Low-dose *N*-Acetylcysteine Protects Rats against Endotoxin-mediated Oxidative Stress, But High-dose Increases Mortality

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We evaluated the effect of the antioxidant *N*-acetylcysteine (NAC) on oxidative stress, lung damage, and mortality induced by an endotoxin (lipopolysaccharide, or LPS) in the rat. Continuous intravenous infusion of 275 mg NAC/kg in 48 h, starting 24 h before LPS challenge, decreased hydrogen peroxide (H_2O_2) concentrations in whole blood ($p < 0.01$). This decrease was accompanied by fewer histologic abnormalities of the lung and decreased mortality ($p < 0.025$), compared with rats receiving LPS alone. *N*-Acetylserine, which has no sulfhydryl group, did not protect rats against LPS toxicity. Improved survival was not associated with an increase in pulmonary reduced glutathione, nor with inhibition of serum tumor necrosis factor (TNF) activity. *In vitro*, TNF production and DNA binding of nuclear factor kappa B (NF-κB) in human Mono Mac 6 cells was only inhibited at concentrations of NAC above 20 mM. High-dose NAC treatment (550 and 950 mg/kg in 48 h) decreased lung GSH ($p < 0.05$) and resulted in a significantly smaller number of surviving animals when compared with the low-dose NAC group ($p < 0.025$). *In vitro*, NAC increased hydroxyl radical generation in a system with Fe(III)-citrate and H_2O_2 by reducing ferric iron to its catalytic, active Fe^{2+} form. We conclude that low-dose NAC protects against LPS toxicity by scavenging H_2O_2, while higher doses may have the opposite effect. Sprong RC, Winkelhuyzen-Janssen AML, Aarsman CJM, van Oirschot JFLM, van der Bruggen T, van Asbeck BS. Low-dose ***N***-acetylcysteine protects rats against endotoxin-mediated oxidative stress, but high-dose increases mortality. AM J RESPIR CRIT CARE MED 1998;157:1283–1293.

N-Acetylcysteine (NAC), which is a scavenger of hydrogen peroxide (H_2O_2), hypochloric acid (HOCl), and hydroxyl radical (·OH) *in vitro* (1), has been successfully used in various models (2, 3) of adult respiratory distress syndrome (ARDS). Evaluation of the protective effects of NAC against lung damage induced by the endotoxin lipopolysaccharide (LPS) has been mainly directed to the hemodynamic effects of LPS, such as increased pulmonary arterial pressure (3, 4). Although sufficient studies support the involvement of reactive oxygen species (ROS) in ARDS (5–7), direct evidence for an antioxidant effect of NAC in LPS toxicity is still lacking. Recently, diminished peroxide levels in the expired breath of NAC-treated rats were reported in an interleukin (IL)-1 model of lung injury (8).

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There is increasing evidence that H_2O_2, directly or indirectly via its reduction product ·OH, acts as a messenger molecule in the synthesis and activation of inflammatory mediators. Oxidant scavengers, such as dimethyl sulfoxide and dimethylthiourea, inhibited LPS-stimulated IL-8 release in human whole blood (9). *N*-Acetylcysteine has been reported to diminish the expression of the vascular cell adhesion molecule VCAM-1 on endothelial cells *in vitro* (10) and to decrease TNF levels in endotoxic mice (2) and dogs (3). Several lines of evidence suggest that TNF gene expression is controlled by the transcription factor nuclear factor κB (NF-κB), which activity can be induced by H_2O_2 (11). Antioxidants have been shown to inhibit the activity of NF-κB in several cell lines, including the human monocytic cell line Mono MAC 6 (11, 12) and murine peritoneal macrophages (13).

Here, we report the effect of NAC in a rat model of LPS toxicity by monitoring oxidative stress, lung damage, and endogenous antioxidant protection in lungs. Oxidative stress was assessed from peroxide concentrations in deproteinized whole blood. The effect of NAC on the inflammatory mediator TNF was evaluated *in vivo* by measuring serum TNF activity, and *in vitro* using LPS-induced TNF production and NF-κB activity in Mono Mac 6 cells.

Toxicity of NAC is low, although adverse effects, including anaphylactic responses, have been observed in accidental high-

dose NAC infusion in humans. No adverse effects of NAC in LPS toxicity have been reported so far. Nevertheless, we found that high doses of NAC aggravate LPS toxicity. To understand this finding, its mechanism was further investigated. Autoxidation of thiols is known to occur in the presence of transition metals, such as iron(III), which then reduces to its catalytically active form Fe^{2+} (14). In addition, autoxidation of sulfhydryl groups may yield superoxide ($O_2^{-}\cdot$) and H_2O_2 (15, 16). Thus, factors necessary for ·OH generation according to the Fenton reaction (17)—i.e., H_2O_2 and Fe^{2+} ions—can be generated during thiol oxidation. *N*-Acetylcysteine, therefore, may also display a similar pro-oxidant activity. Therefore, the iron-reducing capacity of NAC and its potentiating effect on ·OH generation were also studied.

METHODS

Animals. Pathogen-free male Wistar rats weighing approximately 250 g (Iffa-Credo, Brussels, Belgium) were used for this study. They were given water and standard food *ad libitum*. All animal studies performed followed the institutional guidelines concerning the care and handling of animals.

Experimental procedures. After anesthetizing the rats with 0.5 ml/kg Hypnorm intramuscularly (10 mg/ml fluanison and 0.315 mg/ml fentanyl citrate) and 2.5 mg/kg Diazepam intravenously, animals were cannulated. A siliconized silastic cannula was inserted in the jugular vein, the end of the cannula reaching into the right atrium. The other end was passed subcutaneously to the skull, where it was fixed to a metal cannula (0.8 mm). During recovery, the system was filled with 50% polyvinylpyrrolidone and 500 U/ml heparin in saline to prevent occlusion by blood. After 7 d, the system was connected to a miniature single-channel swivel (Alice King Chatham Medical Arts, Los Angeles, CA). Continuous infusion of 2.0 ml NAC in 24 h (*N*-acetyl-L-cysteine, 5 g/25 ml, pH 6.95; Fluimucil infusion solution, Zambon, Amersfoort, The Netherlands) was maintained with a perfusion pump, modified for the use of 8 syringes. Different doses of NAC were given for 48 h: Group 1 received a loading dose of 75 mg/kg in 1 h followed by 100 mg/kg in 24 h (total dose 275 mg/kg in 48 h); Group 2 received a loading dose of 150 mg/kg in 1 h followed by a maintenance dose of 200 mg/kg in 24 h (total dose 550 mg/kg in 48 h); Group 3 received a loading dose of 150 mg/kg in 1 h followed by a maintenance dose of 400 mg/kg in 24 h (total dose 950 mg/kg in 48 h). Control rats received either saline for 48 h or *N*-acetyl-D,L-serine (NAS; Sigma, St. Louis, MO), with a structure similar to that of NAC but lacking the sulfhydryl group, in amounts equimolar to 275 mg NAC/kg in 48 h. The total volume infused in both NAC and control animals was 4 ml and the pH ranged from 6.95 to 6.52 for the NAC-containing solutions, compared to a pH of 5.97 for saline. All solutions were endotoxin-free as measured by the *Limulus amoebocyte* lysate assay. A lethal dose of 5 mg/kg LPS (phenol extract from *Salmonella ryphimurium*, L6511; Sigma) was given intraperitoneally 24 h after NAC infusion was started. This time point is referred to as t = 0 h. Rats were either sacrificed at times stated below or used for survival experiments.

In order to investigate whether the endogenous antioxidant glutathione is important in the defense mechanism against LPS, it was depleted in rats. Therefore, rats were intraperitoneally injected with L-buthionine-[S,R]-sulfoximine (BSO; Sigma) at a dose of 6 mmol/kg each day for 2 d. On the third day, LPS was intraperitoneally injected together with a third dose of BSO. Survival was measured 24 h after LPS injection.

Blood samples taken from the intravenous system were collected in heparin tubes for H_2O_2 determination. Serum was stored at −70° C until assayed for TNF.

Histology. For histological examination, the lungs were removed and fixed *ex vivo* by intratracheal instillation of phosphate-buffered 10% formalin at 20 cm H_2O. Sections were stained with hematoxylin and eosin. Histologic assessment was performed in a blind fashion and judged by three independent observers to assure that the presented data are representative.

Preparation of tissue homogenates. For the determination of pulmonary acid-soluble sulfhydryl and glutathione concentrations, rats were killed with 45 mg/kg of Nembutal (Ceva, Paris, France) mixed with 250 U heparin/kg to prevent blood clotting. Lungs were perfused blood-free *in situ* with saline via the pulmonary artery, and bronchoalveolar lavage was performed eight times with 10 ml saline. Lungs were frozen in liquid nitrogen until homogenization with an Ultra-turrax T25 (IKA Labortechnik, Staufen, West Germany) in two volumes (wt/vol) ice-cold 50 mM phosphate buffer, pH 7.4. After centrifugation (48,000 × *g*, 20 min, 4° C), tissue supernatant was used immediately for glutathione assay or stored at −20° C.

Hydrogen peroxide concentrations in whole blood. Blood samples drawn at t = 0 h and t = 3 h after LPS injection were immediately deproteinized using 2.5 ml ice-cold 5% trichloric acid per 1 ml whole blood. Samples were quickly centrifuged (2,100 × *g*, 10 min) and supernatants were adjusted to pH 7.0 by adding 3 M K-phosphate, pH 13.0. Hydrogen peroxide was measured in deproteinized samples using the oxidation of phenol red by horseradish peroxidase (18). Addition of exogenous catalase (100 μg/ml, thymol-free, from bovine liver; Sigma) to the pH-adjusted deproteinized sample totally abolished peroxidase-mediated oxidation of phenol red, indicating that other blood components do not interact with the H_2O_2 assay.

Total glutathione, reduced glutathione, and protein measurement. Total glutathione, defined as the sum of reduced glutathione (GSH) and oxidized glutathione (GSSG), was determined enzymatically in deproteinized lung supernatants, as described by Tietze (19), and expressed as μmol per g protein according to Bradford (20). Reduced glutathione was measured following the method of Prins and Loos (21).

Tumor necrosis factor levels in sera. Blood samples were drawn exactly 90 min after LPS injection. Preliminary studies with blood samples drawn every 15 min for the first 180 min after LPS injection showed that peak values of TNF occur at 90 min after LPS injection. Tumor necrosis factor was determined in heated (30 min at 56° C) serum using the murine L929-fibroblast cytotoxicity assay (22). L cells were cultured (37° C, 5% CO_2) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and gentamycin (10 μg/ml). Confluent cells were spliced with a trypsin ethylene diaminetetracetic acid (EDTA) solution (Gibco, Paisley, UK). Cells were seeded in a 96-well plate (Nunc, Roskilde, Denmark) at a density of 2×10^4 cells/well. After overnight incubation, various concentrations of RhuTNF (12.5–1600 pg/ml, activity approximately 1 μg TNFα/ml = 40,000 IU/ml; NIBSC, Hertfordshire, UK) and rat sera at different dilutions were added in duplicate. Actinomycin D (1 μg/ml final concentration in a total volume of 200 μl) was added and cells were incubated for 18 h. After fixation with 18.3% glutaraldehyde, cells were stained with 0.05% methylene blue for 30 min. The amount of TNF U/ml represents the reciprocal of the TNF dilution causing 50% cytotoxicity under the conditions described. To address the possibility that NAC in serum inhibits Rhu TNF-mediated L929 cell lysis *in vitro*, causing an artifact in the bioassay, cells were similarly incubated in triplicate with Rhu TNF (0–625 pg/ml) in the presence of NAC at concentrations ranging from 0–150 mM. The NAC-mediated protection was measured as an increase in the concentration of Rhu TNF that was needed to induced 50% lysis (LC50). The lowest concentration of NAC at which an inhibition of the LC50 of L929 cells by TNF was observed was 10 mM. In the presence of 10 mM NAC, the LC50 of L929 cells was reached at 273.6 ± 175.9 pg TNF/ml, compared with 70.9 ± 22.7 pg TNF/ml when NAC was not added to the incubation medium ($p = 0.026$), and at 82.1 ± 34.5 pg TNF/ml in the presence of 1 mM NAC (mean ± SD, n = 7).

Tumor necrosis factor production by Mono Mac 6 cells in vitro. In order to investigate whether NAC reduces TNF production *in vitro*, Mono Mac 6 cells (1×10^6 cells/ml) were preincubated with NAC at concentrations of 0–30 mM in IMDM supplemented with 10% FBS for 30 min (37° C, 5% CO_2). After 2 h of incubation with 30 ng LPS/ml, the amount of TNF released in the supernatant was measured using ELISA.

Nuclear extracts and electrophoretic mobility shift assay (EMSA). Mono Mac 6 cells (10^6 cells/ml) were preincubated with 0–30 mM NAC in IMDM supplemented with 10% FBS for 30 min at 37° C, then subsequently incubated with 30 ng LPS/ml for 2 h. Nuclear extracts were made according to Schreiber and colleagues (23). Electrophoretic mobility shift assays were performed as described by Dignam and colleagues (24). Binding of NF-κB to the ^{32}P-labeled double-stranded oligonucleotide representing the −605 NF-κB site of the hu-

man TNF gene (23) was carried out with 10 μg nuclear protein. Samples were separated on nondenaturing polyacrylamide gels in 1× TBE buffer (89 mM Tris-HCl, 89 mM borate, 2 mM EDTA, pH 8.3). Supershift assays with a polyclonal antibody directed against the p50 subunit of NF-κB (Santa Cruz Biotechical Inc., Santa Cruz, CA) were performed to investigate whether the DNA binding factor was indeed NF-κB.

Ferricytochrome c *reduction assay.* The reduction of ferricytochrome *c* (75 μM, horse heart type II; Sigma) to ferrocytochrome *c* in the presence of various concentrations of NAC was performed in Hanks balanced salt solution (HBSS, pH 7.4). The change in absorbance at 550 nm was continuously monitored for 30 min using a diode array spectrophotometer (Hewlett Packard 8452A; Hewlett Packard, Palo Alto, CA).

Hydroxyl radical generation during autoxidation of N*-acetylcysteine.* The generation of ·OH was determined by the oxidation of 2-keto-4-methylthiobutyric acid (KMB) to ethylene (25). Briefly, ethylene was assayed by gas chromatography (Model GC-9a; Shimadzu, Kyoto, Japan) using a stainless steel column packed with Carbosieve G (Supelco, S.A., Gland, Switzerland) and a flame ionization detector. The oven was heated from 145° C to 195° C (programmed to rise at 6° C/min). Nitrogen (N_2) was used as a carrier (flow rate, 50 cm^3/min). Glucose oxidase (100 mU/ml; Sigma), 5 mM glucose, 50 μM ferric citrate, 10 mM KMB, and various concentrations of NAC were incubated at 37° C in a shaking water bath. The assay was performed in HBSS. The glass tubes were sealed with rubber stoppers. After 30 min, a sample of 0.5 ml of headspace gas was taken using an airtight syringe and immediately analyzed. Ethylene production was determined by comparing it with a calibration chromatogram of a known amount of ethylene standard (Supelco S.A.) injected into the column.

Statistical analysis. Statistical significance was determined using Student's *t* test, with significance defined as $p < 0.05$. Unless otherwise stated, the standard deviation was taken as an estimate of variance. Statistical differences in the survival of rats were determined using Chi-Square distribution.

RESULTS

Effects of Various *N*-Acetylcysteine Concentrations on Lipopolysaccharide-induced Mortality

In the first series of experiments, the protective effect of NAC against LPS toxicity was evaluated 24 h after endotoxin challenge. No deaths were recorded after this time point. Intraperitoneal injection of rats with 5 mg LPS/kg induced extensive hemorrhages in lung, liver, gut, and kidney. Survival of control rats (n = 39) that received saline and were challenged with LPS was 53% (Table 1). In contrast, when rats (n = 29) were treated with NAC by continuous intravenous administration at a concentration of 275 mg NAC/kg in 48 h, starting 24 h before LPS injection, there was a significant decrease in mortality (83% survival; $p < 0.025$). Treatment with an equimolar concentration of NAS, which has a structure similar to NAC but lacks the sulfhydryl group, was used to evaluate whether that group is crucial for protection. Survival after LPS challenge (40%, n = 25) was not influenced by NAS treatment. The protective effect of NAC was only noticed in the group of animals treated with 275 mg NAC/kg in 48 h. Survival of rats receiving 550 mg/kg in 48 h (63%, n = 46) was not significantly improved, whereas a marked increase in mortality (21%, n = 25) was observed at a dose of 950 mg NAC/kg in 48 h. At these doses, a significantly ($p < 0.025$) smaller number of rats survived the LPS challenge than survived after receiving 275 mg NAC/kg in 48 h. This difference could not be attributed to a direct toxic effect of NAC itself, since there were no deaths among animals (n = 10) receiving 950 mg of NAC/kg in 48 h alone.

To validate the protective role of antioxidants against LPS toxicity, the effect of glutathione depletion on LPS-induced mortality was investigated. Treatment with BSO for 2 d reduced total glutathione in the lungs (6.0 ± 1.5 μmol/g protein, n = 8, versus 13.3 ± 3.2, n = 14 in saline-treated rats, $p < 0.01$) and significantly ($p < 0.005$) enhanced LPS-induced mortality (6% survival, n = 16).

TABLE 1

EFFECT OF *N*-ACETYLCYSTEINE AND *N*-ACETYLSERINE ON THE SURVIVAL OF ENDOTOXIN-CHALLENGED RATS

Treatment	Number of Surviving Rats*	Survival† (%)
Saline	21 (39)	53
NAC (mg/kg in 48 h)		
275	24 (29)	83‡
550	29 (46)	63§
950	7 (25)	32§
NAS (mg/kg in 48 h)		
337	10 (25)	40§
BSO	1 (16)	6§

Infusion with NAC or NAS (equimolar concentrations of 275 mg NAC/kg in 48 h) was started 24 h before rats were challenged with an intraperitoneal injection of 5 mg LPS/kg. Buthionine sulfoximine was given intraperitoneally at a dose of 6 mmol/Kg each day for 2 d. Then LPS was given on the third day with another dose of BSO. Survival was 24 h after LPS challenge.

* Data represent the number of surviving rats, with the total number of rats in parentheses.

† Data denote the percent survival.

‡ $p < 0.025$ when compared with saline treated rats, tested with chi-square statistics.

§ $p < 0.025$ when compared with rats treated with 275 mg NAC/kg in 48 h.

Lung Histology of *N*-Acetylcysteine; and *N*-Acetylserine Treated Rats after Lipopolysaccharide Challenge

Lungs from LPS-injected rats treated with either NAC, NAS, or saline were investigated for histologic abnormalities. Control animals, which were treated with saline but not with LPS, had no histologic abnormalities in their lungs (Figure 1A). Lungs of control LPS-treated rats receiving a continuous infusion of saline showed thickening of the alveolar capillary membrane, shedding of bronchiolar epithelium, and diffuse alveolar damage characterized by alveolar and interstitial hemorrhage and edema as well as extensive interstitial and alveolar infiltration with leukocytes (Figure 1B). A similar array of histological changes was observed in NAS-treated rats, in rats treated with 550 and 950 mg NAC/kg in 48 h, and in rats treated with 275 mg NAC/kg in 48 h that died from LPS (not shown). Rats treated with NAS and saline that survived the LPS challenge also showed intra-alveolar edema and hemorrhage, infiltrated leukocytes, and thickening of the alveolar capillary membranes (not shown). In marked contrast, animals treated with 275 mg NAC/kg in 48 h that survived showed no lung lesions (Figure 1C).

Effect of *N*-Acetyclysteine on Lipopolysaccharide-induced Oxidative Stress

Because the oxidant scavenger NAC protects against LPS toxicity *in vivo*, reactive oxygen species (ROS) involvement is most likely. Further investigations were therefore conducted to test the theory that NAC protects against LPS toxicity by scavenging oxygen metabolites. First, oxidative stress was monitored in LPS-challenged rats by measuring H_2O_2 levels in deproteinized whole blood using the oxidation of phenol red by horseradish peroxidase at t = 0 h at t = 3 h after LPS challenge. This method specifically measures H_2O_2, since addition of catalase to the deproteinized pH-adjusted sample totally abolished the oxidation of phenol red (not shown). As shown in Figure 2, a baseline peroxide concentration (at t = 0 h) of 226 ± 93 μM (n = 28) is present in deproteinized blood of

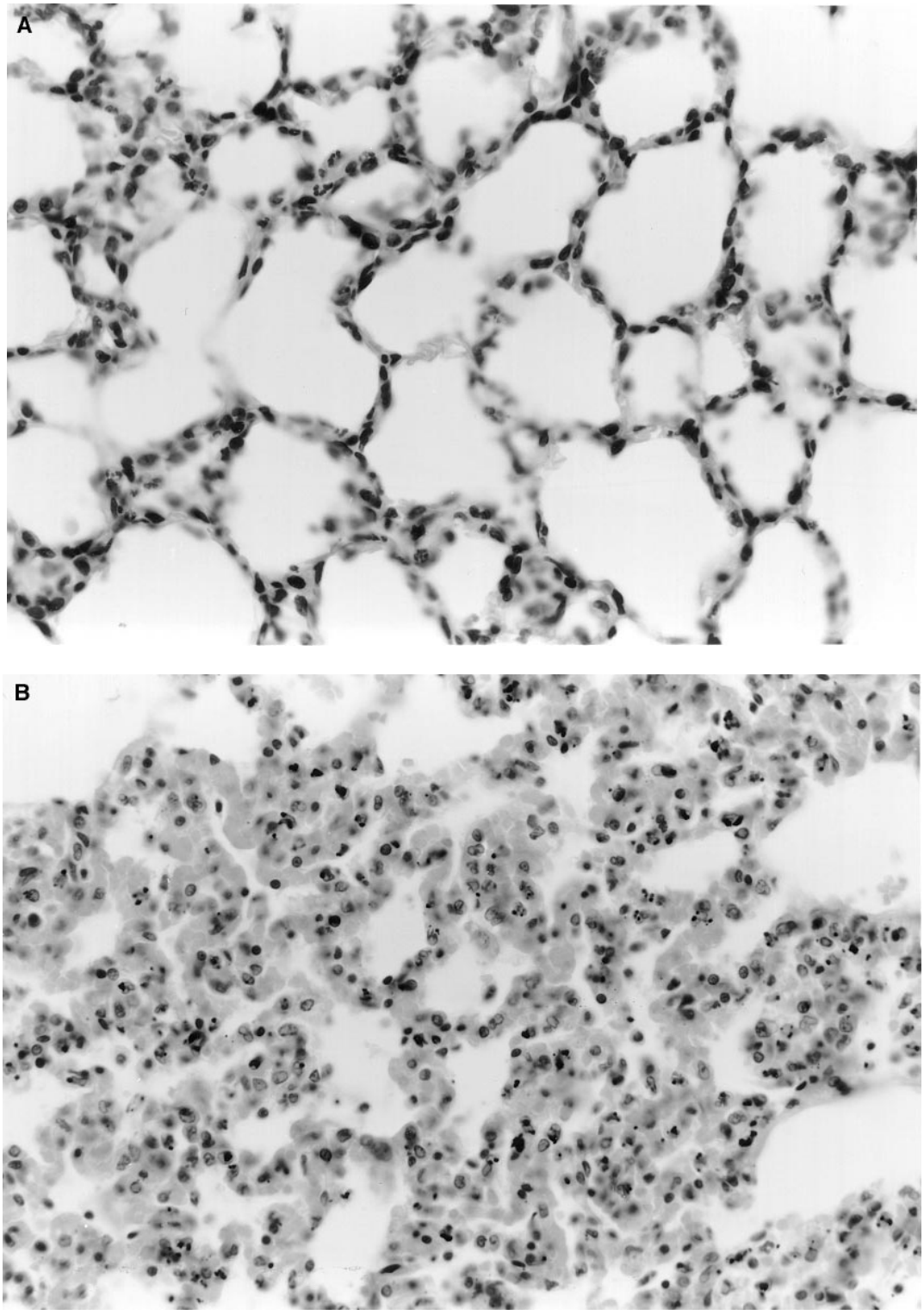


Figure 1. Histology of representative lung sections of rats treated with NAC and NAS and challenged with LPS. Lungs were removed *ex vivo* and fixed by intratracheal instillation of phosphate-buffered 10% formalin at 20 cm H_2O. Sections were stained with hematoxylin–eosin. Original magnification: ×100. (*Panel A*) Lung histology of a control (untreated) rat showing normal alveolar architecture. (*Panel B*) Lung tissue showing extensive alveolar damage of saline-treated animals that died within 24 h after LPS. (*Panel C*) Lung histology of a rat treated with 275 mg NAC/kg in 48 h sacrificed 24 h after LPS injection.

control saline-treated rats. Three hours after LPS injection, H_2O_2 levels were significantly elevated, up to 388 ± 125 μM ($p < 0.01$, $n = 18$), and remained increased for at least 6 h, suggesting that LPS induces oxidative stress in rats. As shown previously by carbon monoxide treatment of whole blood to prevent ROS generation during trichloroacetate treatment (due to autoxidation of heme-ferrous-oxygen complexes), the H_2O_2 increase after LPS challenge can be ascribed to endoge-

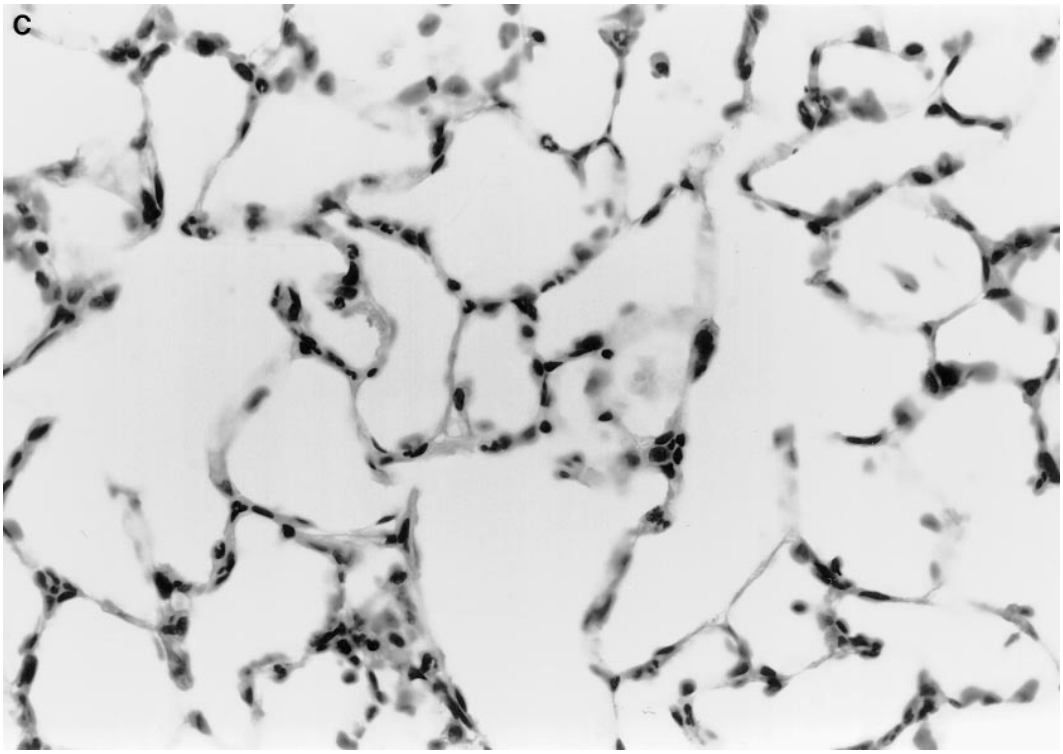


Figure 1. *Continued.*

nous H_2O_2 generation (26). When rats were treated with 275 mg NAC/kg in 48 h, baseline H_2O_2 levels (144 ± 49 μM, n = 16) measured 24 h after NAC infusion (at t = 0 h) were significantly ($p < 0.01$) lower than in control saline-treated rats. Although NAC-treated rats showed a slight increase in the peroxide concentrations at t = 3 h after LPS injection (257 ± 56 μM, n = 19), circulating H_2O_2 concentrations in saline-treated LPS-challenged rats were significantly ($p < 0.01$) higher. In contrast, an equimolar concentration of NAS could not reduce H_2O_2 levels in either control (239 ± 83 μM, n = 6) or LPS-treated rats (349 ± 71 μM, n = 9), implying an important role for the sulfhydryl group of NAC in its antioxidant activity. When higher doses of NAC were administered, no decrease in whole-blood H_2O_2 was observed (not shown). At 550 mg NAC/kg in 48 h, the H_2O_2 concentrations before and after LPS injection were 278 ± 93 μM and 389 ± 192 μM (n = 4), respectively. In addition, treatment with 950 mg NAC/kg in 48 h did also not result in diminished baseline H_2O_2 concentrations (241 ± 125 μM, n = 5) but, on the contrary, increased LPS-induced oxidative stress (528 ± 80 μM, n = 3).

Effect of *N*-Acetylcysteine on Total and Reduced Glutathione Concentrations

N-Acetylcysteine may act as a precursor of glutathione, which plays an important role in the defense of the lung against oxidants. Therefore, the effect of NAC on pulmonary total glutathione and GSH concentrations was measured several times after LPS injection. Because total glutathione is the sum of reduced (GSH) and oxidized (GSSG) glutathione, and the measurement is specific whereas the method we used for measuring GSH also measures non-GSH sulfhydryls, the combination of both determinations allows proper analysis of the glutathione status, including GSSG formation, which is always a result of oxidation of GSH. Treatment with 275 mg NAC/kg in 48 h did not result in augmentation of the total glutathione (Figure 3A) and GSH (Figure 3B) in the lungs during the first 24 h of infusion (measured at t = 0 h) when compared with saline-treated rats. When LPS was injected, pulmonary total glutathione levels increased significantly ($p < 0.05$) after 6 h in both saline-treated rats and animals receiving 275 mg NAC/kg in 48 h. Similarly, GSH concentrations were augmented significantly ($p < 0.05$) in both groups of rats 6 h after LPS injection. Reduced glutathione equaled total glutathione levels in saline-treated rats, whereas reduced glutathione levels in rats receiving 275 mg NAC/kg in 48 h were markedly higher than total glutathione levels, suggesting the presence of non-GSH sulfhydryls. After 12 h of LPS injection, pulmonary GSH significantly ($p < 0.02$) dropped in animals treated with 275 mg NAC/kg in 48 h to levels comparable to those at t = 0 h, suggesting oxidation of both the initial LPS-enhanced GSH and NAC-related non-GSH sulfhydryls.

Because 950 mg NAC/kg in 48 h aggravated LPS toxicity, we also investigated the influence of this dose on pulmonary glutathione levels. In contrast to treatment with the lower dose of NAC, animals treated with 950 mg NAC/kg in 48 h showed a significant ($p < 0.02$) increase in pulmonary total glutathione after 24 h of infusion, whereas GSH was not significantly increased. This finding indicates the presence of augmented GSSG by oxidation primarily of enhanced GSH as a result of high-dose NAC-induced oxidative stress, since the animals had not yet received LPS. When LPS was injected (t = 0 h), total glutathione content of the lung was not altered at either 6 h or 12 h after LPS challenge, but—obviously as a result of LPS-induced oxidative stress and GSSG formation—pulmonary GSH was significantly ($p < 0.05$) decreased.

Effect of *N*-Acetylcysteine on Tumor Necrosis Factor Activity

Because many symptoms of LPS toxicity can be ascribed to the cytokine TNF and a decrease in TNF activity has been de-

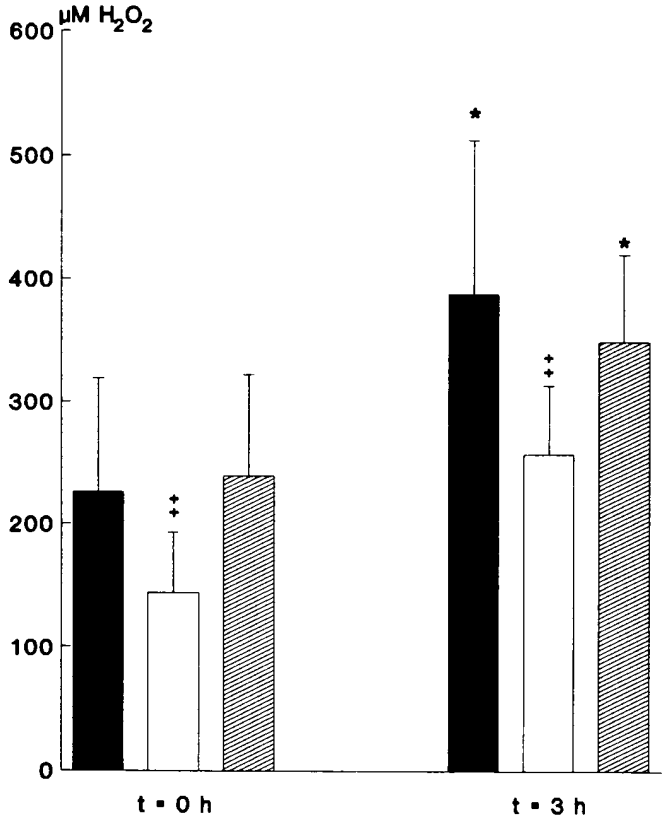


Figure 2. Blood H_2O_2 levels from rats treated with saline (*closed bars*), 275 mg NAC/kg in 48 h (*open bars*), or an equimolar concentration of *N*-acetylserine (*hatched bars*). Samples were taken 24 h after NAC infusion, just before LPS injection (t = 0 h) and 3 h after LPS injection (t = 3 h). Data are presented as the mean ± SD of μM H_2O_2 of values obtained from at least six rats. *$p < 0.05$ when compared with values at t = 0 h; ‡$p < 0.01$ when compared with values obtained from saline-treated rats.

scribed in endotoxin mice (2) and dogs (3), we assessed serum levels of bioactive TNF to investigate interference of NAC with TNF production. Tumor necrosis factor activity was not detectable in serum of control rats (< 8 U TNF/ml serum, detection limit). After injection with LPS, TNF activity was quickly elevated, with peak values at 90 min ($16.7 \times 10^3 \pm 9.7 \times 10^3$ U/ml, n = 12). Peak values of TNF were not altered in rats infused with either 275 mg NAC/kg in 48 h ($17.1 \times 10^3 \pm 8.1 \times 10^3$ U/ml, n = 11) or 950 mg NAC/kg in 48 h ($14.2 \times 10^3 \pm 7.9 \times 10^3$ U/ml, n = 8). As shown in the METHODS sections, the bioassay was not influenced by serum NAC concentrations below 10 mM. At higher serum concentrations of NAC, we observed inhibition of RhaTNF-mediated L929 cell lysis.

In order to elucidate further the effect of NAC on TNF, *in vitro* studies with Mono Mac 6 cells were performed. Although this seems to be an indirect approach to examining the *in vivo* events in rats, human Mono MAC 6 cells are an appropriate model for investigating the effect of antioxidants on TNF production, since it has been shown that antioxidants act in a similar way on NF-κB activation and TNF production in murine (13) and rat (26) peritoneal macrophages. *N*-Acetylcysteine at concentrations of 0–10 mM did not alter TNF production in LPS-stimulated Mono Mac 6 cells (Figure 4). At concentrations of 20 mM and 30 mM, however, NAC markedly reduced TNF production (Figure 4).

Effect of *N*-Acetylcysteine on Nuclear Factor-κB Activation

In order to study whether the effects of NAC on TNF production of LPS-stimulated Mono Mac 6 cells were due to a reduction in NF-κB activity, binding of NF-κB to the NF-κB consensus locus of the human TNF promoter was determined. As shown in Figure 5, NAC at concentrations of 10 mM and lower did not attenuate NF-κB activation, while concentrations of 20 and 30 mM markedly reduced NF-κB DNA-binding. Incubation of the nuclear extracts with an antibody against the p50 part of NF-κB resulted in a supershift of the upper band (not shown), indicating that the transcription factor located in the upper band is indeed NF-κB.

Pro-oxidant Activity of *N*-Acetylcysteine in a Cell-free System

Thiols are very reactive and highly susceptible to oxidation to their disulfide form in the presence of a transition metal (16), and NAC at high concentrations worsened LPS toxicity; therefore, we considered the possibility that NAC might act as a pro-oxidant. In order to explore this idea, the conversion of ferricytochrome *c* to ferrocytochrome *c* by NAC was monitored. *N*-Acetylcysteine dose-dependently reduced ferricytochrome *c* (Figure 6). Cytochrome *c* reduction was not affected by superoxide dismutase, or SOD (not shown), indicating that $O_2^-\cdot$ is not involved in the NAC-mediated reduction of ferricytochrome *c*. Thus, in the presence of NAC, catalytically active iron can be formed which, in the presence of H_2O_2 may favor the generation of ·OH. To further investigate this possibility, we measured the formation of ethylene from KMB in a cell-free system consisting of the H_2O_2-generating system glucose plus glucose oxidase and 50 μM Fe(III)-citrate, in the absence or presence of NAC. In the absence of NAC, 65 ± 14 pmol ethylene (mean ± SEM; n = 5) was detectable. *N*-Acetylcysteine (10 mM) without H_2O_2 and iron did not generate ethylene from KMB (less than 75 pmol ethylele), whereas the amount of ethylene produced by NAC plus H_2O_2 was 274 ± 127 pmol ethylene (n = 8). However, when 10 mM NAC was added to the complete system, we measured a significant ($p < 0.01$) increase in the ethylene concentration (3,639 ± 790 pmol ethylene, n = 9; Figure 7A), suggesting production of ·OH. In the absence of H_2O_2, that is, the reaction between 50 μM Fe(III)-citrate and 10 mM NAC, ·OH (6,957 ± 1,409 pmol ethylene, n = 8) was also generated (Figure 7B). This reaction could be prevented by the addition of catalase (100 μg/ml; 1,437 ± 494 pmol ethylene, n = 8, $p < 0.005$), indicating H_2O_2 formation, presumably by the reaction of $O_2^-\cdot$ with NAC (*see* Reference 16). Indeed, when SOD (100 μg/ml) was added, the formation of ethylene markedly increased (9,481 ± 1,786 pmol ethylene, n = 8). These data suggest the reduction of Fe(III)-citrate by NAC to catalytically active Fe^{2+} ions, which catalyze ·OH generation from H_2O_2 in the Fenton reaction.

DISCUSSION

In the present study, we show that continuous infusion with the oxidant scavenger NAC improved survival of endotoxemic rats and resulted in fewer histologic abnormalities in the lung. Other investigators have consistently shown that NAC, when administered before LPS challenge, protects animals against the hemodynamic (3, 4) and lethal (2) effects of LPS. We have now demonstrated that the NAC-mediated protection against LPS toxicity is associated with decreased H_2O_2 concentrations in whole blood. This is in line with another study in which NAC decreased expired H_2O_2 in rats treated with IL-1 (8).

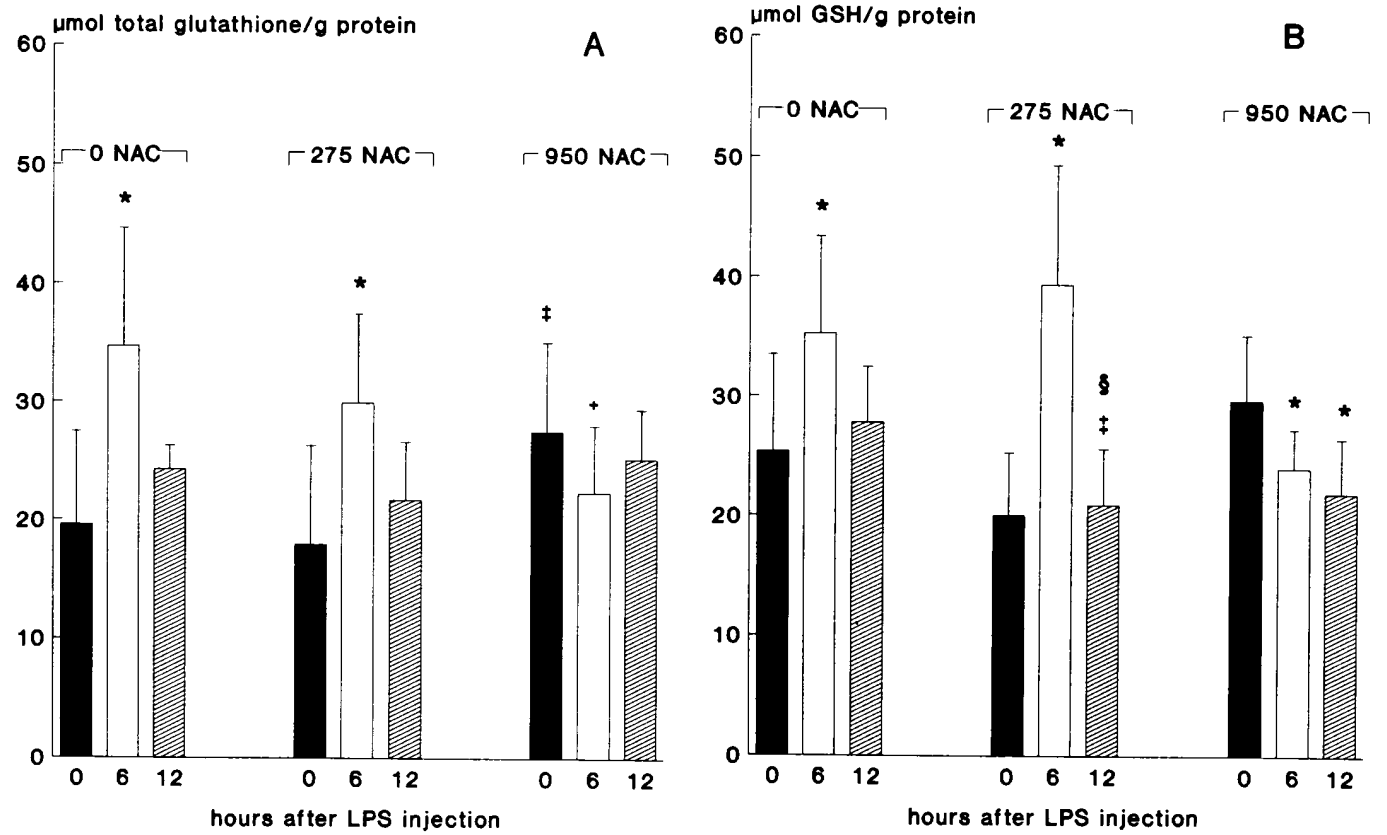
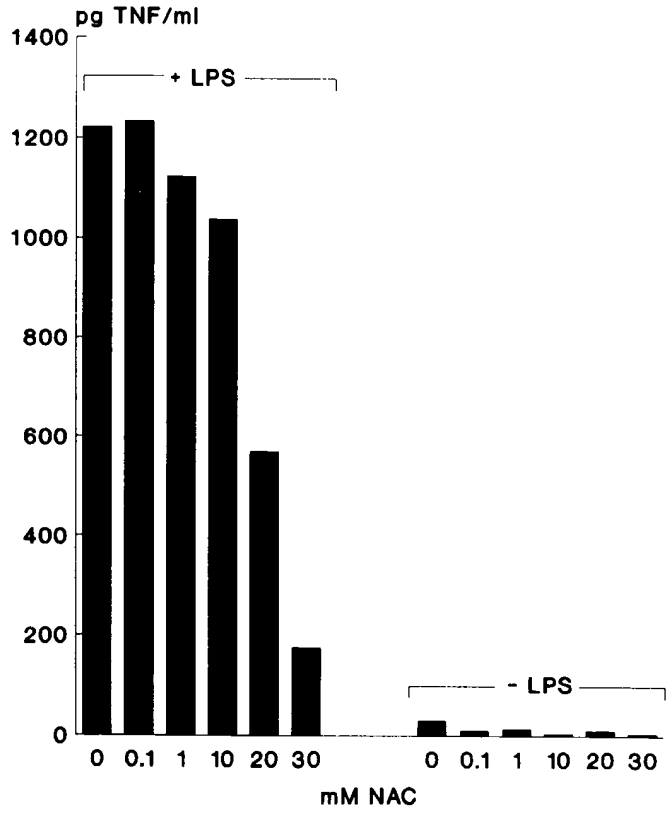


Figure 3. Effect of NAC on the concentrations of total pulmonary glutathione (*A*) and GSH (*B*) in rats treated with endotoxin. Continuous intravenous infusion with NAC started 24 h before rats were challenged with an intraperitoneal injection of 5 mg LPS/kg (at t = 0 h). Total glutathione (GSH + GSSG) and GSH concentrations were measured in rat lung homogenates. Animals receiving no NAC were intravenously infused with saline. Data represent means ± SD of values from at least 16 rats in the 0 h group and 5 rats in the 6 h and 12 h groups. Statistical significance was determined using Student's *t* test; *$p < 0.05$ when compared with t = 0 h; ‡$p < 0.05$ when compared with saline-treated rats at the same time point; §$p < 0.02$ when compared with t = 6 h.



The protective effect of NAC can be attributed to its sulfhydryl group because NAS, which lacks that group, did not improve survival and had no effect on whole-blood H_2O_2 levels. The NAC-mediated protection is probably a result of direct scavenging of toxic oxygen species and is not due to increased glutathione synthesis, since NAC (275 mg/kg in 48 h) had no effect on the concentration of pulmonary and hepatic (not shown) total glutathione. In addition, NAC did not influence the level of GSH. However, GSH increased (markedly more than total glutathione) in rats treated with 275 mg NAC/kg in 48 h at 6 h after LPS injection. This may be due to non-GSH sulfhydryls, either from NAC itself or its deacetylated form cysteine, interfering with the spectrophotometric assessment of reduced DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)], since this phenomenon was not observed in the saline-treated group of animals. These observations agree with another study (27), in which NAC had no effect on the concentration of total glutathione in plasma. *N*-acetylcysteine only enhanced the concentration of GSH in glutathione-deficient states, such

Figure 4. Effect of NAC on TNF production. The concentration of TNF was measured in the supernatant of Mono Mac 6 cells (10^6 cells/ml), preincubated with 0–30 mM NAC for 30 min in IMDM supplemented with 10% FBS, and subsequently incubated with (+LPS) and without (−LPS) 30 ng LPS/ml for 2 h at 37° C. The data of one experiment, representative of three performed, are shown.

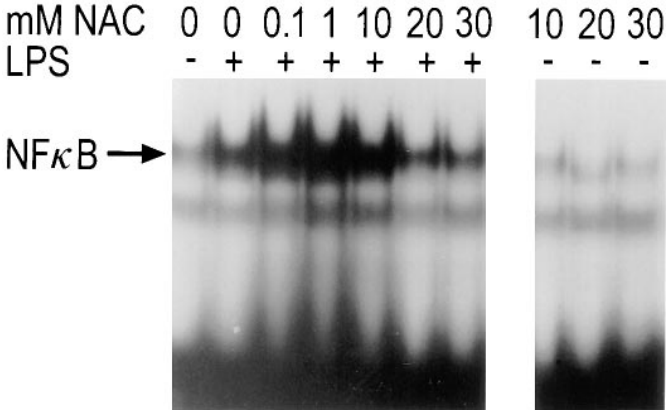


Figure 5. Effect of NAC on NF-κB activation. Activation of NF-κB was measured with an EMSA in nuclear extracts of Mono Mac 6 cells (10^6/ml), which were preincubated with 0–30 mM NAC for 30 min and subsequently incubated with 30 ng LPS/ml for 2 h at 37° C. Nuclear factor κB activation was measured in control unstimulated cells, in LPS-activated cells, in LPS-activated cells preincubated with 0.1, 1, 10, 20, and 30 mM NAC, and in cells exposed to 10, 20, and 30 mM NAC only.

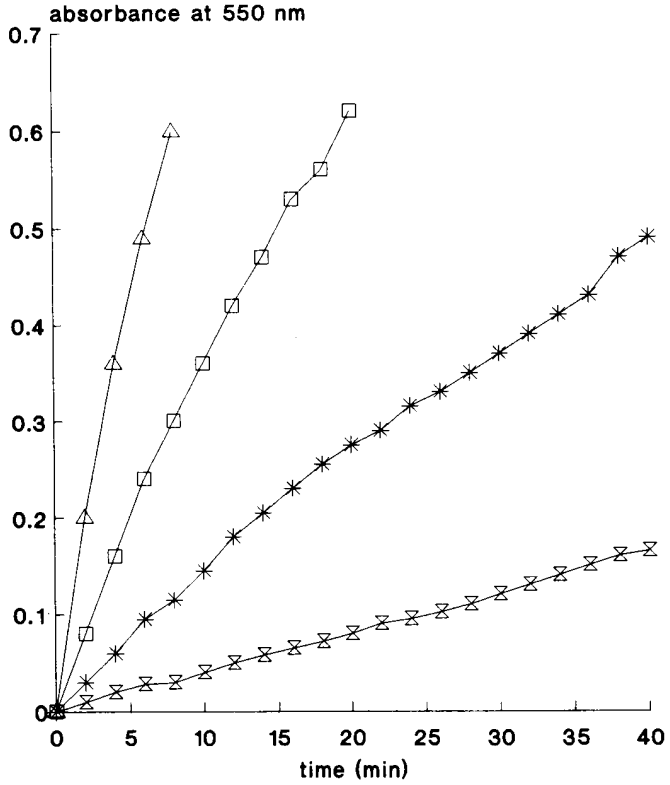


Figure 6. Concentration-dependent reduction of ferricytochrome *c* by NAC. Data are expressed as the increase in absorbance at 550 nm. *Double triangles* represent 0.1 mM, *asterisks* 0.5 mM, *squares* 1 mM, and *triangles* 10 mM NAC. The data of one experiment, representative of three performed, are shown.

as idiopathic pulmonary fibrosis (28), chronic obstructive pulmonary disease (29), and experimental models of sepsis (2) and ischemia/reperfusion (30). The observation that NAC inhibited the decrease in liver GSH in mice treated with LPS and the glutathione synthesis inhibitor BSO (2) suggests that NAC prevents oxidation of GSH by its own oxidant scavenging capacity, rather than by enhancing the synthesis of GSH.

The augmentation of total glutathione observed in both saline- and NAC-treated animals 6 h after administration of LPS suggests an increased synthesis in response to LPS-mediated oxidant exposure, as has been shown to occur in lungs (31) and in endothelial cells (32) after oxidant stress. It is conceivable that the same condition, oxidative stress, is also responsible for the subsequent fall in GSH in both saline- and low-dose NAC-treated rats as measured 12 h after the LPS challenge. This is consistent with the general opinion that oxidation of GSH is a parameter of oxidative stress.

The observed decrease in H_2O_2 levels after low-dose NAC treatment could result in protection against LPS-mediated oxidative stress by interfering with several peroxide-mediated pathways. First, less substrate is available for the generation of the highly toxic ·OH and of ROS generated by myeloperoxidase-catalyzed reactions. Second, inflammatory responses may be blunted, since a second messenger function of H_2O_2 and H_2O_2-derived oxidants, such as ·OH, has been suggested in the synthesis and activation of inflammatory mediators, including IL-8 (9) and TNF (2, 3). The activity of this latter cytokine has been reported to be mediated by the redox-controlled transcription factor NF-κB. Our *in vitro* studies with Mono Mac 6 cells also support the hypothesis that NF-κB and subsequent TNF production can be diminished by NAC. In our rat model of LPS toxicity, however, serum TNF activity was not decreased in NAC-treated animals. As shown in our *in vitro* studies, inhibition of NF-κB activity and TNF production could only be inhibited by high concentrations of NAC ($>$ 10 mM). These results are consistent with those reported by others (11) who showed that high concentrations of NAC (20 and 30 mM) inhibited the activation of NF-κB by H_2O_2 as well as its activation by TNF and PMA. At the highest dose of 950 mg/kg in 48 h in our study, assuming no disappearance from the circulation, the plasma NAC concentration would accumulate to 45 mM (calculated from data in reference 33). However, these concentrations were probably not achieved because the NAC terminal half-life after intravenous administration is 2 to 2.5 h (34, 35). The fact that NAC had no effect on TNF in our study, while it decreased TNF activity in mice (2) and dogs (3), might be explained by the higher doses of NAC used in those studies (1 g/kg orally administered NAC in mice [2] and a bolus injection of 150 mg/kg followed by 20 mg/kg/h in dogs [3]). Lower doses of NAC were not effective in decreasing TNF activity in mice (2). The highest dose used in our study (total dose 950 mg/kg in 48 h) was lower than those used by others. In addition, it has recently been reported that NAC dose-dependently suppresses LPS-induced NF-κB activation in rat lung tissue (36), suggesting TNF-α inhibition as well. The authors indeed observed a diminished inflammatory response as measured by cytokine-induced neutrophil chemoattractant mRNA expression and LPS-induced neutrophilic alveolitis. They administered NAC as a single intraperitoneal injection at concentrations of 200 to 1,000 mg/kg 1 h before the endotoxin challenge. This may have led to peak NAC plasma concentrations of about 3–15 mM at the time of LPS injection (calculated from data in references 34, 35), close to our *in vitro* NAC concentrations at which inhibition of NF-κB and TNF was observed. However, it is also possible that NAC was already partly oxidized before entering the blood stream. In our study, LPS was injected 24 h after the start of intravenous NAC at dosages accumulating to 175–550 mg/kg after a period of 24 h; therefore, it is possible that higher concentrations would have been necessary to achieve an inhibitory ef-

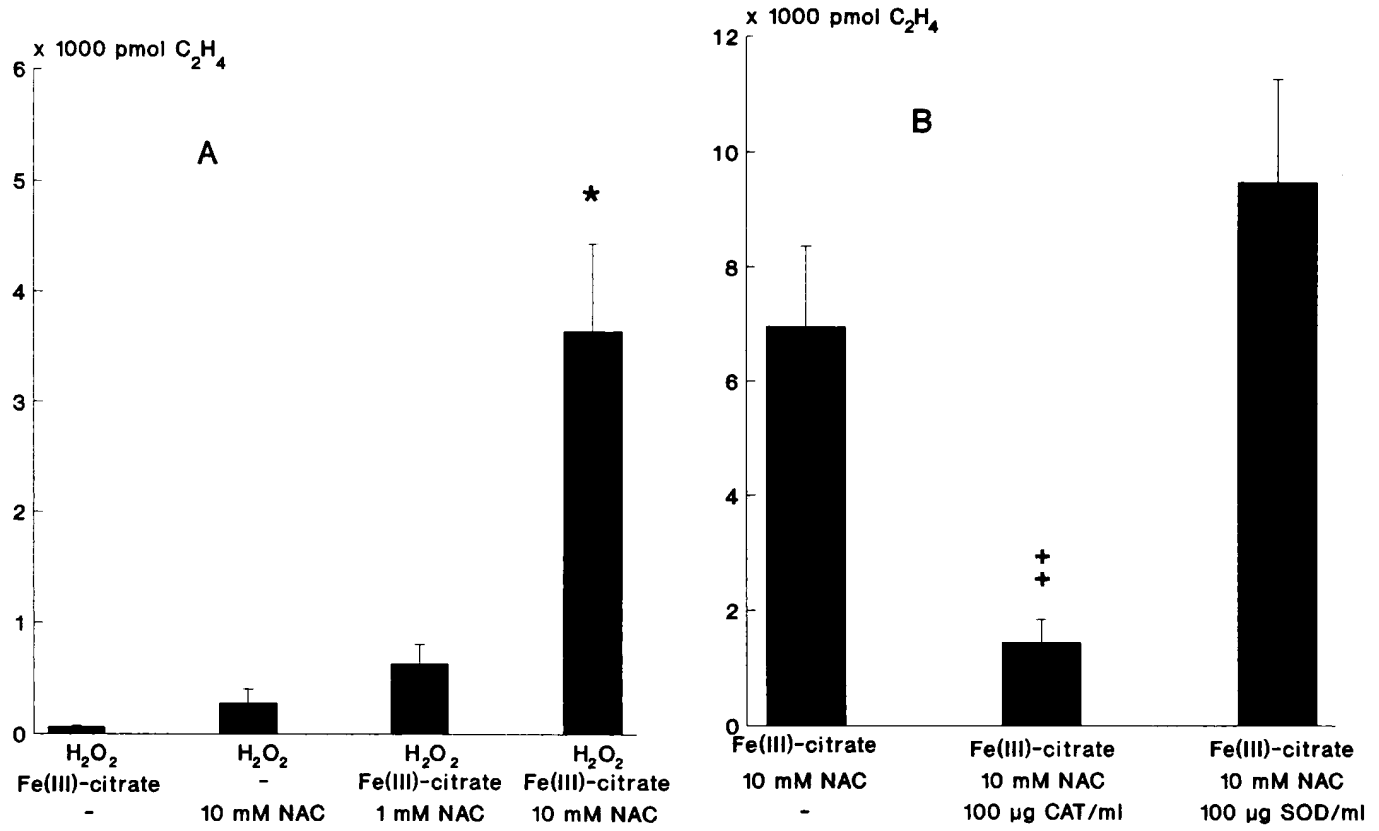


Figure 7. Catalytic effect of NAC on ·OH generation by H_2O_2 plus iron [50 µM Fe(III)-citrate], as measured by ethylene (C_2H_4) formation from 2-keto-4-methylthiobutyric acid at 37° C for 30 min. Hydrogen peroxide was generated by 5 mM glucose plus 100 mU glucose oxidase/ml. (*A*) Hydroxyl radical formation in the absence or presence of 1 or 10 mM NAC. (*B*) Effect of catalase (100 µg/ml) and SOD (100 µg/ml) on ·OH generation during the reaction between NAC and Fe(III)-citrate in the absence of H_2O_2. Data are presented as means ± SEM of pmol $C_2O_4 \times 1{,}000$ of values obtained from at least five experiments. *$p < 0.01$ when compared with values obtained with H_2O_2 and Fe(III)-citate alone; ‡$p < 0.005$ when compared with values obtained with Fe(III)-citrate and NAC alone.

fect on serum TNF levels. As discussed below, when we increased the dose of NAC, protection against LPS toxicity was apparently overruled by pro-oxidant effects. Comparison of the two studies suggests that these adverse reactions do not play a role when high-dose NAC is administered as an intraperitoneal injection. Alternatively, NAC at a concentration of 10 mM and higher may have inhibited recombinant-TNF-induced L929 cell lysis (*see* METHODS). Thus, depending on local tissue concentrations, NAC may reduce TNF cytotoxicity.

Increasing the NAC dose to 550 and 950 mg/kg in 48h did not result in improved survival and reduction of circulating H_2O_2. At these doses, H_2O_2 concentrations just before the LPS challenge (24 h after NAC treatment) were not significantly different from H_2O_2 concentrations in animals treated with saline, which contrast with the inhibition of the baseline H_2O_2 level in the 275 mg NAC/kg in 48 h group. Although treatment with 950 mg NAC/kg/48 h for 24 h significantly increased pulmonary total glutathione, GSH was not enhanced. This suggests the presence of oxidized GSH (i.e., GSSG) that was formed under the influence of high-dose NAC, since the animals had not yet received LPS. Furthermore, the failure of protection against LPS paralleled a lack of increase in pulmonary total glutathione levels, as was noticed 6 h after LPS injection in low-dose NAC-treated animals and which was a result of the LPS challenge itself. These observations, together with the decrease in GSH at 6 h and 12 h after LPS injection, strongly suggest further oxidation of GSH by oxidative stress induced by high-dose NAC (950 mg/kg in 48 h) as well as by LPS. A pro-oxidant effect of NAC might indeed account for the decrease in survival of LPS-challenged rats receiving the highest dose, since in those animals a drug-associated increase in while blood H_2O_2 was observed. This effect of NAC is supported by the considerable literature reporting that low-molecular-weight thiols are pro-oxidants as well as antioxidants (14–16). Pro-oxidant activity is the result of transition metal-dependent autoxidation yielding $O_2^{-}\cdot$, H_2O_2, and the reduced form of the transition metal, which may behave as a catalyst in free radical reactions. In the present study we show the ability of NAC to reduce iron to the ferrous oxidation state in a cell-free system, which results in ·OH generation in the presence of H_2O_2. In the absence of H_2O_2 (i.e., the reaction of NAC with ferricitrate), ·OH was also generated. Inhibition of ethylene formation by catalase provides evidence for the generation of H_2O_2 in this reaction. Consequently, addition of SOD, which favors H_2O_2 formation, resulted in increased ·OH production. Thus, thiols can serve as electron donors for metal-catalyzed ·OH generation and oxidative damage of biological structures.

The finding that high-dose NAC did not reduce the level of circulating H_2O_2 but, on the contrary, enhanced LPS-induced oxidative stress is supported by our *in vitro* observations. However, the exact cause of high-dose NAC–associated mortality is not completely clear. Histological examinations did not reveal an enhancement of lung injury in relation to the in-

creased mortality. This might indicate that in a specific treatment group some animals were able to resist toxicities induced by LPS and high-dose NAC, whereas other animals died, due to natural differences in resistance against endotoxemia and oxidative stress. Furthermore, it is unlikely that the increased mortality was a result of acidosis-related hemodynamic changes or volume overload–related pulmonary edema, which could synergize with LPS-related events, since there were no relevant differences in the pH of NAC-containing solutions and saline or in the total volume infused in low- and high-dose NAC regions or saline controls. *N*-acetylcysteine–related toxicity can probably also not be ascribed to peak NAC levels, as LPS was injected 24 h after the start of the infusion and at that time a steady-state concentration of NAC in body fluids might be expected.

In our study and those of others (2–4), LPS was injected after steady-state concentrations of NAC have been achieved. This may limit the therapeutic value of NAC in sepsis. However, other investigators showed that infusion of NAC after intratracheal administration of IL-1 attenuated lung damage in rats (8). In addition, recent clinical trials of NAC suggest that it may shorten the duration of active lung injury (37, 38).

In summary, our data indicate that NAC's protection against endotoxin-related mortality can be attributed to its capacity to directly reduce oxidative stress, rather than augmenting endogenous glutathione stores or reducing TNF production. Because low-dose NAC powerfully protects against LPS toxicity, this drug and other scavengers and antioxidants may be effective in the treatment of acute lung injury induced by gram-negative sepsis. However, at high concentrations, NAC, possibly by its capacity to reduce iron to the catalytically active form, increases oxidative stress and LPS toxicity. Therefore, antioxidant therapy must be critically controlled in order to minimize its unfavorable effects.

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