Effect of *N*-Acetylcysteine on the Pulmonary Response to Endotoxin in the Awake Sheep and upon In Vitro Granulocyte Function

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Abstract. Oxygen free radicals released during endotoxemia may contribute to the lung injury of the adult respiratory distress syndrome (ARDS). As this syndrome occurs frequently after gram-negative sepsis in humans, we studied the effect of intravenous *N*-acetylcysteine (NAC), a free radical scavenger, upon the endotoxin (E)-induced model of ARDS in awake sheep. In vivo studies demonstrated that NAC attenuates the endotoxin-induced rise in pulmonary artery pressure (62±3 torr with E control vs. 43±3 torr for E + NAC), and markedly diminishes the rise in lymph flow at 1 h (8.5±1.2 vs 4.5±0.6 ml/15 min) and 4 h (5.0±0.6 vs. 3.3±0.4 ml/15 min), respectively, for E control vs. E + NAC. NAC also markedly attenuated the alterations in lung mechanics after endotoxemia. Dynamic compliance at 2 h after endotoxemia was 44±6% of base line for E vs. 76±10% of base line for E + NAC. Resistance to airflow across the lung at 1 h postendotoxin was 811±280% of base line for E vs. 391±233% of base line for E + NAC. NAC substantially reduced the 1 h postendotoxin rise in lymph concentrations of thromboxane B_2 (8.29±3.28 vs. 2.75±1.93 ng/ml for E vs. E + NAC) and 6-keto-prostaglandin-$F_{1\alpha}$ (0.91±0.27 vs. 0.23±0.12 ng/ml for E vs. E + NAC). In addition, in vitro studies were performed which revealed NAC to be a potent free radical scavenger in both biologic and nonbiologic free radical generating systems. NAC decreased phorbol-stimulated granulocyte aggregation in a concentration-dependent manner in vitro. Minimal effects were observed, however, upon leukocyte degranulation at the concentrations of NAC achieved during the in vivo tests. Thus, NAC significantly attenuated all monitored pathophysiologic changes in the endotoxin model of ARDS in sheep, possibly by its ability to scavenge toxic oxygen free radicals. A direct impairment of the ability of inflammatory cells to generate oxygen radicals cannot be ruled out.

Introduction

The adult respiratory distress syndrome (ARDS)[1] is a lung disorder characterized by diffuse bilateral pulmonary infiltrates, decreased lung compliance, and hypoxemia. All of these occur in the absence of heart failure, as defined by a normal pulmonary capillary wedge pressure. The course is frequently one of progressive deterioration of lung function, with death resulting from respiratory failure or a superimposed complication. It is postulated that activated granulocytes and possibly other cells sequestered in the lungs of these patients actively and progressively damage the capillary endothelium, which results in increased vascular permeability, and nonhydrostatically mediated pulmonary edema. As this syndrome frequency occurs in humans after gram-negative sepsis, an animal model of ARDS that uses an infusion of gram-negative bacterial endotoxin has been de-

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1. *Abbreviations used in this paper:* $\Delta AaPO_2$, alveolar-to-arterial oxygen pressure difference; ABG, arterial blood gases; AA, arachidonic acid; ARDS, adult respiratory distress syndrome; BSS, balanced salt solution; C_{dyn}, dynamic compliance; C_{lp}, clearance of lung lymph protein; D_5W, 5% dextrose in water; E, endotoxin; FRC, functional residual capacity; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; LA, left atrium; L/P, lymph-to-plasma concentration ratio; NAC, *N*-acetylcysteine; PA, pulmonary artery; P_{ao}, airway opening pressure; PMA, phorbol myristate acetate; P_{PA}, pulmonary artery mean pressure; P_{pl}, pleural pressure, P_{tp}, transpulmonary pressure; PVR, pulmonary vascular resistance; $\dot{Q}_L$, lymph flow; R_L, resistance to airflow across the lung; SA, left carotid artery; TxB_2, thromboxane B_2; V, tidal volume; $\dot{V}$, flow; WBC, white blood cell; ZAP, zymosan-activated plasma.

veloped in sheep (1). In the unanesthetized sheep there is a biphasic response to the endotoxemia. Within 1 h there is an early phase that is characterized by pulmonary hypertension, an increase in the flow of protein-poor lung lymph, and marked alterations of lung mechanics and gas exchange (2). These changes are prevented by pretreatment with agents that block the cyclooxygenase pathway of arachidonic acid metabolism (3). Subsequently, there is a late phase response at 3–4 h, which is characterized by the development of pulmonary edema with an increase in the flow of protein-rich lung lymph in spite of normal or near normal pulmonary artery and left atrial pressures, and improving but still impaired lung mechanics and gas exchange.

Previous studies have suggested that granulocytes are important mediators of the late phase changes, and possibly contribute to the early phase abnormalities as well. Evidence for this is threefold. First, histologic studies reveal granulocyte sequestration in the lung after endotoxemia associated with endothelial cell damage (4). Secondly, granulocyte-depleted sheep have a markedly blunted late phase (5), and an attenuation of the early phase alterations in lung mechanics (6). Finally, phorbol myristate acetate (PMA), a known granulocyte activator, is capable of producing a similar biphasic response (7, 8). The mechanisms by which granulocytes contribute to these changes are not fully understood but may relate to their ability to release a variety of oxygen radicals, including the superoxide anion (O_2^-), singlet oxygen, and hydroxyl radicals (OH^- and $HOCl^-$).

Free radical-generating systems are capable of producing endothelial cell damage in vitro (9, 10). Activated granulocytes and tissue macrophages are also capable of generating oxygen free radicals, thereby raising the possibility that these cells contribute to acute lung injury (11, 12). Endothelial cell damage in in vitro models can be blocked by the addition of enzymes or free radical scavengers, such as catalase (10, 13, 14), superoxide dismutase (12, 15, 16), dimethyl sulfoxide (17, 18), and dimethylthiourea (10, 19, 20). These agents work by differing mechanisms but the latter two contain sulfhydryl groups that are thought capable of scavenging free radicals.

NAC has been used extensively and successfully in humans in the treatment of acetaminophen (paracetamol) toxicity (21). Acetaminophen itself is not toxic, but the metabolism of acetaminophen by the liver involves the production of small amounts of hydroxylated free radicals, which can bind covalently to hepatic proteins. These highly reactive radicals will preferentially bind to glutathione, thereby converting it to a nontoxic conjugate of cysteine, but the detoxifying mechanisms can be exhausted if cysteine or glutathione stores are depleted (22). The sulfhydryl bond of cysteine seems to be the important element because similar results have been obtained with methionine and cysteamine (23, 24). These compounds, along with NAC, are thought to work by providing substrate to replenish glutathione stores, or by competitively binding directly to the free radical species. Because previous clinical experience has demonstrated that NAC is an effective and nontoxic free radical scavenger, we chose to study its effect in endotoxin infusion in sheep.

We postulated that the treatment of sheep with NAC before an infusion of endotoxin would decrease the ensuing lung damage either by increasing intracellular substrates for the glutathione reductase pathway, or by the direct scavenging of free radicals by the NAC molecule itself. If oxygen radicals are involved in the pathophysiology of this animal model of ARDS, then regardless of the source of these radicals, NAC should attenuate the endotoxin-induced lung injury.

Methods

Experimental preparation. Four thoracotomies (two on each side) were performed during a single anesthesia period in each sheep. Silastic catheters were placed directly in the left atrium (LA), proximal pulmonary artery (PA), left carotid artery (SA), and left jugular vein. A Swan-Ganz flow-directed thermodilution tip catheter was also positioned in the proximal pulmonary artery via the left jugular vein. In addition, the efferent vessel of the caudal mediastinal lymph node was cannulated with a silastic catheter, which was brought out through the right side of the chest. Contaminating abdominal and systemic lymphatics were disrupted by ligating the caudal aspect of the lymph node at the level of the inferior pulmonary ligament, and by bilaterally interrupting diaphragmatic lymph vessels (25, 26). Pleural balloons (3) were placed in the pleural space via one of the right thorocotomies. Tracheostomies were performed either during the same anesthesia or at a second operation. Tracheostomy tubes (No. 10 Shiley) were inserted only during the experiments.

Measurements of pressure, protein content, and lymph flow. Pressures from the PA, LA, and SA lines were monitored continuously by using physiological pressure transducers, series 1280-C (Hewlett-Packard Co., Palo Alto, CA), in conjunction with amplifiers (Validyne Engineering Corp., Northridge, CA) and a model 2800 8 channel pen recorder (Gould, Inc., Instruments Div., Cleveland, OH). Protein analysis was performed by the modified biuret method on an automated analysis system (AutoAnalyzer, Technicon Instruments Corp., Tarrytown, NY) (27). Lymph was collected over EDTA and indomethacin in plastic collection tubes; and the volume was recorded every 15 min.

Measurement of arterial blood gases (ABG), alveolar-to-arterial oxygen pressure difference ($\Delta AaPO_2$) and white blood cell (WBC) counts. Heparinized samples of arterial blood were drawn anaerobically into a plastic syringe from either the SA or LA line at base line, 15 min after beginning the endotoxin infusion, and every 30 min thereafter. The samples were stored in ice for subsequent measurement of arterial P_{O_2}, P_{CO_2}, and pH with an Instrumentation Laboratory, Inc. (Lexington, MA) Blood Gas Machine, model 513. $\Delta AaPO_2$ was calculated by using the alveolar gas equation, and by assuming a respiratory exchange ratio of 0.8. Blood for WBC count was drawn every 30 min in conjunction with the ABG sample as noted above. The samples were collected on EDTA and analyzed with a Coulter Diluter, model WRD2, and Coulter Counter, model ZBI.

Measurement of lung mechanics. Awake sheep were studied while standing in a specially constructed whole-body pressure-compensated integrated-flow plethysmograph. The plethysmograph is constructed of 1-cm-thick clear plexiglass and has a volume of 285 liters. The sheep's tracheostomy tube was connected to an external valve, which was used to obstruct the airway during determinations of functional residual capacity (FRC) via flexible noncollapsible tubing. This permitted the animal to move without interfering with measurements. A loosely fitting sling was placed under the sheep to prevent it from lying down while in the

plethysmograph and such that it was not restrictive with regard to pulmonary mechanics measurements. A constant bias flow was used to reduce the effective deadspace of the tubing. Tidal volume (V) was measured by pressure compensating the integrated signal from the plethysmographic pressure transducer. Flow ($\dot{V}$) was obtained by electrically differentiating the volume signal. Airway opening pressure (P_{ao}) was measured by a multiple side hole catheter, which was positioned 0.5 cm past the distal end of the tracheostomy tube. Pleural pressure (P_{pl}) was measured directly from the balloon in the pleural space. Transpulmonary pressure (P_{tp}) was the pressure difference between P_{pl} and P_{ao}. All pressure signals were measured using Validyne Engineering Corp. pressure transducers and Validyne Engineering Corp. amplification equipment. The signals from pressure transducers, catheters, and silastic envelopes were tuned at 6 Hz to eliminate phasic distortion. These methods have been described in detail elsewhere (28).

Before each measurement of lung mechanics, the sheep's lungs were inflated to 40 cm H_2O P_{ao} using the bias flow and an occluded airway. Simultaneous $V/\dot{V}$ and V/P_{tp} curves were then recorded during spontaneous respiration on a dual-beam storage oscilloscope (Tektronix, Inc., Beaverton, OR) and photographed for calculation of dynamic compliance (C_{dyn}) and resistance to airflow across the lung (R_L). C_{dyn} was calculated as V divided by P_{tp} at points of zero flow and expressed in liters per centimeter H_2O at BTPS. R_L was calculated by using the method of von Neergaard and Wirz (29) by dividing P_{tp} by $\dot{V}$ at midtidal volume and was expressed as centimeters H_2O (liters per second)$^{-1}$ at BTPS. FRC was measured by using Boyle's law by the method of DuBois et al. (30). The airway was manually obstructed at end expiration. The sheep continued to make respiratory efforts against the obstruction for one to three breaths and a graph of the change in plethysmographic volume against the change in P_{ao} was traced on the oscilloscope and photographed for calculation of FRC.

Measurement of thromboxane and prostacyclin metabolites. Thromboxane B_2 (TxB_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) were measured in lung lymph specimens collected during base line, after NAC loading (where applicable) and at 30, 60, and 240 min after endotoxin infusion or after beginning NAC maintenance infusion in the NAC control experiments. Analysis was performed by radioimmunoassay using rabbit anti-TxB_2 and anti-6-keto-$PGF_{1\alpha}$ antibodies obtained from Dr. Lawrence Levine (Brandeis University, Waltham, MA). The anti-TxB_2 antibody cross reacts <1% with prostaglandin E_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ and <3% with prostaglandin D_2. The 6-keto-$PGF_{1\alpha}$ antibody cross-reacts <3% with prostaglandin D_2, and <1% with prostaglandin E_2, prostaglandin D_2, and TxB_2. Authentic prostaglandins and TxB_2 were generously supplied by Dr. John Pike (Upjohn Company, Kalamazoo, MI). Radiolabeled (5,6,8,9,11,12,14,15-^{3}H)-TxB_2 and (5,6,8,9,11,12,-14,15-^{3}H)-6-keto-$PGF_{1\alpha}$ were purchased from New England Nuclear (Boston, MA). Bovine γ-globins, Trizma buffer, and ammonium sulfate were purchased from Sigma Chemical Co. (St. Louis, MO).

The radiolabeled ligand (≃4,000 cpm per tube) was first mixed with bovine γ-globins (10 mg/ml in Trizma, pH 7.4). To 100-μl aliquots of this mixture were added 100-ml aliquots of sample or appropriate unlabeled standard dilutions. The binding reaction was initiated by addition of 100 μl of the appropriate antibody, which was diluted to yield 60% binding of the label. The binding reaction continued for 60 min at 37°C and was terminated by precipitation of the immune complexes with ammonium sulfate at a final concentration of 50% of saturation. After centrifugation at 2,500 rpm at 4°C for 10 min, 300 μl of the supernatant was counted in Aquasol (New England Nuclear). Each sample was assayed in duplicate, and duplicate determinations differed by <10%. The detection limit of both assays was <20 pg.

In vitro studies. WBC were harvested from chronically instrumented but otherwise healthy sheep, which had not been experimented upon within 48 h. 30 ml of whole blood in 1,000 U heparin was layered over 15 ml of Ficoll-Hypaque in a 50-ml polypropylene tube (Histopaque, Sigma Chemical Co.) and centrifuged at 400 rpm for 40 min and allowed to stop with the brake off. The resultant plasma layer and top three-fourths of the Ficoll-Hypaque layer was discarded. The granulocyte layer was then aspirated and resuspended in a hypotonic lysing solution consisting of 8.9 g NH_4Cl, 1.0 g $KHCO_3$, and 30 mg EDTA per liter of distilled water. This was allowed to react for 5 min before resedimenting the remaining cells at 200 rpm for 5 min. The cells were then washed in 0.9 N NaCl solution before being suspended in Hanks' balanced salt solution (BSS) with Ca^{++} and Mg^{++} at a final concentration of 1×10^7 cells/ml. The cells were routinely >95% viable by trypan blue dye exclusion, and consisted of ~80–90% granulocytes. No erythrocytes or platelets were seen at the time of counting.

Measurement of aggregation. Aggregation studies were performed with a Payton Dual Channel model 300 BD Aggregometer coupled with a Dual Pen Recorder, model 585 (Linear Instruments Corp., Reno, NV). The aggregometer was set at 37°C, 400 rpm's, level 0.5, and a range of four. The recorder was set at 50 mV and a paper speed of 1 cm/min. 350 μl of leukocyte suspension was placed in a small siliconed glass cuvette with a stir bar, inserted in the aggregometer, and allowed to reach 37°C. The aggregometer was then standardized by setting the light transmittance to 0% on the paper recorder using the zero control, then replacing the cell cuvette with one containing only Hanks' physiologic buffered saline and a stir bar and adjusting the output level to 100% light transmittance. The cell cuvette was then placed back into the aggregometer and the cells were stimulated with 50 μl of either PMA (40 ng/ml final concentration) or zymosan-activated plasma (ZAP, full strength). To determine the effect of NAC upon aggregation, the leukocytes were suspended in NAC (variable concentrations) and either incubated at 37°C for 30 min, or stimulated immediately. The results are reported as the maximum percent change in light transmittance (%).

Measurements of chemiluminescence. Free radical production was measured by chemiluminescence (31) as NAC was found to spontaneously reduce cytochrome C, which made that assay invalid. The biologic system consisted of a leukocyte suspension (4 ml) in Hanks' BSS (2.5×10^6 cells/ml final concentration), which was stimulated with either PMA (40 ng/ml final concentration) or ZAP (10%) with and without the presence of NAC (2.8 mg/ml). The nonbiologic system consisted of 3,150 μl of xanthine solution (xanthine, 12 mg; EDTA, 5 mg; Hepes solution, 12 μl; all in 250 ml 0.9 N saline), 400 μl human plasma, and 400 μl of either Hanks' BSS or NAC (2.8 mg/ml final concentration) in BSS. The reaction was initiated by the addition of 50 μl of xanthine oxidase solution (0.96 U/ml). All reactions were run in a darkened room at room temperature with dark adapted polyethylene scintillation vials and reagents. Measurements were taken on an LS-250 Liquid Scintillation System (Beckman Instruments, Inc., Fullerton, CA) in the off-coincidence mode with only a single photomultiplier tube activated and a window from 0 to 10. Background counts were taken from empty scintillation vials and subtracted from all subsequent measurements. Data are reported in counts per minute.

Measurements of β-glucuronidase and lysozyme. Sheep granulocytes do not contain lysozyme (32). Therefore, β-glucuronidase release was used as a measure of sheep leukocyte degranulation. Cytochalasin B-treated (5.0 μg/ml × 10 min at 37°C) leukocyte suspensions (8×10^6 cells/ml) were preincubated at 37°C for 30 min with gentle agitation with either NAC (2.8 mg/ml) or BSS in polypropylene tubes (Falcon Labware, Div. Becton, Dickinson, & Co., Oxnard, CA). Subsequently, either ZAP (10%) or PMA (40 ng/ml final concentration) was added

and the cells were further incubated for 20 min. The suspensions were then centrifuged at 400 rpm for 10 min; and the supernates were decanted and placed on ice (4°C). Controls of resting cells and Triton X-100 (0.4%) disrupted cells were also run. β-glucuronidase activity was assayed by the hydrolysis of *p*-nitrophenyl-β-D-glucuronide to *p*-nitrophenol and β-D-glucuronic acid as previously described (33). Briefly, 200 μl of either β-glucuronidase standard or leukocyte supernatant was added to 500 μl of substrate and 500 μl of acetate buffer. The reaction was run at 37°C for 16 h. The content of β-D-glucuronic acid was determined spectrophotometrically at 410 nm, and the results are reported as the percent of total enzyme release.

Release of lysozyme from human granulocytes, prepared in an identical fashion as for sheep cells, was assayed as described by Gordon et al. (34). The decrease in turbidity of a suspension of *Micrococcus lysodeckticus* caused by supernatants of granulocytes at rest and exposed to PMA or ZAP with or without preincubation with NAC (2.8 mg/ml) as described in the β-glucuronidase section, was determined by continuous spectrophotometric recording at 540 nm. Results are expressed as the percentage of total lysozyme content, which was determined using supernatants of granulocytes lysed with Triton X-100.

In vivo experimental protocol. After 6–7 d postoperative recovery time, 10 yearling sheep were studied awake and standing in the plethysmograph. The sheep received either endotoxin (E) alone or in combination with NAC in random order on separate days, which was followed by a third study consisting of NAC alone. The sheep were allowed 3–4 d recovery time between each experiment.

For all experiments, stable values (±10%) of vascular pressures, lymph flow, and lung mechanics were obtained for at least 1 h (four measurements) as a base line. for the E-control experiment, the sheep then received an intravenous infusion of 200 ml of 5% dextrose in water (D_5W) over 1 h as the vehicle control, followed by another 50 ml D_5W over the subsequent hour. *Escherichia coli* endotoxin (055:B5), 1 μg/ml in D_5W, was then infused via a central line at a dose of 0.2–0.5 μg/kg over 15 min. The sheep continued to receive D_5W intravenously at a rate of 50 ml/h for the next 4 h until the experiment was completed.

In the combined NAC-plus-E experiments, after base line, the sheep received NAC intravenously in a loading dose of 150 mg/kg in 200 ml D_5W over 1 h, followed by a maintenance dose of 20 mg/kg in 50 ml D_5W each hour for 5 h until the experiment was ended. After the first hour of maintenance NAC infusion, the sheep received E in an identical manner as described above. An identical dose of E was given to the sheep for both the E-control and NAC-plus-E experiments.

The NAC-control experiments were carried out similarly to the NAC-plus-E experiments, only the E was omitted. The animals were given 3–5 d between each experiment to recover. All sheep received both E and combined NAC-plus-E experiments, while only six sheep received the NAC-control experiment. Of these six sheep, only three had a patent lymph cannula on the day of the NAC study. Lung mechanics also were obtained on only three sheep during the NAC-control experiment, while hemodynamic measurements were obtained on all NAC-control sheep.

Statistics. Physiologic data are presented as the mean value±standard error. To test statistical significance of differences over time in each of the three groups, E-plus-vehicle, E-plus-NAC, and NAC alone, and to test the significance of differences between groups, we used a split-plot design with randomized complete blocks for three-way analysis of variance. The null hypothesis was rejected for $P < 0.05$. Kramer's adaptation of Duncan's Multiple range test (35) was then used to test for significance of differences within groups at the $P < 0.05$ level. The in vitro data were analyzed by the paired *t* test at the $P < 0.05$ level. The null hypothesis was rejected for $P < 0.05$.

Results

Endotoxin response. E alone caused a marked increase in the pulmonary artery mean pressure (P_{PA}) reaching a peak from 30 to 45 min after endotoxemia before decreasing rapidly, reaching base line values by the third hour postendotoxemia (Table I, Fig. 1). Cardiac output fell significantly at 1 h post-

Table I. Effect of NAC on the Hemodynamic and Lymph Data of E-treated Sheep

	Pulmonary artery pressure (n = 10)	Pulmonary vascular resistance (n = 10)	Cardiac output liters · min^{-1} (n = 10)	Lymph flow (n = 7)	Lymph/plasma protein concentration ratio (n = 7)	Lymph protein clearance (n = 7)
	torr	*cm H_2O (liters/s)$^{-1}$*	*liters/min*	*ml/15 min*	*% base line*	
Base line						
E	21±1	4.0±0.3	5.2±0.4	2.0±0.3	1.00	1.2±0.2
E + NAC	20±1	3.4±0.3	4.9±0.5	2.3±0.4	1.00	1.2±0.2
NAC	22±1	4.0±0.8	5.9±1.0	2.0±0.4	1.00	1.3±0.3
1 h post-E						
E	62±3*	17.2±1.9*	4.0±0.4*	8.5±1.2*	0.74±0.09*	3.4±0.3*
E + NAC	43±3*‡	9.4±1.2*‡	5.5±0.8‡	4.5±0.6*‡	0.94±0.08‡	2.1±0.1*‡
NAC	23±2	4.6±1.0	6.7±1.5	1.7±0.5	0.97±0.01	1.1±0.3
4 h post-E						
E	26±1	5.0±0.8	4.8±0.4	4.9±0.6*	1.08±0.09	3.4±0.7*
E + NAC	25±1	5.1±0.8	5.2±0.6	3.2±0.3	1.17±0.08	1.9±0.2*‡
NAC	22±2	4.6±1.3	5.4±1.4	1.8±0.6	1.00±0.06	1.2±0.3

All values are mean±SEM * The value is significantly different from the base-line value ($P < 0.05$). ‡ The value is significantly different from the time matched endotoxin control value ($P < 0.05$).

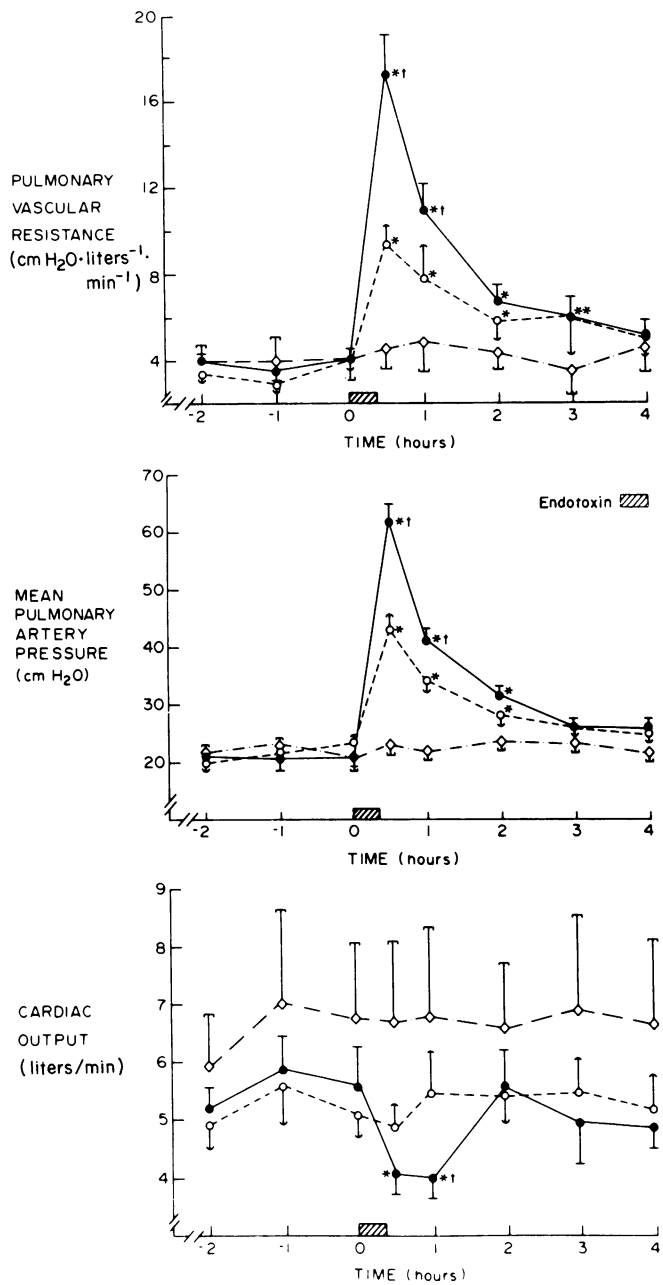

Figure 1. Effect of NAC on E-induced changes in pulmonary artery pressure, PVR, and cardiac output. Data are expressed as the mean±SEM. n = 10 for E-plus-vehicle and E-plus-NAC; n = 6 for NAC control. The 0.5-h data points represent the highest pressure readings obtained over the time period 15–45 min post-E. All other points represent the mean values for the time period immediately preceeding the data point. The data from the E-plus-vehicle control experiments are represented by •; the data from the E-plus-NAC experiments are represented by ○; and the data from the NAC-control experiments are represented as ◇. * indicates that the data point is significantly different ($P < 0.05$) from base line. † indicates that the data point is significantly ($P < 0.05$) different from the time-matched E-plus-NAC data point.

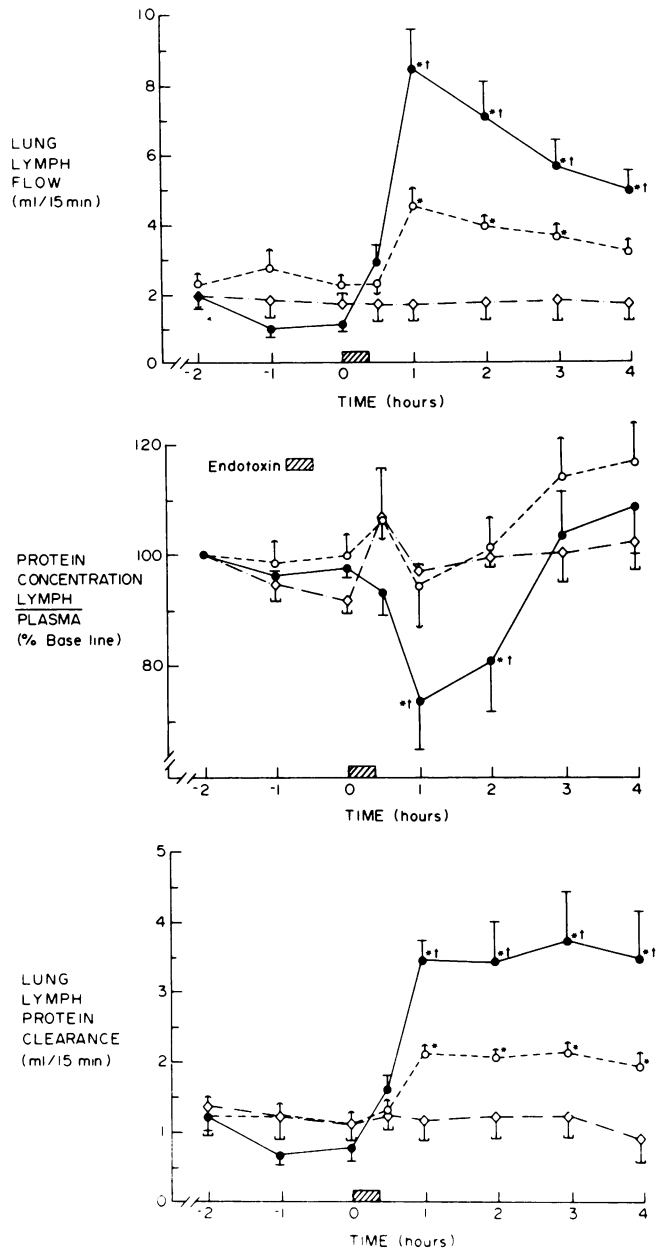

Figure 2. Effect of NAC on E-induced changes in $\dot{Q}_L$, L/P, and the clearance of lymph protein (C_{lp} = $\dot{Q}_L$ × L/P). Data are expressed as the mean±SEM. n = 7 for E-plus-vehicle and E-plus-NAC; n = 3 for NAC control. The 0.5-h data points represent the peak $\dot{Q}_L$ and L/P measured for the time period 15–45 min post-E. All other data points represent the mean values for the time period immediately preceding the data point. The data from the E-plus-vehicle control experiment are represented by •; the data from the E-plus-NAC experiments are represented by ○; and the data from the NAC-control experiments are represented by ◇. * indicates that the data point is significantly different from control ($P < 0.05$). † indicates that the data point is significantly different ($P < 0.05$) from the time-matched E-plus-NAC data point.

E, but returned rapidly to base-line values. Consequently, there was a large increase in the pulmonary vascular resistance (PVR) at 1 h post-E. While PVR fell rapidly toward base-line values, it remained significantly elevated at the end of 4 h post-E.

The lung lymph was also markedly altered in response to E. The lymph flow ($\dot{Q}_L$) increased significantly by 1 h postendotoxemia. It then decreased gradually but remained significantly elevated at 4 h after endotoxin. The lymph-to-plasma protein (L/P) ratio fell significantly at 1 h postendotoxemia. By 4 h postendotoxin the L/P ratio had increased and was slightly although not significantly greater than at base line. When the data were expressed as the clearance of lung lymph protein, C_{lp} ($\dot{Q}_L \times$ L/P), the C_{lp} increased significantly by 1 h post-E, and remained elevated throughout the experiment (Fig. 2).

Endotoxin also produced marked alterations in lung mechanics (Table II). The C_{dyn} fell markedly by 1 h post-E, then improved slightly over the next hour before falling again. C_{dyn} remained significantly depressed at 4 h. Likewise, R_L increased markedly 1 h postendotoxemia, but then decreased rapidly over the ensuing 2 h, plateauing at a level slightly higher than the original base line, although not significantly so (Fig. 3). The FRC was not altered significantly by endotoxemia.

Gas exchange, as expressed by $\Delta AaPO_2$, worsened after endotoxemia, and reached a nadir at 1 h post-E. This improved with time and was not significantly greater than base line at 4 h post-E (Fig. 4). For reasons that are unclear, the base-line value for this experiment is higher than with the other two experiments (NAC-plus-E; NAC alone). Nonetheless, the $\Delta AaPO_2$ in these sheep worsened with E similarly to experiments previously reported from this laboratory (2, 3).

The peripheral WBC count fell dramatically with endotoxemia, reaching a nadir at 1 h postendotoxemia. It then rose slowly and was not significantly different from base line by the third hour post-E (control = 8,698±1,382 cells/mm^3; 1 h = 2,306±481 cells/mm^3; 4 h = 6,732±1,464 cells/mm^3; Fig. 5).

Finally, E infusion produced a marked increase in lung lymph TxB_2 levels at 1 h, as seen in Fig. 6. Similarly, the 6-keto-$PGF_{1\alpha}$ levels were increased at 1 h, while neither the TxB_2 nor 6-keto-$PGF_{1\alpha}$ levels were significantly different from base line by 4 h post-E (Table III).

NAC-plus-endotoxin. Treatment with NAC before the administration of E attenuated all of the above abnormalities caused by E alone. The pulmonary hypertensive response was significantly blunted as was the increase in PVR post-E, although there were no changes in the timing of the response as seen with E alone. However, NAC completely prevented the decrease in cardiac output observed with E alone (Fig. 1, Table I).

Lymph flow at ½ h post-E was significantly less with NAC-plus-E as compared with E alone. The $\dot{Q}_L$ then decreased gradually with time; similar to E alone, but unlike with E control, it was not significantly different from base line at the fourth hour post-E. The L/P ratio at 1 h post-E did not fall significantly with NAC-plus-E, but tended to be elevated at 4 h, though not significantly. Consequently, C_{lp} was significantly lower 1 h post-E with NAC-plus-E than with E alone. The C_{lp} remained significantly lower throughout the observation period with NAC-plus-E than with E alone (Fig. 2, Table I).

Lung mechanics were also less affected with NAC-plus-E than with E alone. C_{dyn} decreased significantly less, and R_L increased significantly less at 1 h post-E when NAC was present, than with E alone (Fig. 3, Table II). 2 h post-E, the differences

Table II. Effect of NAC on the Lung Function of E-treated Sheep

	Dynamic compliance (n = 6)	Resistance to airflow (n = 6)	Functional residual (n = 6)	$\Delta AaPO_2$ (n = 10)
	liters/cm H_2O	*cm H_2O (liters/s)$^{-1}$*	*liters*	*torr*
Base line				
E	0.071±0.007	0.4±0.1	1.318±0.139	29±5
E + NAC	0.090±0.015	0.5±0.1	1.306±0.099	18±3
NAC	0.053±0.016	0.6±0.1	—	26±2
1 h post-E				
E	0.028±0.005*	3.6±1.3*	1.114±0.111	46±3*
E + NAC	0.046±0.013*	2.1±1.3*‡	1.259±0.196	31±4*‡
NAC	0.049±0.012	0.6±0.1	—	33±4
4 h post-E				
E	0.045±0.010*	1.3±0.5	1.244±0.103	37±5
E + NAC	0.066±0.008	1.1±0.6	1.329±0.124	33±5*
NAC	0.054±0.015	0.7±0.1	—	33±4

All values are mean±SEM. * The value is significantly different from the base line ($P < 0.05$). ‡ The value is significantly different from the time-matched E-control value ($P < 0.05$).

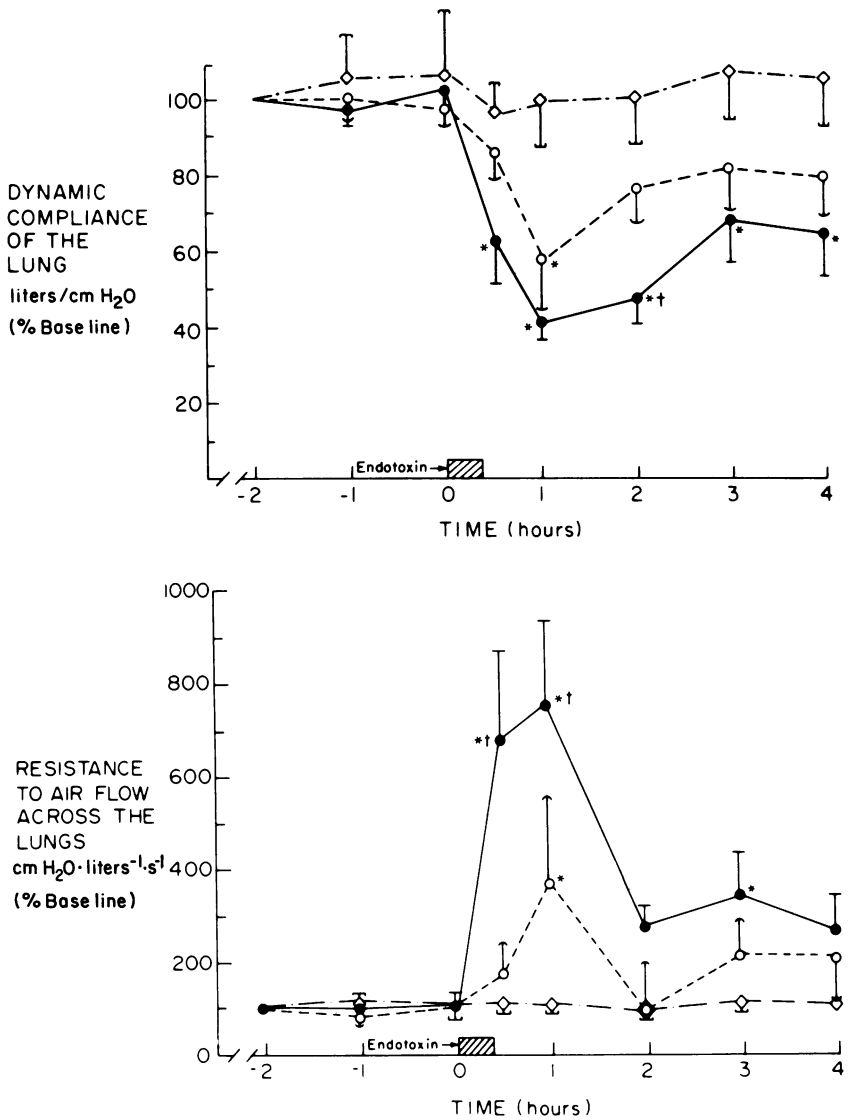

Figure 3. Effect of NAC on E-induced changes in C_{dyn}, and R_L. Data points are expressed as the mean±SEM. n = 6 for E-plus-vehicle and E-plus-NAC; n = 3 for NAC control. The 0.5-h data point represents the peak change from base line seen during the 15–45 min post-E. All other points represent the mean value for the time period immediately preceeding the data point. The data from the E-plus-vehicle experiments is represented by •; the data from the E-plus-NAC experiments are represented by ○; the data from the NAC-control experiments are represented by ◇. * indicates that the data point is significantly different ($P < 0.05$) from base line. † indicates that the data point is significantly different ($P < 0.05$) from the time-matched E-plus-NAC data point.

in C_{dyn} were even greater ($P < 0.05$). At 3 h post-E, neither C_{dyn} nor R_L was significantly different from base line, unlike with E alone in which both remained significantly different (Fig. 3).

The peripheral WBC count fell 1 h post-E when NAC was present, though significantly less so than for E alone (5,934±1,320 cells/mm^3 vs. 2,306±481 cells/mm^3, respectively). This effect persisted even when the counts were expressed as percentage of base line. Further, the recovery from E-induced neutropenia was more rapid when NAC was present, (4 h WBC = 12,322±3,326 cell/mm^3 with E-plus-NAC as compared with 6,732±1,465 cells/mm^3 with E alone) (Fig. 5).

Finally, there was a pronounced effect of NAC upon lung lymph TxB_2 and 6-keto-$PGF_{1\alpha}$ levels. The rise in TxB_2 and 6-keto-$PGF_{1\alpha}$ levels seen 1 h after endotoxemia was significantly less for NAC-plus-E than with E alone in all sheep. Concentrations of TxB_2 and 6-keto-$PGF_{1\alpha}$ in the NAC-plus-E experiments were not significantly different from the base-line value either at 1 or 4 h post-E (Table III, Fig. 6).

NAC alone. None of the measured variables changed significantly during the infusion of NAC alone except for $\Delta AaPO_2$ and peripheral WBC count. The $\Delta AaPO_2$ significantly worsened after NAC in both the NAC alone and in the pre-E portion of the NAC-plus-E experiments (Fig. 4). There was, however, no alteration of either C_{dyn} or R_L after NAC (Fig. 3).

The peripheral WBC count increased dramatically after NAC alone (Fig. 5). This progressed with time and was accompanied by an increase in the percentage of circulating immature granulocytes.

In vitro leukocyte studies. NAC was found to have a dose-dependent effect upon leukocyte aggregation as seen in Fig. 7. This effect was, however, less prominent when the cells were

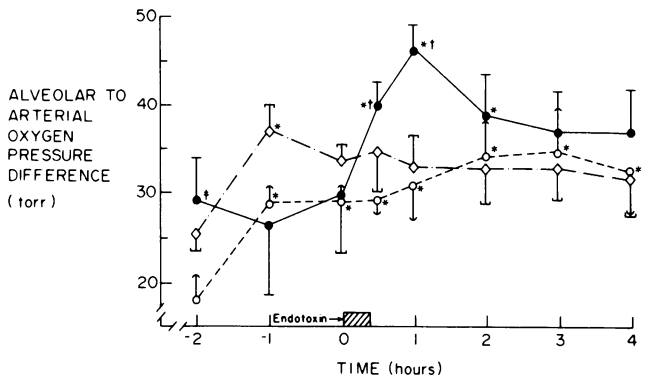

Figure 4. Effect of NAC on E-induced changes in the alveolar-to-arterial oxygen pressure difference. Data points represent the mean±SEM. n = 10 for E-plus-vehicle and E-plus-NAC; n = 4 for NAC control. The data from the E-plus-vehicle are represented by •; the data from the E-plus-NAC experiments are represented by ○; and the data from the NAC-control experiments are represented by ◇. * indicates that the data point is significantly different ($P < 0.05$) from base line. † indicates that the data point is significantly different ($P < 0.05$) from the time-matched E-plus-NAC data point. ‡ indicates that the base-line data point is significantly different ($P < 0.05$) from the time-matched E-plus-NAC base-line data point.

not preincubated with NAC but rather stimulated immediately after the addition of NAC. The attenuation of aggregation may represent a cytotoxic effect of NAC upon leukocytes as trypan blue dye studies revealed excessive uptake by cells incubated with NAC (1×10^{-1} M) for 3 h as compared with cells similarly incubated with Hanks' BSS. Such changes were not, however, seen at lower doses of NAC and for shorter incubation periods. Assuming that NAC was entirely limited to the circulating blood volume and was neither excreted nor metabolized, the maximum calculated concentration of NAC that was achieved during the in vivo tests was 2.8 mg/ml (1.7×10^{-2} M). At this concentration of NAC, and when preincubated with leukocytes in vitro at 37°C for 30 min, there was a slight but significant ($P < 0.05$) decrease in aggregation in response to both PMA and ZAP (Table IV).

In contrast, NAC potentiated leukocyte degranulation. Azurophilic degranulation, as measured by β-glucuronidase activity from sheep granulocytes, was found to be slightly potentiated ($P < 0.05$) by the preincubation of cells with NAC when stimulated with ZAP (Table IV). Degranulation by resting cells was also slightly enhanced by NAC but PMA was found not to cause azurophilic degranulation as is also the case in human cells (36).

Specific granule release was studied in human cells (n = 6) as sheep cells do not contain lysozyme (32). There were no significant differences ($P < 0.05$) in enzyme release in resting cells preincubated with either Hanks' BSS (14±0.8% enzyme release) or NAC (2.8 mg/ml) (15±1.0% enzyme release). In PMA-stimulated cells, however, NAC was found to significantly ($P < 0.05$) potentiate lysozyme release (48±1.5% total enzyme activity) as compared with Hanks' BSS preincubated cells (37±1.3% enzyme activity).

In contrast, NAC reduced leukocyte-generated chemiluminescence in a dose-dependent fashion (Fig. 8, Table IV). This effect was also observed using the nonbiologic xanthine-xanthine oxidase system of free radical generation. The base line count per minute (cpm)±SEM (n = 6) for the xanthine control (8,703±1,150 cpm) was not different from the xanthine-plus-NAC (2.8 mg/ml) system (7,822±472). The xanthine oxidase-stimulated reaction gave significantly ($P < 0.05$) higher counts per minute for the control xanthine-plus-Hanks' BSS (76,049±713 cpm) than did the xanthine-plus-NAC reaction (19,787±683 cpm). Control experiments were performed by measuring uric acid concentrations as an end-product. Equal amounts of xanthine were converted to uric acid whether or not NAC was present in the system, which indicates that NAC did not directly inhibit the xanthine-xanthine oxidase enzyme. Both reactions generated significantly ($P < 0.05$) more chemiluminescence than did the nonstimulated base-line systems.

Discussion

In the sheep model of endotoxin-induced ARDS we have shown that NAC, when delivered before, during, and continuing after

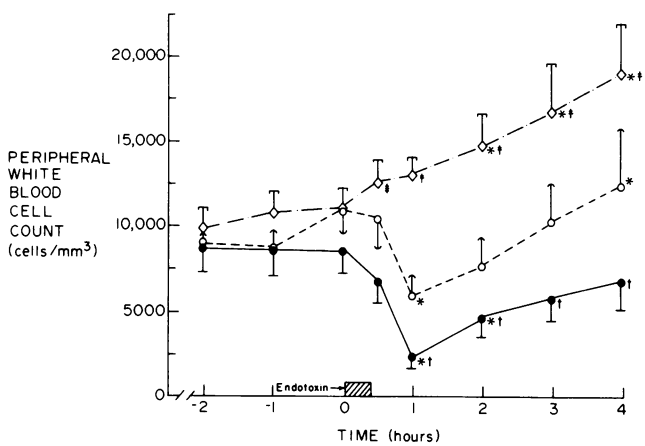

Figure 5. Effect of NAC on the peripheral WBC count. Data points are represented as the mean±SEM. n = 9 for E-plus-vehicle and E-plus-NAC; n = 6 for NAC control. The data points for E-plus-vehicle experiments are represented by •; the data points for E-plus-NAC experiments are represented by ○; and the data points for NAC-control experiments are represented as ◇. * indicates the data point is significantly different ($P < 0.05$) from base line. † indicates that the E-plus-vehicle data point is significantly different ($P < 0.05$) from the time-matched E-plus-NAC data point. ‡ indicates that the NAC-control data point is significantly different ($P < 0.05$) from either the E-plus-vehicle or E-plus-NAC time-matched data points. ‡ indicates that the NAC-control data point is significantly different ($P < 0.05$) from the time-matched E-plus-vehicle data point.

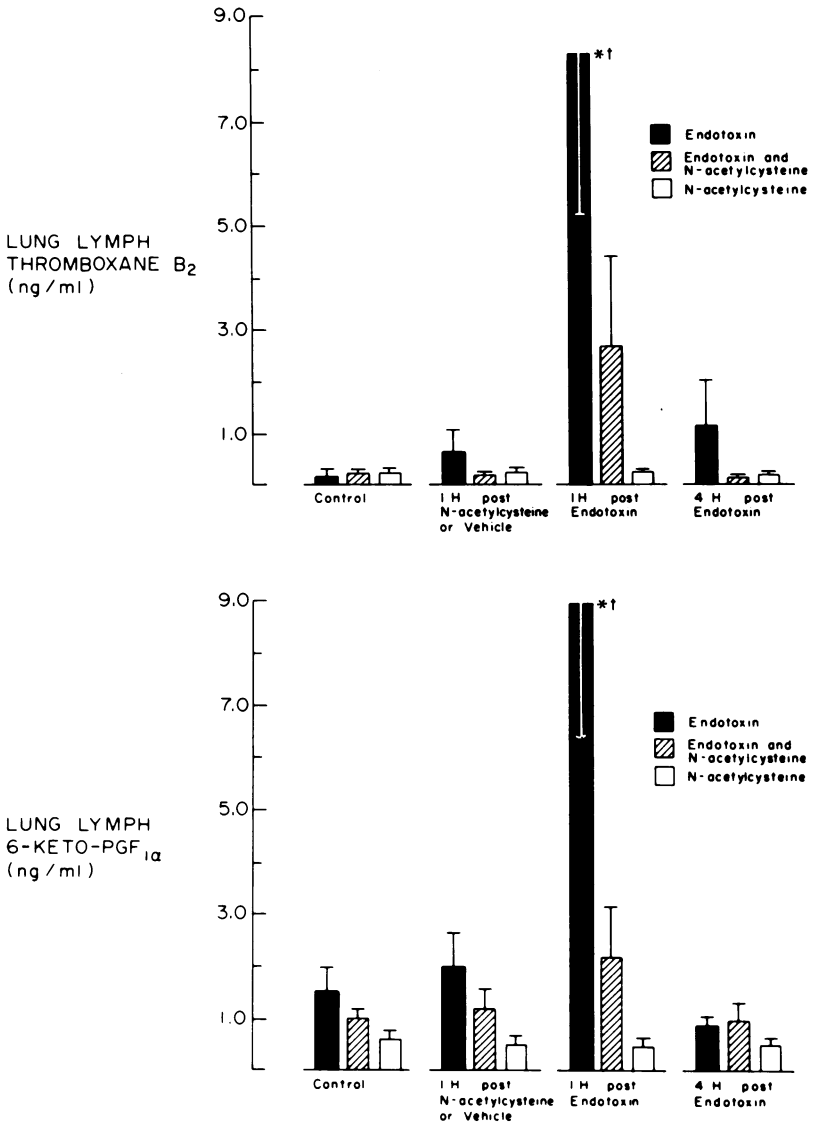

Figure 6. Effect of NAC on E-induced changes in lymph Tx B_2 and 6-keto-PG $F_{1\alpha}$ levels. Data represent the mean±SEM. n = 7 for E-plus-vehicle and E-plus-NAC; n = 3 for NAC control. * indicates that the data are significantly different ($P < 0.05$) from base line. † indicates that the data are significantly different ($P < 0.05$) from either the E-plus-vehicle or E-plus-NAC time-matched data.

an infusion of endotoxin, is capable of attenuating all measured pathophysiologic changes.

NAC attenuated the early-phase rise in pulmonary artery pressure, and the increase in PVR, and prevented the decrease in cardiac output associated with endotoxemia. These findings, supported by the marked attenuation of the E-induced rise in TxB_2 and 6-keto-$PGF_{1\alpha}$, suggest that NAC interfered in the usual post-E cascade of events that lead to increased arachidonic

Table III. Effect of NAC on Arachidonic Acid Metabolites in Lung Lymph of E-treated Sheep

	TxB_2			$PGF_{1\alpha}$		
	E (n = 7)	E + NAC (n = 7)	NAC (n = 3)	E (n = 7)	E + NAC (n = 7)	NAC (n = 3)
Base line	0.10±0.24	0.19±0.07	0.19±0.03	0.15±0.05	0.11±0.03	0.06±0.02
1 h post-E	8.29±3.28*	2.75±1.93	0.23±0.08	0.91±0.27*	0.23±0.12	0.05±0.01
4 h post-E	1.16±0.89	0.14±0.03	0.23±0.05	0.09±0.19	0.10±0.04	0.06±0.02

* The value is significantly different from both the base-line value and time-matched E-plus-NAC value ($P < 0.05$).

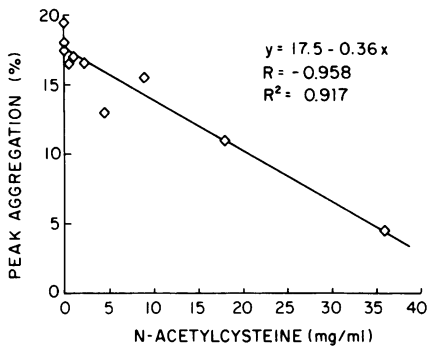

Figure 7. Effect of NAC on the in vitro ability of sheep leukocytes to aggregate in response to ZAP.

acid (AA) metabolism. This could occur either by blockade of critical enzymes in the metabolic pathway, such as cyclooxygenase or phospholipase A, or by otherwise preventing either the release of free arachidonic acid, or its oxidation (37). It is unlikely that NAC inhibits the cyclooxygenase enzyme, since we have found that NAC given either before or subsequent to an infusion of AA is incapable of preventing or abolishing the pulmonary artery pressure rise associated with such infusions (P_{PA} = 22 torr at base line; 40 torr with AA infusion; 38 torr with NAC + AA). This pressure increase seen with arachidonate infusion is felt to be secondary to the conversion of AA to prostaglandin exdoperoxides through the cyclooxygenase pathway and is inhibited by cyclooxygenase inhibitors (38). The effect of NAC on phospholipase A is unknown, though an inhibitory effect on this enzyme would be compatible with our data. Alternatively, and consistent with our hypothesis, NAC may work by decreasing cell membrane damage and the attendant release of phospholipids.

NAC also markedly blunted both the early- and late-phase increase in lung lymph flow. The early-phase attenuation is probably accounted for in part by the accompanying attenuation of the early phase pulmonary hypertension. However, the decrease in lymph flow seen in the animals treated with NAC is out of proportion to that expected to occur for the corresponding drop in pulmonary pressure. This is readily explained if permeability alterations begin to occur during the early phase. Support for the latter concept is found in studies using cyclooxygenase inhibitors in which there persists a significant increase in lymph flow by as early as 1 h post-E, despite only a mild rise in pulmonary artery pressure (3). In addition, histologic studies have revealed electron microscopic evidence of endothelial cell damage by 30 min post-E, although light microscopy does not reveal edema formation until 2 h after endotoxin (4). Thus, it is possible that microvascular permeability increases early in response to endotoxemia, and that NAC attenuated this pathological response. Further support for the ability of NAC to attenuate E-induced permeability changes is found in the late (3–4 h) phase response, where there is a high relatively protein-rich lymph flow, which has been associated with frank nonhydrostatic pulmonary edema (2).

Although this study was not designed to address the mechanism of the observed alterations in lung function, it is clear from other studies in our laboratory that granulocyte depletion can attenuate the sheep's airway response to E (6). It is conceivable that granulocytes contribute to the altered lung mechanics either by inducing vascular and/or airway damage directly, or by initiating the production of mediators that subsequently produce bronchospasm. The former postulate seems unlikely as the physiologic alterations in lung mechanics occur before the demonstration of marked histologic changes; although physiologic changes can precede histologic changes. This suggests that NAC interferes with either the production, release, or action of the proposed bronchospastic mediators. Of these possible mechanisms the first two would be consistent with the concept that NAC attenuates endothelial cell damage, which was suggested by the other data presented here. An antagonism of the bronchospastic mediators or a direct beneficial effect by NAC upon airway function can not be excluded by our data, but seems unlikely. NAC is not a bronchodilator, but rather can induce bronchospasm when given as an aerosol. In this study we found a reproducible deterioration in gas exchange when NAC was infused, but no alteration in lung mechanics. The etiology of the widened $\Delta AaPO_2$ is unknown.

While it is clear that NAC at high concentrations (1×10^{-2}

Table IV. Effect of NAC on In Vitro Sheep Leukocyte Function

	Aggregation (% change light transmittance)		Chemiluminescence (counts per minute)		β-Glucuronidase Activity (% of total)	
Stimulous	Control (n = 8)	NAC (n = 8)	Control (n = 6)	NAC (n = 6)	Control (n = 8)	NAC (n = 8)
Resting	0	0	2,297±544	3,255±881	0	10.7±1.2‡
Phorbol myristate acetate	39.5±0.9	35.6±1.0‡	84,816±27,886*	12,240±1,722*‡	0.3±0.3	12.9±1.6‡
Zymosan activated plasma	16.5±0.6	14.4±0.5‡	11,504±1,540*	5,630±462*‡	76.6±4.9*	88.2±6.0*

All values are mean±SEM. *The value is significantly different ($P > 0.05$) from the resting cell value. ‡The stimulant + NAC value is significantly different ($P < 0.05$) from the matched control stimulant values.

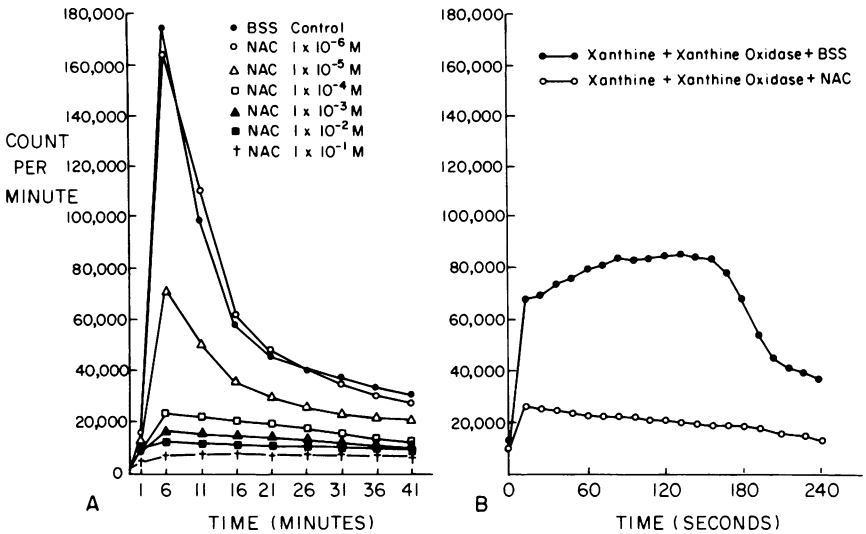

Figure 8. Representative example of the effect of NAC on chemiluminescence generated by (*A*) PMA-stimulated leukocytes; and by (*B*) a xanthine-xanthine oxidase-plasma reaction.

M) can have cytotoxic effects (independent of pH) upon sheep leukocytes as seen in both aggregation and enzyme release studies, it is unlikely that such concentrations were achieved in vivo. NAC has a small molecular weight and has relatively free access to all body tissue fluids. The plasma half-life of NAC has been estimated to be ~80 min. NAC in the plasma appears to be in an equilibrium between free drug and that bound to albumin and body proteins (38, 39). Under normal conditions then, NAC may be excreted via the kidneys only slowly, while under conditions of oxidant stress, it is readily metabolized by cells via the glutathione reductase pathway and the metabolites are rapidly excreted via the kidneys. Thus, it is likely that the NAC concentration to which the leukocytes were exposed in vivo is much less than the 1.7×10^{-2} M (2.8 mg/ml) concentration used during the in vitro experiments. Our data suggest that NAC did not reach high enough concentrations in vivo to affect leukocyte aggregation or degranulation.

Adherence has been shown to be vital in the leukocytes' ability to produce endothelial cell damage—possibly due to the short half-life of the oxygen free radical. Aggregation and adherence phenomena seem to be similar functions of the leukocyte, but subtle differences in extracellular cation requirements are known to exist (40, 41) and a recent report by Dahinden and Fehr (42) has shown that E increases leukocyte adherence to surfaces (petri dishes) without causing aggregation of granulocytes in suspension. It seems that NAC probably had little effect on leukocyte aggregation in vivo; but it is unclear what effect NAC had on adherence since these may be different phenomena. NAC did cause a progressive rise in the peripheral WBC count and tended to blunt the E-induced neutropenia. Differential cell counts revealed an increase in immature granulocytes, which suggested early bone marrow release of WBCs. Demargination effects can not be excluded. The animals had no fever, chills, or other alteration in the monitored physiologic variables that would suggest pyrogenic effects, and the NAC used was certified pyrogen free (Meade-Johnson Pharmaceutical Co., Evansville, IN). Therefore, we believe that the changes in leukocyte counts were caused by a direct effect of the drug itself rather than to contamination by pyrogens. In fact, if NAC is capable of decreasing in vivo granulocyte adherence this would provide an additional explanation for its effect on the endotoxin reaction.

In the in vitro studies, the reduction in chemiluminescence produced by granulocytes stimulated with either ZAP or PMA in the presence of NAC is consistent with either a decreased cellular release of oxygen free radicals or a scavenging effect upon these radicals once they were released. The ability of NAC to also attenuate chemiluminescence of a nonbiologic free radical generating system suggests that NAC is a direct free radical scavenger. Whether NAC also works to decrease cytotoxic effects of free radicals by an enhancement of the cell's cytoplasmic defense systems through replenishment of glutathione and cysteine stores is not known.

Finally, the inability of NAC to interfere with leukocyte degranulation in vitro is significant from a pathophysiologic standpoint because this suggests that NAC did not interfere with in vivo proteolytic enzyme release or function. The relative role of oxygen free radicals vs. activated enzymes in producing granulocyte-mediated endothelial cell damage in vivo is unclear. However, in vitro studies reveal that free radicals alone are capable of producing endothelial cell damage (9, 10).

Summary and conclusions. We have shown that in the awake sheep, E infusion produces a characteristic response and that NAC is able to attenuate all of the pathophysiologic changes monitored during this response. Activated leukocytes are felt to be important in many of these processes through release of toxic oxygen free radicals and activated lysozomal enzymes. NAC inhibits granulocyte aggregation and scavenges free radicals in vitro. It is postulated, therefore, that the beneficial effect of NAC in attenuating the pathophysiologic processes seen in this

animal model of the adult respiratory syndrome is due to its ability to scavenge oxygen free radicals in vivo. There has been, however, no direct measurement of the extent to which these free radicals are actually released in this model of ARDS. In addition, enhancement of cytoplasmic defenses against oxidant injury and a reduction of leukocyte adherence or chemotaxis remain as alternative explanations of NAC's beneficial effects. That NAC does not completely abolish all pathophysiologic responses to endotoxemia could be due to dosage or distribution factors, as well as to the complexity of the endotoxin response, which may involve multiple pathological pathways. While we are encouraged by the relative safety of intravenous NAC, and its potential role in ARDS or other disease states involving oxygen free radicals, we feel that further documentation of its beneficial effect is required before it can be recommended for clinical studies.

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