endothelial permeability in response to H_2O_2. This effect was due to the TNFα-mediated decrease in intracellular GSH content since supplementation of GSH significantly inhibited the TNFα "priming" effect on endothelial permeability. Pretreatment of endothelial cells with the XO inhibitor, oxypurinol, also prevented the TNFα-induced sensitization of endothelial cells after H_2O_2 exposure. We conclude that TNFα-induced decrease in intracellular GSH mediates the increased susceptibility of endothelial cells to H_2O_2. This effect of TNFα on endothelial cells may play a critical role in the high-permeability pulmonary edema associated with endotoxic shock.

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Note added in proof. A study appearing since the acceptance of this manuscript (Marcho, Z., J. E. White, P. G. Higgins, and M.-F. Tsan. 1991. *Am. J. Respir. Cell Mol. Biol.* 5:556–562), has shown that TNF-α enhances endothelial cytotoxicity to hyperoxia (95% O_2), which was the result in part of a reduction in intracellular GSH. This finding is consistent with the present observations concerning the role of TNF-α–induced decrease in GSH in augmenting the increase in vascular endothelial permeability mediated by H_2O_2.

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