Effects of Various Antioxidants on Endotoxin-Induced Lung Injury and Gene Expression

mRNA Expressions of MnSOD, Interleukin-1β and iNOS

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Abstract

Antioxidants have been shown to be effective in attenuating acute lung injury. In this study, we determine the effects of various antioxidants by different mechanisms on the lipopolysaccharide (LPS)-induced changes. LPS was administered intravenously at a dose of 10 mg/kg to anesthetized rats. LPS induced a significant decrease in blood pressure ($P < 0.01$) and increased exhaled nitric oxide (NO) from 3.60±0.18 to 35.53±3.23 ppb ($P < 0.01$) during an observation period of 4 h. Plasma nitrate concentrations also increased from 0.61±0.06 to 1.54 ±0.22 μmol/l ($P < 0.05$). LPS-induced oxygen radical release from white blood cells isolated from rat peripheral blood also increased significantly ($P < 0.001$). After the experiment, the lung weight was obtained and lung tissues were taken for the determination of mRNA expression of inducible nitric oxide synthase (iNOS), tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and manganese superoxide dismutase (MnSOD). Histological examination of the lungs was also performed. In the control group injected with saline solution, mRNA expressions of iNOS, IL-1β, TNF-α and MnSOD were absent. Four hours after LPS administration, mRNA expressions of iNOS, IL-1β, and MnSOD were significantly enhanced, but TNF-α was not discernibly expressed. LPS also caused a twofold increase in lung weight. Pathological examination revealed endothelial cell damage and interstitial edema. Various antioxidants were given 1 h after LPS administration. These agents include SOD, catalase (CAT), SOD + CAT or vitamin C (ascorbic acid). These antioxidants effectively reversed the systemic hypotension, reduced the quantity of exhaled NO and plasma nitrate concentration, and prevented acute lung injury. Administration of various antioxidants also significantly attenuated LPS-induced oxygen radical release by rat white blood cells. LPS induced mRNA expressions of MnSOD and iNOS were significantly depressed by these antioxidants. However, only SOD + CAT and vitamin C inhibited the mRNA expression of IL-1β. These results suggest that oxygen radicals are responsible for LPS-induced lung injury. Antioxidants can attenuate the lung injury by inhibiting mRNA expressions of iNOS and IL-1β.

Key Words: lung injury, lipopolysaccharide, interleukin-1β, manganese superoxide dismutase, catalase, inducible nitric oxide synthase, oxygen radicals, antioxidants, vitamin C

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Introduction

Sepsis is a well known risk factor for acute respiratory distress syndrome (ARDS) and septic shock is a type of sepsis syndrome with hypotension (17). Persistent systemic hypotension resulting in multiple organ dysfunctions is a frequent cause of death among patients who suffer from septic shock (14). Release of endotoxin (lipopolysaccharide, LPS) from the cell wall of gram-negative bacteria, is responsible for inducing a series of physiological or pathological changes that occur in septic shock and multiple organ dysfunction (30). Various molecular mechanisms of inflammation are implicated during septic shock.

Based on the results reported in the literature, researchers believe that reactive oxygen species (ROS) induced by LPS play a key role in hemodynamic and metabolic derangement (12). ROS include superoxide anion (O_2^-), nitric oxide (NO), hydroxyl radical (OH·), peroxynitrite ($ONOO^-$) and their by-products (e.g., hydrogen peroxide, H_2O_2). The proinflammatory properties of ROS include endothelial damage, increased microvascular permeability, formation of leukotriene B_4, recruitment of neutrophils at sites of inflammation, lipid peroxidation, DNA damage, and release of cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) (6). Under normal conditions, the injurious effects of ROS are limited, at least in part, by endogenous antioxidants. Of these cellular defense systems, superoxide dismutase (SOD) and catalase (CAT) have been extensively studied. SOD catalyzes the conversion of superoxide to H_2O_2. Catalase is an enzyme responsible for converting H_2O_2 to oxygen and water. The radicals and antioxidants in the body usually maintain a dynamic balance. In disease states, the balance can be destroyed and overproduction of oxygen radicals then induces tissue damage.

There are three isoforms of endogenous SOD that have been cloned, sequenced, and expressed (35). Nonetheless, LPS specifically induces the manganous form of SOD (MnSOD), which is cytoprotective (35). Furthermore, the endotoxic effects of LPS directly activate monocytes and macrophages, leading to the release of proinflammatory cytokines such as TNF-α and IL-1β (4). Both IL-1β and TNF-α have also been implicated as important mediators in the pathogenesis of endotoxemia and endotoxin-induced acute lung injury (14, 32).

In addition to increased generation of ROS, endotoxemia also results in the activation of inducible nitric oxide synthase (iNOS) and increases circulating nitric oxide (NO), thus, inducing a decrease in blood pressure (4). Furthermore, plasma levels of vitamin C are reduced in patients with sepsis or septic syndrome (11). Administration of vitamin C may be helpful to patients because antioxidant vitamin C can scavenge superoxides and peroxynitrites, and improve endothelial function (24). In this study, we tried to determine by different mechanisms whether antioxidants were effective in preventing LPS-induced lung injury. LPS was administrated to anesthetized rats. We observed and compared the time course of changes in blood pressure and exhaled NO concentrations. At the end of the experiment, lung tissues were taken for determination of mRNA gene expressions of MnSOD, IL-1β, TNF-α, and iNOS using reverse-transcription polymerase chain reaction (RT-PCR). The pathological changes in the lungs were also examined. Various antioxidants including SOD, CAT, SOD+CAT and vitamin C were used and their effects were observed.

Materials and Methods

Experimental Protocols

The animals were randomly divided into different groups: (1) the vehicle group (n = 6) received a vehicle injection of PSS (1 ml). The same volume of PSS was given 1 h after the first injection; (2) the LPS group received LPS (10 ml/kg in 1 ml of PSS); PSS without LPS was then injected 1 h after the administration of LPS; (3) the experimental group (n = 6 for each drug) included three subgroups receiving three antioxidants (SOD, 6,000 U/kg; CAT, 150,000 U/kg; SOD+CAT [SOD, 6,000 U/kg; CAT, 150,000 U/kg]; vitamin C, 7.6 mg/100 g body wt) at 1 h after the administration of LPS. Our pilot experiments revealed that exhaled NO from started to increase 1 h after LPS injection. We wanted to determine whether antioxidants were effective when administered before a significant increase in exhaled NO.

Our pilot experiments also indicated that severe lung edema occurred approximately 4 h after the administration of LPS. Accordingly, an observation period of 4 h was selected for each experiment. During this period, the SAP was continuously monitored and the NO concentration in expired air was determined every 30 min. At the end of the experiments, the animal was killed and the lung excised for weight measurement, histological examination, and mRNA expression of MnSOD, iNOS, IL-1β, and TNF-α.

General Preparation

Male Sprague-Dawley rats (300-350 g) were anesthetized with urethane (1 g/kg intraperitoneally). The trachea was cannulated and each rat was ventilated with a gas mixture containing 95% air and 5% CO_2 using an animal respirator. The tidal volume was kept

at 3 ml and the respiratory rate at 65 to 75 breaths/min. The femoral vein was catheterized for administration of fluids or drugs. The femoral artery was cannulated with a catheter and connected to a pressure transducer (Gould Instruments, Cleveland, OH, USA) for the recording of systemic arterial pressure (SAP).

All rats were randomized to receive either LPS (10 mg/kg dissolved in 1 ml of saline; Klebsiella pneumoniae, Sigma, St. Louis, MO, USA), or a vehicle (equal volume of normal saline) by slow injection into the femoral vein within 2 min. Ten min after injection, the exhaled air was collected in a 3-liter polyethylene bag for 20 min. After this period, the SAP was recorded, and the exhaled air was analyzed for the concentration of NO.

Measurement of NO

The NO concentration was analyzed using a chemiluminescence nitric oxide analyzer (Model 270B, Sievers, Boulder, CO, USA). The fundamental principle used to measure NO is based on the observation that ozone interacts with NO to generate chemiluminescent light that can be measured by a sensitive photomultiplier tube. The specimen (expired air) was aspirated into a gas purge chamber previously evacuated to 3 mmHg to remove the O_2 and stabilize the NO. Signals from the detector were calibrated by calibration kit (Sievers) with establishment of linear calibrated scale of NO amount versus analyzer output (mV). The NO concentration in exhaled air was expressed as parts per billion (ppb).

Measurement of Plasma Nitrate

Blood from the femoral artery of a rat, was centrifuged at 5,000 rpm for 10 min to remove the formed elements, and the plasma was frozen for at least 24 h before analysis. For measurement of plasma nitrate concentration, blood samples were deproteinized using cold ethanol (1:3 vol/vol) and reduced in boiling acidic vanadium (III) chloride. Using this technique, nitrates were quantitatively reduced to NO. The nitrate standard curve (0.1 μM to 4 μM) was constructed by adding small aliquots of aqueous sodium nitrate solution as the reactant to produce chemiluminescence read by the luminometer. The data were fit to a straight line. Only standard curves with correlation coefficients (r) higher than 0.95 were used.

Measurement of Chemiluminescence Generated by LPS -Stimulated White Cells

Ten milliliters of blood were taken from the arterial line and centrifuged at 5,000 rpm for 10 min. The buffy coat was aspirated using a pipette and resuspended into 1 ml of phosphate buffered saline (PBS), and then added upon an equivalent volume of Ficoll-Paque (Density 1.007, Pharmacia Chemical Co., Uppsala, Sweden) and centrifuged at 2,000 rpm for 20 min. The polymorphonuclear neutrophil (PMN)-rich (about 0.5 ml) middle layer was aspirated and an equivalent volume of PBS was added. The suspension was then centrifuged at 5,000 rpm for 10 min. Next, 5 ml of distilled water were added to the pellet to lyse the remaining red cells, and then the suspension was centrifuged again at 5,000 rpm. The pellet was resuspended in 1 ml of PBS and the PMNs were counted. The PMNs were then diluted in PBS to a concentration of 3×10^5 cells/ml.

Chemiluminescence was determined using a luminometer (model 1251, LKB Wallac, Turku, Finland) as described by Hsu *et al*. (15). The PMN suspension (3×10^5 cells/ml in 500 μl of PBS) was incubated with 200 μl of lucigenin (10^{-2} M) and 500 μl of LPS (10 mg/ml) in a 2-ml polystyrene vial. The control preparation (buffer blank) contained 500 μl of cell suspension, 200 μl of lucigenin, and 500 μl of PBS. Various antioxidants were then added to assess their influence on the release of O_2-derived free radicals from leukocytes.

mRNA Isolation and RT-PCR

mRNA was isolated from lung tissue using the mRNA Isolation Kit (Boehringer Mannheim, Indianapolis, IN, USA, Cat. No. 1741 985). Reverse transcription was performed using the reverse transcription kit (SuperScript, Gibco BRL, Gaithersburg, MD, USA). The mRNA (400 ng) isolated from each lung tissue sample was reversely transcribed to cDNA following the manufacture's recommended procedures. RT-generated cDNAs encoding MnSOD, iNOS, IL-1β, TNF-α, and β-actin (an internal standard) genes were amplified using polymerase chain reaction (PCR). RNA without a clear β-actin band in the RT-PCR products was considered technically insufficient and was discarded without further study. Pilot experiments were performed to optimize the cycle number and the amount of cDNA to yield PCR products that were in the linear range of amplification. Sequences of the oligonucleotide primers are listed in Table 1. The PCR was performed in a 50-μl reaction volume containing the following materials: *Taq* polymerase buffer (1 X), deoxynucleotide mixture (0.2 mM each), $MgCl_2$ (1.5 mM), *Taq* DNA polymerase (0.5 U), oligonucleotide primer (0.5 μM each), and RT products. After an initial denaturation at 93°C for 5 min, annealing at 60°C for 3 min and polymerization at 72°C for 3 min, 30 cycles

Table 1 Sequence of the oligonucleotide primers

mRNA	Upstream	Downstream	base pairs
MnSOD	5'-AATTCAGTGGCAGAGGAAAGCTGC-3'	5'-GTGAATTCGTGGTACTTCTCCTCGGTG-3'	194
iNOS	5'-ACAACGTGGAGAAAACCCCAGGTG-3'	5'-ACAGCTCCGGGCATCGAAGACC-3'	563
IL-1β	5'-CAGCTCTCTTTAGGAAGACA-3'	5'-CAAGGAGAAAGTAATGAC-3'	294
TNF-α	5'-CAGGGCAATGATCCCAAAGTA-3'	5'-GCAGTCAGATCATCTTCTCGA-3'	475
β-actin	5'—GGTATGGGTCAGAAGGACTCC-3'	5'-TGATCTTCATCGTGCTAGGACCC-3'	822

of amplification (93°C for 1 min, 60°C for 1 min, and 72°C for 3 min) were performed followed by a 10-min extension at 72°C, and ended at 4°C. Possible contamination of any PCR components was excluded by performing a PCR reaction with these components in the absence of RT product in each set of experiments (a negative control).

Analysis of PCR Products

An aliquot (15 μl) from each PCR reaction was electrophoresed (at 100 V) in a 1.8% agarose gel containing Tris boric acid, and EDTA (Tris 0.0445 M, boric acid 0.0445 M, and EDTA 0.001 M) and 0.2 μg/ml ethidium bromide. A 100-bp ladder (Pharmacia Biotech, Uppsala, Sweden) was used as a molecular size standard. Amplification of β-actin was used to demonstrate the presence of intact mRNA in each sample and to demonstrate the approximate equivalence of mRNA loaded in each RT-PCR. The PCR products were electrophoresed and visualized on 1.8% agarose gel (Life Technologies, Grand Island, NY) containing 0.5 mg/ml ethidium bromide. For estimation of the relative levels of gene expression, type 55 film (Polaroid, Cambridge, MA, USA) was used to photograph the ethidium bromide-stained gels. The photographs were analyzed by densitometry using ImageQuant (Microsoft) software. The relative intensities of the corresponding bands of the RT-PCR products were normalized after dividing them by the relative intensities of the corresponding β-actin bands (20).

Lung Histology

At the end of the experiment, the animal was killed with an overdose of pentobarbital. The lungs were excised and weighted. The left lower lobes were taken for histological examination. The tissues were immersed in 10% formaldehyde fixative for 24 h. The lung lobes were then washed for 8 h with tap water to remove the formaldehyde. For light microscopy, the lung tissue was dehydrated with graded alcohols (70%, 80%, 90%, 95%, and absolute alcohol, each concentration for 45 min), put into xylene for 1 h, and then embedded in paraffin at 60°C. A series of 5.0-μm sections were cut and stained with hematoxylin and eosin. The histological changes were observed using an Axioplan Microscope (Zeiss, Oberkochen, Germany).

Drugs

LPS (K. *pneumoniae*, Sigma, St. Louis, MO, USA) was used to produce endotoxic shock. Inhibition of the oxidant activity was achieved by injection of the following agents: superoxide dismutase (SOD, 3,000 units /mg, Sigma), Catalase (50,000 units /mg, Sigma), ascorbic acid (Vitamin C, Katayama Chem. CO, Osaka, Japan). These agents were dissolved in physiological saline solution (PSS) immediately before intravenous injection.

Data Analysis

Data were reported as means±SEM. One-way analysis of variance and Student's *t*-test were utilized for statistical evaluation. A *P* value less than 0.05 was considered to be significant.

Results

Effect of LPS on SAP and Exhaled NO

Fig. 1 illustrates the time course of changes in SAP and exhaled NO concentration in the vehicle control group of animals (n = 6) injected with 1 ml of PSS and the LPS animals (n = 6) injected with LPS (10 mg/kg). In this control group, the SAP and exhaled NO concentration changed very little during the 4-hour observation period. In the LPS group, the SAP started to decline approximately 30 min after the injection of LPS. The NO content in expired gas did not change significantly within 1 h after LPS administration. Thereafter, it increased rapidly. After 4 h, the mean SAP decreased from 127.4±9.91 to 81.4± 5.86 mmHg ($P < 0.001$), while the exhaled NO level rose from 3.60±0.18 to 35.53±3.23 ppb ($P < 0.001$).

Effects of Antioxidants on SAP and Exhaled NO

Treatment with antioxidants such as SOD, CAT,

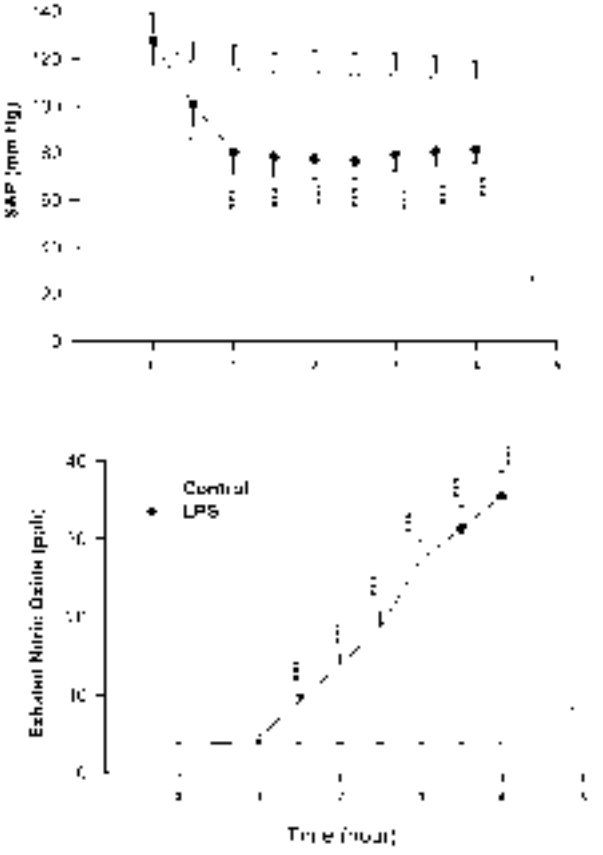

Fig. 1 Time course of changes in systemic arterial pressure (SAP) (a) and exhaled nitric oxide (NO) (b) in the control (n = 6) and the lipopolysaccharide (LPS) (10 mg/kg) groups. *P < 0.05, ***P < 0.001 compared to the control group. Data are mean ± SEM (n = 6).

SOD+CAT or vitamin C, for 1 h after LPS injection prevented systemic hypotension. With intervention by antioxidants, the SAP levels at 4 h after LPS were all significantly higher than those levels after injection of LPS alone (Fig. 2). The antioxidants also significantly reduced the concentration of NO released from the lungs (Fig. 2).

Effects of Antioxidants on Plasma Nitrate Concentration

Plasma nitrate levels in the LPS group were significantly increased from 0.61±0.06 to 1.54±0.22 mol/l (P < 0.05). The increase in plasma nitrate levels in LPS-treated rats was significantly attenuated by the various antioxidants (Fig. 3).

Chemiluminescence and the Effects of Antioxidants

The chemiluminescence from LPS-activated leukocytes increased from 1.39±0.47 to 9.93±0.28 mV (P < 0.001). All of the tested antioxidants significantly attenuated the chemiluminescence from leukocytes (P < 0.05 or P < 0.01) (Fig. 4).

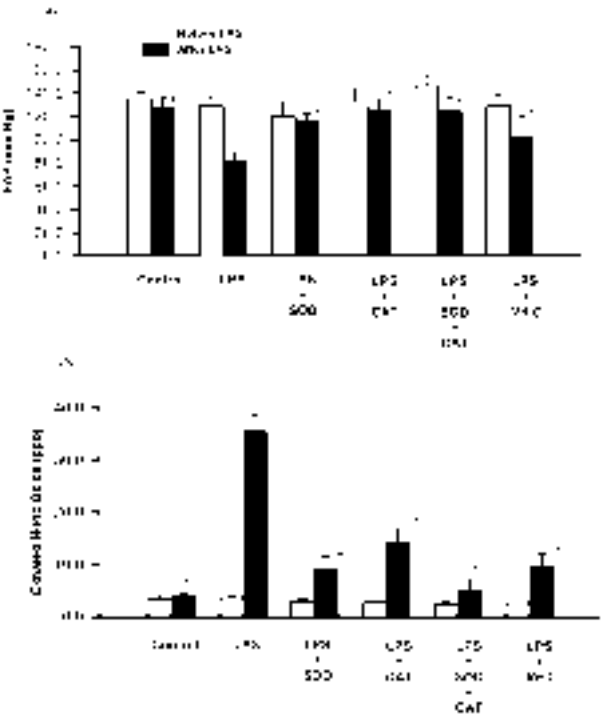

Fig. 2 Effects of various antioxidants on systemic arterial pressure (SAP) (a) and exhaled nitric oxide (NO) (b). *P < 0.05, as compared to the lipopolysaccharide (LPS) only group. Data are mean ± SEM (n = 6).

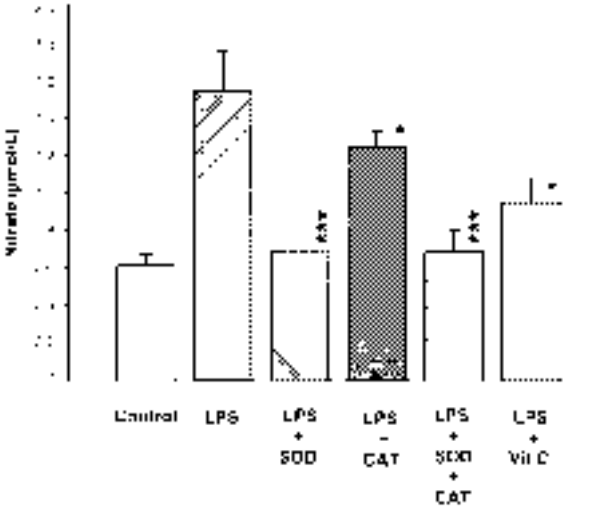

Fig. 3 Effects of various antioxidants on plasma nitrate concentration. *P < 0.05, ***P <0.001, as compared to the lipopolysaccharide (LPS) group. Data are mean ± SEM (n = 6).

mRNA Expression of MnSOD, iNOS, TNF-α and IL-1β in Lung Tissues

In rat lung tissue, expression of MnSOD, iNOS, TNF-α, and IL-1β mRNA (n = 4) was absent or negligible in the control group (n = 4) treated with

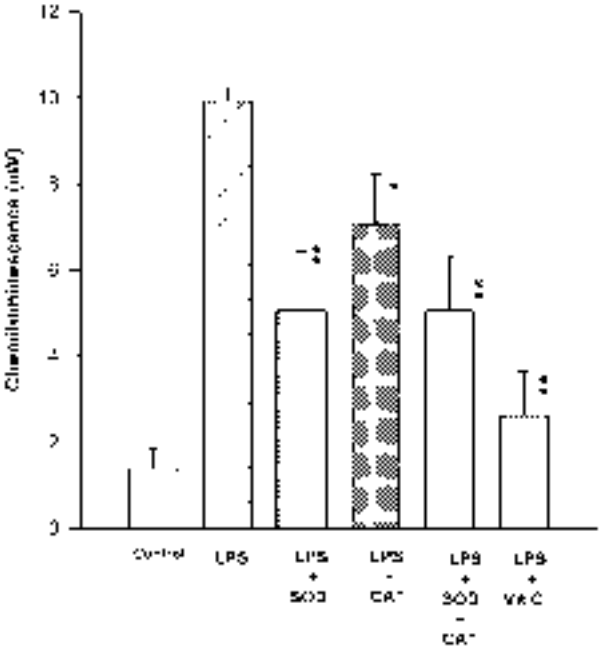

Fig. 4 Effects of various antioxidants on lipopolysaccharide (LPS) induced chemiluminescence of rat white cells. *$P < 0.05$, **$P < 0.01$, as compared to the LPS group. Data are mean ± SEM (n = 6).

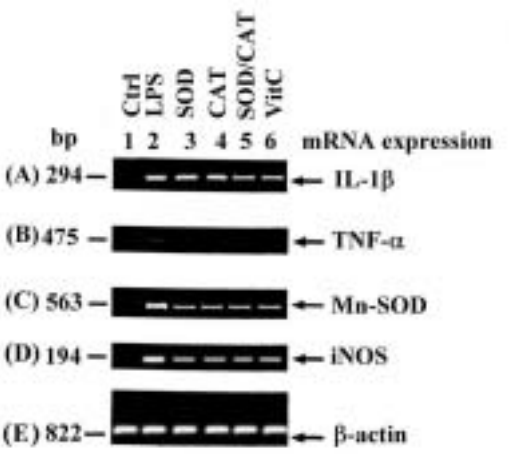

Fig. 5 Effects of various antioxidants on the levels of MnSOD, iNOS, TNF-α, and IL-1β mRNAs in lung tissue. MnSOD, iNOS, and IL-1β expressions were almost absent in the control but were markedly increased after lipopolysaccharide (LPS) injection. TNF-α expression was weak in the control and after LPS injection and with antioxidants. LPS-induced MnSOD and iNOS expressions were suppressed by SOD, CAT, SOD+CAT, and vitamin C. The LPS-induced IL-1β expression was suppressed by SOD+CAT and vitamin C. The expression of β-actin served as the internal control.

saline solution. LPS alone markedly increased the expression of MnSOD, iNOS, and IL-1β mRNA (n = 4) when compared to the control group ($P < 0.05$). The band density of TNF-α mRNA induced by LPS was not obvious. The LPS-induced MnSOD and iNOS mRNA expressions were significantly attenuated by treatment with the different antioxidants. LPS-induced IL-1β mRNA expression was significantly attenuated by treatment with SOD + CAT and vitamin C but not with SOD or CAT alone (Fig. 5).

Pathological Changes in the Lungs

Four hours after LPS administration, the lung weight (318±13 mg, n = 6) was greatly increased over the control value (153±11 mg, n = 6). Treatment with antioxidants significantly reduced the lung weight. The values in the SOD, CAT, SOD+CAT, and vitamin C groups (n = 6 for each group) were 168±11, 171±12, 169±4, 170±12 mg, respectively. These values were significantly smaller than those in LPS group ($P < 0.01$).

Histological examination of the lungs revealed that LPS produced severe tissue injury. The pathological changes included congestion of airways and alveoli, widening of interstitium, leukocyte aggregation in the pulmonary vessels, and interstitial edema (Fig. 6A). Treatment with antioxidants significantly attenuated these pathological changes, including a marked reduction in the inflammatory infiltrate with fewer numbers of both alveolar and interstitial neutrophils as well as reduced septal wall thickening (Fig. 6B, treatment with SOD; Fig. 6C, treatment with CAT; Fig. 6D, treatment with vitamin C).

Discussion

We observed the time course of changes in the SAP and exhaled NO concentration following an intravenous injection of LPS. It is interesting to note that the patterns of changes in SAP and exhaled NO were quite different (Fig. 1). A significant decrease in SAP occurred within 30 minutes after LPS administration and reached the maximum at 1 h. Thereafter, the SAP remained essentially unaltered. The exhaled NO concentration was significantly elevated 1.5 h after LPS injection and continued to rise during the experiment. At 4 h after receiving LPS, the exhaled NO concentration was increased more than eightfold. LPS induces iNOS activity and NO production in various organs (18). Our results indicated that NO formation in the lungs was relatively delayed. However, once the reaction was triggered, continuous formation of NO in the lung occurred. The continuous formation of NO in the lungs appeared to involve a vicious cycle in which interaction of endotoxin, cytokines, and free radicals (including NO) occurred. Finally, severe acute lung injury resulted from endothelial damage, increased vascular

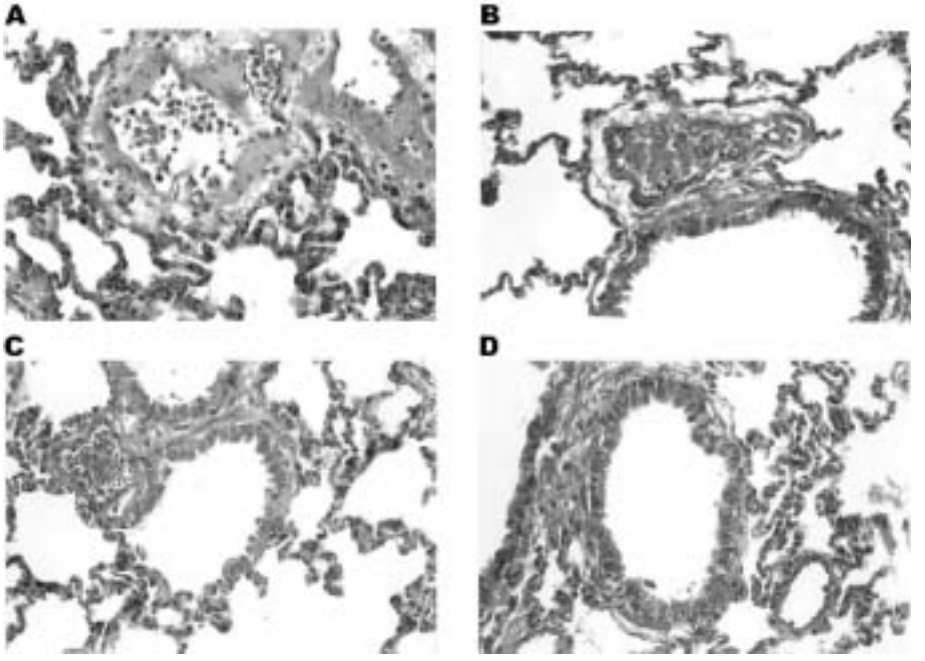

Fig. 6 Pathological changes in the lung caused by lipopolysaccharide (LPS). Note interstitial edema, aggregation of leukocytes in the pulmonary vessels and congestion of the airways and alveoli (A). Histological changes after LPS injection and treatment with SOD (B), CAT (C), and vitamin C (D) at 1 h after LPS injection. Lung pathology was prevented to a great extent by the antioxidants.

permeability, congestion of airways and interstitial spaces, and edema. These changes were prevented by treatment with various antioxidants. These agents were also effective in reversing systemic hypotension and minimizing acute lung injury.

The *in vitro* experiments showed that the LPS-induced respiratory burst of white cells could be scavenged by antioxidants such as SOD, CAT, SOD+ CAT, or vitamin C (Fig. 3). This proves that the antioxidant agents used in this study have antioxidant activity. LPS induced MnSOD mRNA expression in lung tissue in our study. The antioxidants (SOD, CAT, SOD/CAT and vitamin C) significantly decreased MnSOD mRNA expression. Antioxidant enzymes, such as MnSOD, are known to play a central role in the balance of oxygen radicals. Stimuli that increase antioxidant defenses of the cells may give some protection from various insults such as LPS, hyperoxia, and cytokines including TNF-α, IL-1β, IL-6, and radiation (4, 35). Treatment with anti-oxidants may change the whole oxidant state of the lung, and hence, protect the lung from the injurious effect of LPS and attenuating LPS-induced MnSOD mRNA expression. On the other hand, overexpression of MnSOD in human cancer cells abolished LPS-induced activation of nuclear factor κB (NF-κB), and hence, decreases the inflammatory response (23).

Various cytokines, such as IL-1β and TNF-α, have been implicated in endotoxic shock and NO overproduction (14, 32, 35). In cultured macrophages, endothelium, and smooth muscle cells, endotoxin caused rapid expression of IL-1β mRNA (8). The expression and production of TNF-α was also induced by IL-1β and endotoxin (29). Intravenous injection of IL-1β or TNF-α induced hypotension, pulmonary edema, and death similar to the septic shock syndrome (8, 29). The effect became synergetic when IL-1β and TNF-α were administrated together (29). In the present experiment, we found that 4 h after LPS injection, IL-1β mRNA was significantly expressed in rat lung tissue. However, the expression of TNF-α was not discernible. Such findings are consistent with those in the murine model of endotoxic shock, where TNF-α has been shown to increase in the first hour, peaking between 1 and 1.5 h after LPS administration, then returning back to control, unstimulated levels at 4 to 5 h after administration (1, 7). During this time, serum IL-1 was maximum at 4 h post-LPS injection and remained elevated at 24 h postinjection (36).

LPS induced gene expression, resulting in release of inflammatory mediators expression LPS bind to receptor in cell membranes, and activate a number of intracellular transcription factors, including NF-κB protein, activator protein-1 (AP-1), and nuclear factor-interleukin 6 (NF-IL-6). This allows rapid gene induction and the expression of inflammatory mediators, including cytokines, chemokines, lipid mediators, iNOS, enzymes, and adhesion molecules (22).

NF-κB can also be activated in cells by a number of inflammatory stimuli in addition to LPS, including cytokines such as TNF-α and IL-1β, reactive oxidant species (especially hydrogen peroxide), protein kinase C activators, viruses, UV light, and ionizing radiation (2). Feedback loops can be formed that may amplify the response to the initial stimulus or downregulate NF-κB activation. Oxidant species are reported to strongly activate NF-κB; therefore, using antioxidants can inhibit NF-κB activation and thus attenuate the inflammatory response (22). Therefore, strategies to enhance endogenous antioxidant defenses such as administering SOD, CAT, SOD+CAT, and exogenous antioxidant vitamin C effectively attenuated lung injury, possibly *via* inactivation of NF-κB.

Another reactive oxygen species that is closely associated with septic shock is NO. Many investigators considered that overproduction of NO was the major cause of circulatory failure in septic shock (4). However, other investigators did not think that NO itself was the cause of hemodynamic dysfunction in septic shock. They thought that perhaps it was interaction of NO with other reactive species that resulted in the circulatory failure in septic shock. Furthermore, if the increased formation of NO from iNOS was responsible for the hypotension seen after LPS injection, one might have expected an exacerbation of hypotension with SOD treatment (e.g., the SOD might have increased the amount of NO by preventing its reaction with superoxide). In fact, this exacerbation did not occur in our study. The benefit of treatment with SOD would be protection against organ injury caused directly by superoxide and $ONOO^-$ in the disease. Recently, one study considered that superoxide reacted with catecholamines, deactivating them *in vitro* (21). Treatment with an SOD mimetic reversed the hypotension in these animals with septic shock, suggesting that deactivation of endogenous norepinephrine by superoxide contributed to this aspect of vascular dysfunction (1). Whether CAT or vitamin C has the same effect is not clear.

Circulating concentrations of vitamin C are markedly depleted in patients with sepsis, systemic inflammatory response syndrome, and ARDS (11, 31). This indicates that oxidative stress plays an important role in these critical illnesses. *In vitro* studies showed that vitamin C inhibited replication of bacteria and prevented hydrogen peroxide injury to cultured microvascular endothelial cells (24, 25). Administration of vitamin C decreased impairment of cardiorespiratory dysfunction caused by LPS in sheep and improved survival after LPS administration in mice (10, 28). In the present study, we found that vitamin C was very effective in inhibiting gene expression, reversing hypotension, and attenuating acute lung injury. Vitamin C is a powerful electron donor, reacting with both superoxide and hydroxyl radicals (24). The protective effect of vitamin C is likely, at least in part, due to its antioxidant properties, because vitamin C inhibits the generation of oxidizing free radicals in endotoxin-exposed myocardium and neutrophils, and decreases endotoxin-induced oxidative modification of liver proteins (5, 9, 33).

Both ROS and NO are produced by macrophages and neutrophils after LPS administration (35). We also found that antioxidants suppressed NO production, including exhaled NO and plasma NO concentration, in rats. Antioxidants such as pyrroline dithiocarbamate and N-acetyl-L cysteine inhibit NF-κB and iNOS expression, suggesting that both are regulated by reactive radicals (26, 34). One study showed CAT with activity against H_2O_2, but not SOD against O_2^- inhibited iNOS expression and NF-κB activation, suggesting that H_2O_2-dependent oxidative stress is involved in the transcriptional induction of iNOS *via* NF-κB activation in LPS-stimulated macrophages (13). Nonetheless, we found that all antioxidants in our study suppressed NO production, including exhaled NO and plasma NO concentrations, and iNOS gene expression in rats. The reason for different result for SOD in our study is not clear. It might have been due to our more complex whole animal model rather than the use of a simple cell line.

Our experiment showed that LPS induced much gene expression of IL-1β, iNOS and MnSOD. ROS are reported to be involved in the mechanism of LPS induced inflammation, in particular in NF-κB activation (35). Binding sites for transcriptional regulatory factor NF-κB are present in the promoters of IL-1β, iNOS, and MnSOD, and activation of NF-κB is important in modulating the expression of these and other immunoregulatory mediators (22). Previous studies showed that inhibition of NF-κB activation prevented endotoxin-induced proinflammatory cytokine expression and neutrophil accumulation in the lungs (3, 19). Antioxidants may block these LPS-induced gene expressions by inhibiting ROS formation and consequently inactivating NF-κB. This suggests that LPS may activate NF-κB through a common mechanism involving the synthesis of ROS. ROS are not only injurious by-products of cellular metabolism but also serve as messengers mediating, directly or indirectly, the release and regulation of inflammatory mediators. Only SOD+CAT inhibited IL-1β mRNA, rather than SOD only or CAT only. Therefore, it appeared that both superoxide and hydrogen peroxide induced IL-1β mRNA expression in our experiment. Further investigation is needed to confirm this result.

Although the use of SOD, CAT, and vitamin C as antioxidant intervention in human disease was proved to be ineffective, in a number of animal models and in our study model, they were protective. For

example, the infusion of SOD attenuated endotoxin-induced lung injury in awake sheep (16). Infusion of CAT in unanesthetized sheep attenuated endotoxin-induced acute lung injury (27). Vitamin C reduced the endotoxin-induced lung injury in awake sheep (10). Furthermore, the acute lung injury induced by LPS was attenuated by all antioxidants in our experiment and was evidenced by the pathological lung changes.

In summary, our study had demonstrated that SOD, CAT, SOD+CAT, and vitamin C significantly reversed systemic hypotension, attenuated acute lung injury, and reduced the increase in exhaled NO and plasma nitrate concentration in LPS-injected rats. The LPS-induced chemiluminescence of PMN *in vitro* was also significantly reduced. The increase in gene expression of MnSOD and iNOS in LPS-treated lung tissue was minimized by these antioxidants. SOD+CAT and vitamin C but not SOD or CAT alone inhibited the increase of IL-1β mRNA expression in LPS-treated lung tissue. Whether the NF-κB activation was blocked by these antioxidants and whether ROS have a role in signaling transduction require study.

References

1. Bagby, G.J., Plessala, K.J., Wilson, L.A., Thompson, J.J. and Nelson, S. Divergent efficacy of antibody to tumor necrosis factor-alpha in intravascular and peritonitis models of sepsis. *J. Infect. Dis.* 163: 83-88, 1991.
2. Barnes, P.J. Nuclear factor-κB. *Int. J. Biochem. Cell Biol*. 29: 867-870, 1997.
3. Blackwell, T.S., Blackwell, T.R., Holden, E.P., Christman, B.W. and Christman, J.W. In vivo antioxidant treatment suppresses nuclear factor-κB activation and neutrophilic lung inflammation. *J Immunol*. 157: 1630-1637, 1996.
4. Cadenas, S. and Cadenas, A.M. Fighting the stranger-antioxidant protection against endotoxicity. *Toxicology* 180: 45-63, 2002.
5. Cadenas, S., Rojas, C. and Barja, G. Endotoxin increases oxidative injury to proteins in guinea pig liver: protection by dietary vitamin C. *Pharmacol. Toxicol.* 82: 11-18, 1998.
6. Chabot, E., Mitchell, J.A., Gutteridge, J.M.C. and Evans, T.W. Reactive oxygen species in acute lung injury. *Eur. Respir. J*. 11: 745-57, 1998.
7. Chu, E.K., Ribeiro, S.P. and Slutsky, A.S. Heat stress increase survival rates in lipopolysaccharide-stimulated rats. *Crit. Care Med*. 25: 1727-1732, 1997.
8. Dinarello, C.A. and Wolff, S.M. The role of interleukin-1 in disease. *N. Engl. J. Med*. 328: 106-113, 1993.
9. Dwenger, A., Funck, M., Leuken, B., Schweitzer, G and Lehmann, U. Effect of ascorbic acid on neutrophil functions and hypoxanthine/xanthine oxidase-generated, oxygen-derived radicals. *Eur. J. Clin. Chem. Clin. Biochem.* 30:187-191, 1992.
10. Dwenger, A., Pape, H.C., Bantel, C., Schweitzer, G., Krumm, K., Grotz, M., Lueken, B., Funck, M. and Regel, G. Ascorbic acid reduced the endotoxin-induced lung injury in awake sheep. *Eur. J. Clin. Invest*. 24: 229-235, 1994.
11. Galey, H.F., Davies, M.J. and Webster, N.R. Xanthine oxidase activity and free radical generation in patients with sepsis syndrome. *Crit. Care Med*. 24: 1649-1653, 1996.
12. Godde, H.F. and Webster, N.R. Free radicals and antioxidants in sepsis. *Crit. Care Med*. 21: 1770-1776, 1993.
13. Han, Y.J., Kwon, Y.G., Chung, H.T., Lee, S.K., Simmons, R.L., Billiar, T.R. and Kim, Y.M. Antioxidant enzymes suppress nitric oxide production through the inhibition of NF-κB activation: role of H_2O_2 and nitric oxide in inducible nitric oxide synthase expression in macrophages. *Nitric Oxide* 5: 504-513, 2001.
14. Hotchkiss, R.S. and Karl I.E. The pathophysiology and treatment of sepsis. *N. Engl. J. Med*. 348: 138-150, 2002.
15. Hsu, K., Wang, D., Shen, C.Y., Hsu, Y.S. and Shieh S.D. Protective effect of ketotifen on chemiluminescence of neutrophils. *J. Formosan Med. Assoc*. 87: 939-943, 1988.
16. Koyama, S., Kobayashi, T., Kubo, K., Sekiguchi, M. and Ueda, G., Recombinant-human superoxide dismutase attenuates endotoxin-induced lung injury in awake sheep. *Am. Rev. Respir. Dis*. 145: 1404-1409, 1992.
17. Levy, M.M., Fink, M.P., Marshall, J.C., Abraham, E., Angus, D., Cook, D., Cohen, J., Opal, S.M., Vincent, J.L. and Ramsay, G. SCCM/ESICM/ ACCP/ATS/SIS. 2001 SCCM/ESICM/ACCP/ ATS/SIS International sepsis definitions conference. *Crit. Care Med*. 31: 1250-1256, 2003.
18. Liu, S., Adcock, I.M., Old, R.W., Barnes P.J. and Evans, T.W. Lipopolysaccharide treatment in vivo induces widespread tissue expression of inducible nitric oxide synthase mRNA. *Biochem. Biophys. Res. Commun*. 196: 1208-1213, 1993.
19. Liu, S.F., Ye, X. and Malik, A.B. In vivo inhibition of nuclear factor-κB activation prevents inducible nitric oxide synthase expression and systemic hypotension in a rat model of septic shock. *J. Immunol*. 159: 3976-3983, 1997.
20. Lung, H.L., Ip, W.K., Wong, C.K., Mak, N.K., Chen, Z.Y. and Leung, K.N. Anti-proliferative and differentiation-inducing activities of the green tea catechin epigallocatechin-3-gallate (EGCG) on the human eosinophilic leukemia EoL-1 cell line. *Life Sci*. 72: 257-268, 2002.
21. Macarthur, H., Westfall, T.C., Riley, D.P., Misko, T.P. and Salvemini, D. Inactivation of catecholamines by superoxide gives new insights on the pathogenesis of septic shock. *Proc. Natl. Acad. Sci.* USA 97: 9753-9758, 2000.
22. Macdonald, J., Galley H.F. and Webster, N.R. Oxidative stress and gene expression in sepsis. *Br. J. Anaesth.* 90: 221-232, 2003.
23. Manna, S.K., Zhang, H.J., Yan, T., Oberley, L.W. and Aggarwal, B.B. Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *J. Biol Chem.* 273: 13245-13254, 1998.
24. May, J.M. How does ascorbic acid prevent endothelial dysfunction? *Free Radic. Biol. Med*. 28: 1421-1429, 2000.
25. Metnitz, P.G., Bartens, C., Fischer, M., Fridrich, P., Steltzer, H. and Druml, W. Antioxidant status in patients with acute respiratory distress syndrome. *Intensive Care Med*. 25: 180-185, 1999.
26. Meyer, M., Schreck, R. and Baeuerle, P.A. H_2O_2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J*. 12: 2005-2015, 1993.
27. Milligan, S.A., Heffel, J.M., Goldstein, I.M. and Flick, M.R. Effect of catalase on endotoxin-induced acute lung injury in unanesthetized sheep. *Am. Rev. Respir.Dis.* 137: 420-426, 1988.
28. Nonaka, A., Manabe, T. and Tobe, T. Effect of a new synthetic free radical scavenger, 2-octadecyl ascorbic acid, on the mortality in mouse endotoxemia. *Life Sci*. 47: 1933-1939, 1990.
29. Okusawa, S., Gelfand, J.A., Ikejima, T., Connolly, R.J. and Dinarello, C.A. Interleukin-1 induced a shock-like state in rabbits: Synergism with tumor necrosis factor and the effect of cycloxygnease inhabitation. *J. Clin. Invest*. 81: 1162-1172, 1988.
30. O'Reilly, M., Newcomb, D.E. and Remick, D. Endotoxin, sepsis, and the primrose path. *Shock* 12: 411-420, 1999.
31. Padayatty, S.J. and Levine, M. New insights into the physiology and pharmacology of vitamin C. *C. A. M. J.* 164: 353-355, 2001.

32. Pugin, J., Ricou, B., Steinberg, K.P., Suter, P.M. and Martin, T.R. Proinflammatory activity in bronchoalveolar lavage fluids from patients with ARDS, a prominent role for interleukin-1. *Am. J. Respir. Crit. Care Med*. 153: 1850-1856, 1996.
33. Rojas, C., Cadenas, S., Herrero, A., Mendez, J. and Barja, G. Endotoxin depletes ascorbate in the guinea pig heart. Protective effects of vitamins C and E against oxidative stress. *Life Sci*. 59: 649-657, 1996.
34. Schreck, R., Rieber, P. and Baeuerle, P.A. Reactive oxygen intermediates as apparently widely used messengers in the activation of NF-kappa B transcription factor and HIV-1. *EMBO J*. 10: 2247-2258, 1991.
35. Zelko, I.N., Mariani, T.J. and Folz, R.J. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic. Biol. Med*. 33: 337-349, 2002.
36. Zuckerman, S.H., Shellhaas, J. and Butler, L.D. Differential regulation of lipopolysaccharide-induced interleukin 1 and tumor necrosis factor synthesis: effects of endogenous and exogenous glucocorticoids and the role of the pituitary-adrenal axis. *Eur. J. Immunol*. 19: 301-305, 1989.