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THE PEROXYNITRITE CATALYST WW-85 IMPROVES PULMONARY FUNCTION IN OVINE SEPTIC SHOCK

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Abstract

Systemic inflammatory response syndrome (SIRS) is associated with excessive production of nitric oxide (NO•) and superoxide (O₂⁻), forming peroxynitrite (ONOO⁻), which in turn, acts as a terminal mediator of cellular injury by producing cell necrosis and apoptosis. We examined the effect of the ONOO⁻ decomposition catalyst WW-85 in a sheep model of acute lung injury (ALI) and septic shock. Eighteen sheep were operatively prepared and randomly allocated, either to the sham, control, or WW-85 group (n=6 each). Following a tracheotomy, ALI was produced in the control and WW-85 group by insufflation of four sets of 12 breaths of cotton smoke. Then, a 30 mL suspension of live *Pseudomonas aeruginosa* bacteria (containing 2–5×10¹¹ cfu) was instilled into the lungs according to an established protocol. The sham group received only the vehicle (30 mL saline). The sheep were studied in awake state for 24 hrs and ventilated with 100% oxygen. WW-85 was administered 1 h post injury as bolus infusion (0.1 mg/kg), followed by a continuous infusion of 0.02 mg•kg⁻¹•h⁻¹ until the end of the 24-h experimental period. Compared to injured but untreated controls, WW-85-treated animals had significantly improved gas exchange, reductions in airway obstruction, shunt formation, lung myeloperoxidase-, lung malondialdehyde-, lung 3-nitrotyrosine concentrations, and plasma nitrate-to-nitrite (NOx) levels. Animals treated with WW-85 exhibited less microvascular leakage and improvements in pulmonary function. These results provide evidence that blockade of the nitric oxide - peroxynitrite pathway improves disturbances from septic shock, as demonstrated in a clinically relevant ovine experimental model.

Keywords

Airway obstruction; ARDS; nitric oxide; nitrotyrosine; smoke inhalation

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INTRODUCTION

Every year, more than 750,000 patients in the U.S. develop sepsis, and ~ 30–40% of these patients die. More than 75% of these deaths occur within the first 24 hrs after establishing the diagnosis (1). Systemic inflammatory response syndrome (SIRS) is associated with an excessive production of reactive nitrogen species (RNS), such as nitric oxide ($\text{NO}\bullet$). Furthermore, it is proposed that in septic shock, the release of reactive oxygen species (ROS) from macrophages, e.g. superoxide (O_2^-) is increased (2). Subsequently, peroxynitrite (ONOO^-) is formed by a rapid reaction between $\text{NO}\bullet$ and O_2^- . ONOO^- , in turn, acts as a terminal mediator of cellular injury under various pathophysiological conditions of oxidative and nitrosative stress, and is able to induce cell necrosis and apoptosis (3). The reactivity and decomposition of ONOO^- is determined by the chemical environment and the ratio of O_2^- versus $\text{NO}\bullet$. It has been shown that ONOO^- may initiate toxic oxidative reactions in vitro and in vivo (4). Initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, inhibition of membrane Na^+/K^+ -ATPase activity, inactivation of membrane sodium channels, and other oxidative protein modifications contribute to the cytotoxic effect of ONOO^- . In addition, ONOO^- is a potent trigger of DNA strand breakage, with subsequent activation of the nuclear enzyme poly-ADP-ribosyl-polymerase (PARP), leading to cell dysfunction and pro-inflammatory responses (4).

Previous studies suggest that the peroxynitrite-PARP pathway importantly contributes to the cellular injury in septic shock (3). This large and rapidly expanding body of evidence on the role of ONOO^- in septic shock and a wide variety of human diseases has naturally led to a search for drugs that can detoxify this powerful oxidant. In multiple previous studies, ONOO^- neutralization in animal models of shock, critical illness and inflammatory conditions has been attempted by using a variety of compounds of various structural classes (2). Many of these compounds were suboptimal because they interacted with other reactive species, inhibited NOS and/or they were not catalytic in their reactions with ONOO^- (2). Recently, a potent, catalytic peroxynitrite decomposition catalyst has been synthesized, which showed efficacy in a variety of animal models of disease, including heart transplantation, interleukin-induced systemic inflammation, ventilator-induced lung injury and spinal cord injury (5–8). Using this compound, we have now attempted to better defining the pathogenetic role of peroxynitrite and its catalyst WW-85 in the current model of circulatory shock (9).

MATERIALS AND METHODS

Animal Care and Use

The Institutional Animal Care and Use Committee at the University of Texas Medical Branch (UTMB) at Galveston approved this study. The Investigational Intensive Care Unit (IICU) at UTMB is an Association for Assessment and Accreditation of Laboratory Animal Care International approved facility (AAALAC). The guidelines of the National Institutes of Health (NIH) for the care and use of experimental animals were carefully followed. The animals were individually housed in metabolic cages and were studied in awake state.

Surgical preparation

Eighteen female Merino sheep, weighing 36 ± 2 kg, were anesthetized with isoflurane (Vedco Inc., St. Joseph, MO) using an inhalation mixture of 1–2 Vol% in oxygen, and endotracheally intubated. The sheep were ventilated with a mixture of room air and O_2 (FiO_2 0.5). Under aseptic conditions, the animals were chronically instrumented for hemodynamic monitoring as previously described (9). Briefly, the right femoral artery was

cannulated, and a polyvinylchloride catheter (Intracath™, 16-G, 24-inch, Becton Dickinson Vascular Access, Sandy, UT) was positioned in the descending aorta. A 7-French Swan-Ganz™ thermodilution catheter (model 93A-131-7F, Edwards Critical Care Division, Irvine, CA) was inserted into the right jugular vein through an 8.5-French percutaneous introducer sheath (Edwards Lifescience, Irvine, CA) and was advanced into the common pulmonary artery. Through a left thoracotomy at the level of the 5th intercostal space, a Silastic® catheter (0.062 inch inner diameter, 0.125 inch outer diameter, Dow-Corning, Midland, MI) was positioned in the left atrium. The animals were given a seven-day recovery period. During this time, they had free access to food and water.

Measured variables

One day before the experiment had begun; catheters were connected to pressure transducers (Model PX***3X3, Baxter Edwards Critical Care Division, Irvine, CA) with continuous flushing devices. Electronically calculated mean pressures were recorded on a monitor with graphic and digital displays (model 7830A, Hewlett Packard, Santa Clara, CA). Cardiac output (CO) was determined with the thermal dilution technique and displayed on a cardiac output computer (COM-1, Baxter Edwards Critical Care Division, Irvine, CA). Pressures were measured while the sheep were standing and calm. Zero calibrations were taken at the olecranon joint on the front leg while the animals were standing. Core body temperature was measured with the thermistor of the Swan Ganz™ catheter. Ten mL of 5 % dextrose solution at 1–2 °C served as the thermal indicator. Arterial blood gas samples were analyzed at 37 °C using a conventional blood gas analyzer (Synthesis 15, Instrumentation Laboratories, Lexington, MA) and were corrected for core body temperature. Carboxyhemoglobin (COHb) was measured using a CO-Oximeter (model IL482, Instrumentation Laboratory, Lexington, MA) (10).

Experimental protocol

Following a baseline measurement in the healthy state, sheep were randomly allocated to one of the three groups (each n=6): sham (uninjured, untreated), control (injured, untreated), and WW-85 (injured, treated with WW-85). Thereafter, a tracheotomy was performed under ketamine anesthesia (5 mg/kg). In addition, a Foley® urinary retention catheter was placed in the urinary bladder of all animals to monitor fluid balance. Anesthesia was then maintained using 1.5–2.5% halothane (Vedco Inc., St. Joseph, MO) in O₂. The control and WW-85 animals were subjected to smoke inhalation injury and bacterial challenge according to an established protocol (11). Each animal in both groups was insufflated with four sets of 12 breaths of cotton smoke (< 40 °C). The smoke was generated and delivered by a modified bee smoker that was filled with 40 g of burning cotton toweling and attached to the tracheotomy tube via a modified endotracheal tube. The tube contained an indwelling thermistor from a Swan-Ganz™ catheter to measure the airway temperature during the procedure. The sham group received four sets of 12 breaths of room air through the bee smoker. Arterial COHb plasma concentrations were determined after each set of smoke or air inhalation and served as an index of lung injury. After smoke inhalation, an experimental bacterial solution was instilled into the lung lobes of control and WW-85 animals, using a bronchoscope (Model PF-P40, Olympus America Inc. Melville, NY). Live *Pseudomonas aeruginosa* (Strain 12/4/4), which was isolated and cultured from a male burn patient at Brooke Army Medical Center (San Antonio, TX), was suspended in 30 mL of saline and instilled into the right lower and middle lobes (10 mL each) as well as the left lower lobe (10 mL). The high amount of bacteria used ($2-5 \times 10^{11}$ cfu) suppresses the growth of other species and gives highly reproducible *Pseudomonas aeruginosa* pneumonia with bacterial translocation into the blood stream and consecutive development of septic shock (9, 10, 12, 13). The sham group received only the vehicle (30 mL of saline) that was instilled in the same way. Anesthesia was then discontinued and the sheep were allowed to awaken (9).

All animals were mechanically ventilated (Servo-Ventilator 900C, Siemens, Elema, Sweden) with a tidal volume of 15 mL/kg and an initial respiration rate of 30/min for the duration of the 24-h study period. In this context it is important that sheep have higher lung compliance than man, and therefore, require higher tidal volumes than humans. Ventilator settings were periodically adjusted to maintain the arterial pCO₂ of the blood 10% below of baseline values to allow mechanical ventilation in the awake state. Positive end-expiratory pressure (PEEP) remained on a fixed level of 5 cm H₂O to avoid ventilation-related differences in the study groups. These ventilator settings were chosen in accordance with those originally described for this model by Murakami *et al.* (9).

All animals were fluid resuscitated, initially starting with an infusion rate of 2 mL•kg⁻¹•h⁻¹ lactated Ringer's solution. The fluid rate was then adjusted, depending on the filling pressures and hematocrit (Hct), to prevent hemoconcentration. Fluid resuscitation was limited to a maximum of 1 L/h, since we have noted in previous studies that fluid administration at a rate of more than 1 L/h does not affect Hct in our sheep model because of vascular leakage associated with septic shock (13). Since sheep don't perspire and the inhaled gases were humidified, the fluid balance was calculated as the difference of fluid input and urinary output (mL). Hematocrit was assessed in heparinized microhematocrit capillary tubes (Fisher Brand, Pittsburg, PA). Values < baseline indicated hemodilution, and values > baseline reflected hemoconcentration. During the experiment, the animals had free access to food, but not to water to accurately determine fluid intake.

One hour post injury, WW-85 was dissolved in saline and was intravenously administered with an initial bolus of 0.1 mg/kg followed by a continuous infusion of 0.02 mg•kg⁻¹•h⁻¹ until the end of the 24-h experimental period. WW-85 is an iron-pyridyl-porphyrin, substituted with a benzoic acid group as described in US patent 7432369 (Williams and Southan, 2008). WW-85 was synthesized and provided for the studies by Inotek Pharmaceuticals (Beverly, MA, USA). The sham and control group received the same amount of normal saline as vehicle (5).

The concentration of NO_x (total amount of nitric oxide metabolites) in the plasma was measured intermittently. Plasma samples were subjected to NO_x reduction using vanadium (III) as a reducing agent in a commercial instrument (model 745, Antek Instruments, Houston, TX). The resulting NO was measured with a chemiluminescent NO analyzer (model 7020, Antek) and was recorded by dedicated software as the NO content (in µM) (9).

Necropsy

After completion of the experiment, the animals were anesthetized with ketamine (15 mg/kg) and sacrificed by lethal intravenous injection of 60 mL saturated potassium chloride. Immediately after death, lung tissue was excised and inflated with 10% formalin during necropsy. Fixed samples were embedded in paraffin, sectioned into 6 µm pieces, and stained with hematoxylin and eosin. A pathologist who was unaware of the group assignment analyzed the samples according to the airway obstruction scoring system of Cox *et al.* (14).

Measurement of malondialdehyde and myeloperoxidase

Malondialdehyde (MDA) is a major end-product of oxidation of polyunsaturated fatty acids, and is frequently measured as an indicator of lipid peroxidation and oxidative stress. Myeloperoxidase (MPO) quantitates the infiltration and accumulation of polymorphonuclear neutrophils (PMNs) following an inflammatory stimulus (15).

MDA and MPO was extracted from tissue as recently described (5). Briefly, MDA formation was measured as thiobarbituric acid reactive material. Lung tissue MDA levels

were quantified with a commercially available assay (Northwest Life Science Specialties, Vancouver, WA). The level of lipid peroxides was expressed as MDA in nmol per mg protein, measured by a commercially available assay (FlukaBioChemika, Buchs, Switzerland).

The activity of MPO was determined directly in tissue homogenates. MPO concentrations were evaluated on homogenized lung with a commercially available assay. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance at 450 nm for 10 minutes. MPO activity was reported as mU/mg tissue (CytoStore, Calgary AB, Canada)

Measurement of 3-nitrotyrosine

The lung 3-nitrotyrosine content, a reliable marker of nitrosative stress by reactive nitrogen species such as ONOO⁻, was determined using western blot analysis (13). Briefly, lung tissue was homogenized in phosphate buffered saline containing 1 mM phenylmethylsulfonylfluoride and protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA). The lysate was centrifuged at 12,000 *g* for 5 minutes to remove cellular debris. Protein concentration in the supernatant was determined by Bradford assay (BioRad, Hercules, CA, USA). Eighty micrograms of protein were resuspended in sample loading buffer (Tris-HCl, glycerol, SDS and 2-mercaptoethanol), boiled and resolved in 4–20% SDS-PAGE (BioRad). Protein was electrophoretically transferred to a polyvinylidene difluoride membrane in Tris-glycine-methanol buffer at 35 V overnight at 4 °C. After transfer, the membrane was rinsed twice in water and then blocked in 5% nonfat milk in TBS buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.05% Tween 20). The blot was then probed with Anti-Nitrotyrosine antibody 1:2,000 (Calbiochem, La Jolla, CA, USA) in 5% bovine serum albumin, 0.1% TBS-Tween 20 buffer overnight at 4 °C. The blot was washed 5 times in TBS-Tween 20 buffer and then incubated with Horseradish peroxidase conjugated anti-rabbit IgG for an hour. After washing, immunoreactive bands were detected by using the enhanced chemiluminescence kit (Amersham Biosciences, Inc., Piscataway, NJ, USA) and exposure to an X-ray film. Relative density of bands was then scanned and quantitated using Image-Quant software (Molecular Dynamics, Sunnyvale, CA, USA).

Measurement of bloodless lung-wet-to-dry weight-ratio

For the determination of bloodless lung wet-to-dry weight-ratio, which was calculated according to the equation described by Pearce *et al.* (16), lung tissues from the right upper and middle lobe were harvested during necropsy as described previously (13).

Statistical analysis

For statistical analysis, Sigma Stat 2.03 software (SPSS Inc., Chicago, IL) was used. After confirming normal distribution (Kolmogorov-Smirnov test), a two-way analysis of variance (ANOVA) for repeated measurements with appropriate Student-Newman-Keuls *post hoc* comparisons was used to detect differences within and between groups. Significance was assumed when *P* was less than 0.05. Data are presented as means ± standard errors of the mean (SEM).

RESULTS

Injury and survival

The arterial COHb determined immediately after smoke exposure averaged 63 ± 5 % in the control group, and 66 ± 3 % in the WW-85 group. The sham group, which was not exposed to smoke inhalation, showed a COHb level of 4 ± 1 % after receiving 4 sets of 12 breaths of

room air through the bee smoker. The core body temperature in the control and WW-85 group significantly increased over time compared to the sham group and BL ($P = 0.05$, TABLE 1). The COHb levels as well as the increase in core body temperature in the control and WW-85 groups were not statistically different from one another and reflected the consistency of the injury in each group. With aggressive fluid administration, all animals survived the 24-h study period.

Pulmonary results

While the $\text{PaO}_2/\text{FiO}_2$ -ratio remained stable in the sham group, it decreased significantly in the control and WW-85 group ($P = 0.001$ vs. BL and vs. sham). The $\text{PaO}_2/\text{FiO}_2$ -ratio in the WW-85 group did not fall to the same extent as in the control group and showed attenuated worsening over the entire duration of the experiment ($P = 0.001$). The control group met the criteria for ARDS ($\text{PaO}_2/\text{FiO}_2$ less than 200) within 3 hrs, whereas the WW-85 group met the criteria for ARDS only at 24 hrs (FIG. 1).

Likewise, pulmonary shunt fraction (Qs/Qt) remained stable in sham animals but was significantly increased vs. BL and vs. sham in the control and WW-85 groups over time ($P = 0.001$). Qs/Qt in the WW-85 group was lower than in the control group during the entire experiment (FIG. 1).

A significant increase in peak airway pressure (PAW_{PEAK}) was noted in both injured groups vs. BL and vs. sham over time ($P = 0.05$). In the WW-85 group, PAW_{PEAK} tended to be lower than in the control group during the entire experiment and was significantly lower at 12 hrs post injury ($P = 0.05$, TABLE 1). While pause airway pressure ($\text{PAW}_{\text{PAUSE}}$) remained on BL levels in the sham group, it was significantly increased in the control and WW-85 group ($P = 0.05$ vs. sham and vs. BL). There was no statistical difference between the two injured groups for these variables (TABLE 1).

Lung histology

Lung histology 24 hrs after smoke inhalation and bacterial challenge (FIG. 2) showed a increase in airway obstruction scores of the bronchi of both injured groups compared with sham animals ($P = 0.001$). Bronchi obstruction in the WW-85 group tended to be lower than in controls. The obstruction scores of bronchioli in control animals displayed an increase compared with sham animals ($P = 0.001$). Treatment with WW-85 reduced obstruction of the bronchioli as compared with the control group ($P = 0.002$), and showed no statistical difference in comparison with the sham group.

Lung tissue analysis

Lung MPO activity was higher in the injured control group than in the sham group ($P = 0.029$). MPO activity in the WW-85-treated group was comparable to the sham group (n.s.), but was lower than in the control group ($P = 0.017$, FIG. 3).

MDA formation in lung homogenates was higher in the injured control group than in the sham group ($P = 0.008$). MDA formation in the WW-85 treated group was similar as in the sham group (n.s.), but was lower than in the control group ($P = 0.039$, FIG. 3).

3-NT analysis by western blot, shown in figure 4, displayed a significant higher protein concentration in the control group than in the sham group ($P = 0.05$). Lung tissue 3-NT concentrations in the WW-85 group were similar to those in the sham group (n.s.), but were significantly lower than the control group ($P = 0.05$).

Lung bloodless wet-to-dry weight-ratio in the control and WW-85 groups showed similar (n.s.) changes after 24 h (6.7 ± 0.5 and 6.3 ± 0.2), respectively. Both injured groups showed a significant ($P = 0.05$) increase in wet-to-dry weight-ratio as compared with the sham group (4.9 ± 0.2).

Systemic hemodynamics

Central venous pressure (CVP), mean pulmonary artery pressure (MPAP), pulmonary artery occlusion pressure (PAOP), and left atrial pressure (LAP) remained stable in sham animals and showed an increase in both injured groups vs. BL and the sham group over time (TABLE 1). There was no difference in CVP between controls and sham, or controls and WW-85. MPAP was significantly lower in the WW-85 group as compared to the control group at 3 and 6 hrs post injury ($P = 0.05$). PAOP in WW-85 animals displayed a significant reduction as compared to controls at 24 hrs after the insult ($P = 0.05$). LAP was significantly higher in both injured groups than in the sham group ($P = 0.05$). No statistical difference was detectable between the injured groups.

Cardiac output (CO, TABLE 1) in sham animals remained stable over time. In the control group, CO increased over time and was significantly higher compared with the sham group (beginning at 12 h, and vs. BL beginning 18 h post injury). In WW-85-treated sheep, CO slightly increased over time, a statistical difference to BL, however, was not detectable. CO was lower in the WW-85 group as compared to the control group.

P_{ONC} in the sham group remained on BL level during the 24-h study period. P_{ONC} decreased over time in both injured groups ($P = 0.001$). However, P_{ONC} in the WW-85 group did not fall to the same extent as in the control group and was higher after 24 hrs ($P = 0.003$, FIG. 5).

Plasma NOx levels in the sham group remained on BL levels during the entire experiment. In the control group, NOx levels increased over time compared to the sham group ($P = 0.035$ vs. BL, FIG. 5), and the WW-85 group ($P = 0.031$ FIG. 5).

Hematocrit (Hct) remained on BL levels in sham and WW-85 animals. Hct in the control group was significantly higher than in sham at 12 and 18 hrs, and in WW-85 at 12 – 24 hrs post injury ($P = 0.05$ each).

DISCUSSION

In the present study, the ONOO⁻ decomposition catalyst WW-85 significantly improved pulmonary function in sheep suffering septic shock, by improved PaO₂/FiO₂-ratio and Qs/Qt, as well as reduction of airway obstruction, plasma NOx levels, lung tissue- MPO, -MDA, and 3-NT. In addition, WW-85 significantly improved most hemodynamic parameters.

The sheep model used in the current study is suitable for studying the effects of ALI associated with smoke inhalation injury and septic shock, because it closely mimics the pathophysiology seen in human ALI and sepsis (9), and matches the sepsis criteria as described by Bone *et al.* (17). The question may arise as to whether CO-Hb may have potentially influenced the model and the drug effects. While it is clear that exposure to exogenous CO can be protective in various models of critical illness (18), in the current experiments, clearly, a significant degree of damage occurred. Therefore, any protective effect of the CO-related mechanisms must be secondary to other, parallel pathways of injury that are operative in the current experimental model. As far as potential interactions of the test compound with CO-related mechanisms, we do not believe that this is the case, since

CO-Hb levels were comparable in the sepsis and the sepsis+WW-85 groups. However, CO was used to induce lung injury, but CO washes out of the blood within the first three hours of ventilation with 100% oxygen (19).

The cardio-pulmonary effects of the ONOO⁻ decomposition catalyst WW-85 have, up to the current study, not been tested in large animal models of septic shock. Our group previously investigated the effects of WW-85 in a model of IL-2 induced lung injury (5). The models differ significantly, whereas the IL-2 model can be used to measure pulmonary transvascular fluid flux in non-ventilated sheep with stable hemodynamics, sheep in the sepsis model develop ARDS, require mechanical ventilation and develop the typical hypotensive, hyperdynamic response to septic shock.

WW-85 reduced the formation of plasma NO_x in the current model of smoke inhalation injury associated with septic shock. This is presumably due to the fact that inducible nitric oxide synthase (iNOS) expression (and subsequent NO production) is a continuous process in SIRS and by interrupting NF-κB activation, ONOO⁻ decomposition catalysts block this process, and therefore block the development of NO induced hypotension and pulmonary vascular leakage (20–22). However, reduced NO_x levels at 24 hours did not lead to reduced pulmonary shunt formation after 24 hours, shunt was only improved in the early stage of injury of the present study. The improvement in PaO₂/FiO₂-ratio therefore might most likely be attributed to the significant decrease in airway obstruction. Our study suggests that downstream actions of iNOS-dependent ONOO⁻ might explain some of the effects previously attributed to NO. However, ONOO⁻ itself (23), like other oxidants (24–26), can abrogate NF-kappa-B activation through oxidative inactivation of the upstream kinase IKK in the NF-kappa-B pathway (27–30). ONOO⁻ is a potent nitrating species and initiator of lipid peroxidation and apoptosis. Immunodetection of nitrotyrosine has been often used as evidence of ONOO⁻ formation in biological tissue (31). More recently, it has been shown that protein nitration may, under some conditions, arise independently of ONOO⁻ via the action of MPO and nitrite (32, 33). In addition, the enzyme superoxide dismutase (SOD) is a specific catalyst of the reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ and thus decreases the local concentration of O₂⁻ and consecutively ONOO⁻. SOD exists in two major isoforms: as a copper zinc (Cu/Zn)- containing enzyme and as a manganese (Mn)-containing enzyme. Because of its longer circulating half life compared with Cu/Zn SOD, a recombinant human MnSOD was used in previous studies of systemic inflammation by our group (34). MnSOD improved fluid balance and oxygenation when given as pre-treatment (35). Treatments post injury, however, showed some positive effects but only in selected dosages (36, 37), at higher dosages the enzyme may actually result in injury (38). Ischiropoulos and colleagues (39) have shown that SOD may increase ONOO⁻ production. The small margin of effective SOD administration, and the faster reaction of NO with O₂⁻ resulting in the production of ONOO⁻ decreased the importance of SOD in this model. However, the role of SOD still remains controversial.

While the precise pathways contributing to nitration in SIRS have not yet been determined with certainty, a role of iNOS in nitrotyrosine formation is supported by multiple studies (4). In deed, we were able to identify a significant reduction by WW-85 in lung 3-NT levels in the present study. Studies using a selective iNOS dimerization inhibitor also showed decreased nitrotyrosine levels and apoptosis (40). Since this intervention also decreased iNOS expression, it is possible that decreased nitration resulted secondarily from decreased production of NO, a substrate for ONOO⁻ formation. Other studies using iNOS knockout mice showed decreased apoptosis and lack of nitrotyrosine formation when iNOS^{-/-} donor and recipients were used compared to presence of nitrotyrosine when iNOS^{+/+} allografts were used (41). These findings are complemented by studies of Pieper *et al.* (6) showing limitation of NO by two mechanisms a) by inhibiting iNOS activity but not expression, and

b) decreasing iNOS expression by immunosuppressant therapy in rat cardiac transplants, both decreased protein nitration. These findings support a role of iNOS in nitrotyrosine formation. On the other hand, one cannot exclude the possible nitration of proteins via iNOS-dependent but ONOO⁻-independent pathways. Indeed, MPO in the presence of nitrite and H₂O₂ can cause nitration of proteins (32), what may be excluded in our study, since MPO was significantly reduced by WW-85. Based upon our current knowledge, it cannot be excluded that WW-85 may also contribute to ONOO⁻-independent pathways of protein nitration. Our results, however, are in line with previous findings using iron-based metalloporphyrinic agents as ONOO⁻ decomposition catalysts, which resulted in decreased myocardial MPO activity in septic rats (42), and decreased MPO activity in lung reperfusion injury (21). Nevertheless, our findings using the ONOO⁻ decomposition catalyst WW-85 are significant in providing evidence that MPO-derived nitration is not a major source of nitrotyrosine formation in this model of ALI.

There are a number of metalloproteinic peroxynitrite decomposition catalysts (including MnTyPyP and FP-15) that have been used in vivo to delineate the pathophysiological role of peroxynitrite. These compounds provide beneficial effects in various models of ischemia, shock and neuroinjury (20). WW-85 is another catalytic antioxidant that catalyzes the breakdown of ONOO⁻. WW-85 has been shown to improve transplant rejection in a rat allograft model, and these effects were associated with a significant reduction in tissue nitrotyrosine content (6). In a mouse model of spinal cord injury, WW-85 significantly reduced inflammation and tissue injury, associated with ameliorated recovery of limb function (7). Most recently, our group could show that WW-85 is a useful agent to attenuate IL-2 induced deterioration in pulmonary microvascular permeability (5).

It is well known that ALI is frequently associated with alterations in non-pulmonary organ function. Non-pulmonary organ failure may in part explain why the respiratory complications and the development of septic shock resulting from inhalation injury have become the major cause of mortality in these patients (43). There are several lines of evidence supporting the view that overproduction of NO by iNOS contributes significantly to the circulatory shock. Not only NO itself, but also its downstream biological effects may play a role in vasodilatory hypotension (44). Peroxynitrite is capable of inducing endothelial dysfunction and vascular hyporeactivity (3). Recent data also showed that ONOO⁻ is implicated in the inactivation of alpha-1-adrenoreceptors (45) and norepinephrine (46) and that O₂⁻ deactivates catecholamines resulting in loss of their vasopressor activity (47). These findings are in accordance with the results of the present study, which clearly show that the reduction of nitrotyrosine and myeloperoxidase is linked to improved global hemodynamics.

PARP activation lays downstream of peroxynitrite formation in many pathophysiological conditions. Furthermore, PARP activation has been shown to play a significant pathogenetic role in various forms of circulatory shock (48), including the current experimental model (9). It is conceivable, therefore, that by effectively neutralizing ONOO⁻, WW-85 may have indirectly inhibited PARP in the current experiments, and this effect may have contributed to the benefit seen in the present study. Indeed, in several previous reports, in many models of critical illness, WW-85 has been shown to reduce the activation of PARP in various tissues (5–8).

Taken together, the results of the present study provide further evidence for the pathogenetic role of peroxynitrite in critical illness. In specific, the results demonstrate that the peroxynitrite decomposition catalyst WW-85 is effective in attenuating pulmonary dysfunction in a clinically relevant experimental model of ALI and septic shock.

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Figure 1. Changes in P_{aO_2}/F_{iO_2} -ratio (left panel), and changes in pulmonary microvascular shunt fraction (Q_s/Q_t , right panel) over 24 hrs. Data are presented as mean $\pm$ S.E.M. of 6 animals per group. Significance: $P < 0.05$, #vs. baseline, *vs. control, $\dagger$ vs. sham.

Figure 2. Histological airway obstruction scores. Changes in bronchi (left panel) and in bronchioli (right panel) of 6 animals per group. Data are expressed as obstructed percentage of airway lumen $\pm$ S.E.M. Significance $P < 0.05$, *vs. control, †vs. sham.

Figure 3. Lung myeloperoxidase (MPO, mU/mg, left panel) and lung malondialdehyde (MDA, nmol/mg, right panel) levels. Data are expressed as mean $\pm$ SEM of 6 animals per group. Significance: $P < 0.05$, *vs. control, [†]vs. sham.

Figure 4.

Lung 3-nitrotyrosine levels (densitometric value, arbitrary unit). Data are expressed as mean $\pm$ SEM of 6 animals per group. Significance: $P < 0.05$, *vs. control, $\dagger$ vs. sham. Cytosolic protein isolated from ovine lung homogenates was immunoblotted using antibody specific for protein containing 3-Nitrotyrosine. The arrow shows a representative band (protein) in the immunoblot recognized positive for 3-Nitrotyrosine by the antibody.

Figure 5. Changes in plasma oncotic pressure (P_{ONC} , mmHg, left panel), and changes in plasma nitrate-to-nitrite levels (NOx , μM , right panel). Data are presented as mean $\pm$ SEM of 6 animals per group. Significance: $P < 0.05$, #vs. baseline, *vs. control, †vs. sham

Table 1

Variables	Group	Values (mean ± SEM)					
		BL	3h	6h	12h	18h	24h
TEMP (°C)	Sham	38.8 ± 0.2	39.4 ± 0.1 [#]	39.5 ± 0.0 [#]	39.1 ± 0.2	39.2 ± 0.2	39.3 ± 0.2
	Control	39.2 ± 0.1	40.2 ± 0.3 [#]	40.4 ± 0.4 [#]	40.4 ± 0.2 [#]	40.5 ± 0.3 [#]	40.1 ± 0.3 [#]
	WW-85	39.3 ± 0.1	40.0 ± 0.1 [#]	40.2 ± 0.2 [#]	40.6 ± 0.2 [#]	40.7 ± 0.1 [#]	40.7 ± 0.1 [#]
PAW _{PEAK} (mm Hg)	Sham	20 ± 0	20.4 ± 1.6	22.0 ± 1.6	22.6 ± 2.2	21.0 ± 1.1	20.2 ± 1.9
	Control	20 ± 0	24.4 ± 1.6	26.3 ± 1.3	30.7 ± 1.9 ^{##†}	33.3 ± 1.6 ^{##†}	36.2 ± 2.1 ^{##†}
	WW-85	20 ± 0	21.5 ± 0.7	24.2 ± 1.2	25.0 ± 1.9 ^{#*}	29.7 ± 1.1 ^{##†}	30.5 ± 2.0 ^{##†}
PAW _{PAUSE} (mm Hg)	Sham	20 ± 0	18.7 ± 1.4	19.1 ± 1.3	19.7 ± 1.2	18.1 ± 1.1	16.9 ± 2.1
	Control	20 ± 0	20.5 ± 0.7	23.4 ± 0.9	26.2 ± 0.6 ^{##†}	30.1 ± 1.1 ^{##†}	32.6 ± 1.4 ^{##†}
	WW-85	20 ± 0	18.4 ± 0.7	20.9 ± 1.3	23.6 ± 1.1 ^{##†}	26.0 ± 1.9 ^{##†}	29.6 ± 2.4 ^{##†}
CVP (mm Hg)	Sham	8.0 ± 1.0	10.6 ± 1.7	10.8 ± 1.3	11.4 ± 1.7	10.0 ± 1.4	9.8 ± 1.2
	Control	6.8 ± 0.9	9.3 ± 1.2 [#]	11.0 ± 1.1 [#]	12.5 ± 1.2 [#]	13.8 ± 1.2 [#]	13.5 ± 1.2 [#]
	WW-85	6.0 ± 1.1	9.9 ± 1.7 [#]	11.9 ± 1.4 [#]	12.3 ± 0.6 [#]	13.6 ± 0.8 ^{##†}	13.0 ± 1.1 ^{##†}
MPAP (mm Hg)	Sham	22.7 ± 0.8	26.2 ± 0.7	25.8 ± 1.1	26.3 ± 0.9	26.0 ± 1.8	26.8 ± 2.0
	Control	21.2 ± 0.7	27.3 ± 1.7 [#]	30.0 ± 2.1 ^{##†}	29.2 ± 0.6 [#]	32.5 ± 0.8 ^{##†}	33.2 ± 1.5 ^{##†}
	WW-85	19.3 ± 0.6	21.7 ± 1.8 ^{##†}	24.6 ± 1.5 ^{#*}	28.0 ± 1.0 [#]	29.6 ± 1.1 [#]	33.3 ± 1.7 ^{##†}
PAOP (mm Hg)	Sham	12.0 ± 1.4	14.2 ± 1.5	15.8 ± 0.8 [#]	14.4 ± 1.6	14.8 ± 0.9 [#]	14.2 ± 0.8
	Control	10.2 ± 0.7	15.3 ± 1.6 [#]	16.8 ± 0.7 [#]	17.8 ± 1.1 [#]	17.8 ± 0.9 [#]	19.7 ± 0.3 ^{##†}
	WW-85	9.0 ± 0.6	14.3 ± 1.5 [#]	15.3 ± 1.3 [#]	18.3 ± 1.1 [#]	18.6 ± 1.5 [#]	16.4 ± 1.4 ^{#*}
PVRI (dynes•cm ⁻⁵ •m ⁻²)	Sham	155 ± 27	211 ± 28	155 ± 17	220 ± 29	188 ± 22	231 ± 32
	Control	157 ± 9	193 ± 26	187 ± 23	147 ± 15 [†]	175 ± 19	155 ± 25 [†]
	WW-85	140 ± 6	110 ± 22 ^{*†}	125 ± 19	115 ± 9 [†]	134 ± 11	210 ± 21 ^{#*}
LAP (mm Hg)	Sham	6.5 ± 1.2	10.7 ± 0.9 [#]	10.5 ± 1.0 [#]	10.5 ± 1.3 [#]	10.3 ± 1.1 [#]	10.5 ± 1.0 [#]
	Control	6.7 ± 0.9	10.5 ± 0.9 [#]	13.5 ± 1.7 [#]	13.7 ± 1.5 [#]	14.8 ± 2.1 ^{##†}	15.5 ± 2.1 [†]
	WW-85	6.7 ± 0.5	11.3 ± 1.0 [#]	13.1 ± 1.2 [#]	16.0 ± 1.7 [#]	17.9 ± 2.2 ^{##†}	16.3 ± 2.0 ^{##†}

Variables	Group	Values (mean ± SEM)					
		BL	3h	6h	12h	18h	24h
HR (beats per min)	Sham	96.0 ± 0.5	93.5 ± 6.3	106.2 ± 12.5	102.2 ± 7.3	87.5 ± 8.1	88.7 ± 6.9
	Control	88.3 ± 5.5	124.7 ± 3.6 ^{#†}	134.2 ± 7.5 ^{#†}	134.2 ± 6.1 ^{#†}	139.2 ± 6.9 ^{#†}	135.5 ± 8.9 ^{#†}
	WW-85	95.3 ± 2.6	105.3 ± 4.0 ^{#*}	112.2 ± 5.7 ^{#*}	120.2 ± 5.0 ^{#*†}	119.7 ± 9.2 ^{#*†}	114.5 ± 6.4 ^{#*†}
CO (L/min)	Sham	5.13 ± 0.14	4.72 ± 0.22	4.75 ± 0.23	4.63 ± 0.24	4.38 ± 0.22	4.03 ± 0.15 [#]
	Control	5.28 ± 0.37	4.97 ± 0.65	5.38 ± 0.50	5.80 ± 0.31 [†]	6.52 ± 0.45 ^{#†}	6.91 ± 0.57 ^{#†}
	WW-85	5.32 ± 0.24	5.26 ± 0.36	5.54 ± 0.27	6.10 ± 0.32 [†]	6.12 ± 0.46 [†]	6.02 ± 0.38 [†]
MAP (mm Hg)	Sham	99.2 ± 4.5	100.3 ± 3.8	100.5 ± 3.1	101.2 ± 2.9	105.8 ± 2.6	102.7 ± 2.8
	Control	104.3 ± 3.2	97.3 ± 6.8	97.3 ± 5.7	79.7 ± 3.4 ^{#†}	82.2 ± 3.7 ^{#†}	78.2 ± 6.2 ^{#†}
	WW-85	95.27 ± 2.8	96.0 ± 1.6	98.5 ± 3.3	92.7 ± 2.4 ^{*†}	93.5 ± 4.1 ^{*†}	92.3 ± 4.4 ^{*†}
HCT (%)	Sham	24.7 ± 09	26.0 ± 1.5	26.0 ± 1.7	24.3 ± 1.4	23.8 ± 1.1	25.5 ± 1.2
	Control	28.5 ± 2.4	28.8 ± 2.0	29.7 ± 2.1	30.3 ± 1.5 [†]	30.3 ± 2.1 [†]	30.3 ± 2.5
	WW-85	25.4 ± 1.4	27.1 ± 2.1	27.6 ± 2.6	25.0 ± 2.9 [*]	24.1 ± 2.2 [*]	25.3 ± 2.2 [*]

TEMP, temperature; PAWP/PEAK, peak airway pressure; PAWP/PAUSE, pause airway pressure; CVP, central venous pressure; MPAP, mean pulmonary artery pressure; PAOP, mean pulmonary artery pressure; PVRl, pulmonary vascular resistance index; LAP, left atrial pressure; HR, heart rate; CO, cardiac output; MAP, mean arterial pressure; HCT, hematocrit; Data are presented as mean ± standard error of mean;

Significance was assumed as p .05;

[#] vs. BL,

^{*} vs. control,

[†] vs. sham.