ANTIOXIDANTS & REDOX SIGNALING
Volume 9, Number 11, 2007

DOI: 10.1089/ars.2007.1765

Forum Original Research Communication

Stilbazulenyl Nitrone Decreases Oxidative Stress and Reduces Lung Injury After Hemorrhagic Shock/Resuscitation and LPS

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ABSTRACT

Multiorgan failure is a major cause of late morbidity and mortality after trauma. Reactive oxygen species generated during shock/resuscitation contribute to tissue injury by priming the immune system for an exaggerated response to subsequent inflammatory stimuli such as LPS. Stilbazulenyl nitrone (STAZN) is a novel second-generation azulenyl nitrone that has been shown to have potent antioxidant properties in a rat model of brain ischemia. We hypothesized that STAZN may confer protection against lung injury after shock/resuscitation and LPS by reducing oxidative stress and lowering the production of NF-κB–dependent pro-inflammatory cytokines. Sprague–Dawley rats were submitted to a two-hit model of lung injury involving hemorrhagic shock/resuscitation and subsequent intratracheal LPS injection, with and without intraperitoneal injections of STAZN. STAZN reduced overall lung injury in response to LPS alone and also after shock/resuscitation plus LPS. STAZN also reduced plasma levels of 8-isoprostane, a proxy measure of oxidative stress, indicating its antioxidant activity *in vivo*. The effect of STAZN was, at least in part, related to its effect on nuclear translocation of NF-κB and generation of the pro-inflammatory cytokine TNF-α. Azulenyl nitrones such as STAZN represent a promising novel class of antioxidants for treating organ injury. *Antioxid. Redox Signal.* 9, 1971–1977.

INTRODUCTION

CIVILIAN TRAUMA CONTINUES to represent a significant health care problem. In the United States, it ranks first in terms of hospital days and years of life lost and fourth in overall disease-related mortality (6). Although the majority of early deaths caused by trauma occur as a result of hemorrhage or central nervous system injury, almost two thirds of late deaths (>1 week after trauma) occur because of the development of multiorgan failure (16). The lungs are among the earliest and most commonly affected organs in critically ill patients after hemorrhagic shock, affecting up to 50% of patients (15). The prevailing paradigm for the development of multiorgan failure in this context is the so-called "two-hit hypothesis," whereby the global ischemia/reperfusion caused by shock/resuscitation primes cells of the immune system, including neutrophils and macrophages, for an exaggerated response when faced with a late subsequent inflammatory stimulus such as infection (14). In this state, these inflammatory cells are poised to initiate tissue inflammation and injury out of keeping with the magnitude of the delayed stimulus. This scenario is well described in experimental settings whereby sequential shock/resuscitation followed by delayed stimulus leads to synergistic organ injury. In humans, Botha and colleagues (3) showed that neutrophils recovered from trauma patients were "primed" and exhibited augmented superoxide production, compared with neutrophils from healthy volunteers, when exposed to the agonist formyl-methionyl-leucyl-phenylalanine.

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Oxidants can be generated during critical illness by activated inflammatory cells as described, or by distant tissues that have undergone ischemia/reperfusion. The gut is a well-known source of such oxidants, as ischemic intestinal mucosa produces xanthine oxidase, an enzyme that on reoxygenation converts hypoxanthine and xanthine into superoxide and peroxide (9). Work by several research groups, including our own, has implicated oxidative stress generated during ischemia/reperfusion as being central to the priming process. For example, with a rodent model of lung injury, we previously showed that shock/resuscitation induces a systemic oxidative stress and that generation of these oxidants renders alveolar macrophages more responsive to intratracheal LPS. Alveolar macrophage priming was characterized by heightened nuclear translocation of the oxidant-sensitive transcription factor NF-κB and increased expression of NF-κB–dependent genes such as TNF-α, cytokine-induced neutrophil chemoattractant (the rat orthologue of IL-8), and tissue factor (4). The observation that antioxidant supplements in the resuscitation fluid prevented macrophage priming and resultant lung injury provided support for the role of oxidants in this process. Similarly, in humans, the use of an antioxidant "cocktail" in trauma patients was shown to reduce alveolar cytokines, to affect ventilation parameters positively, and to lessen the incidence of multiple organ failure (12).

Stilbazulenyl nitrone (STAZN) is a novel second-generation azulenyl nitrone with significantly enhanced potency as a chain-breaking antioxidant compared with other members of the α-phenyl nitrone family (1). It was found to have great promise in a study by Ginsberg *et al*. (5, 8) as a lipophilic neuroprotective agent in a rat model of focal brain ischemia. In that study, STAZN conferred a 64 to 97% reduction in mean cortical infarct volume, a reduction far greater than that achieved by any other antioxidant used for that purpose. Based on these observations and the putative role of antioxidants in lung injury, we hypothesized that STAZN may confer protection against lung injury after shock/resuscitation and LPS by reducing oxidative stress and lowering the production of NF-κB–dependent pro-inflammatory cytokines. The following study reveals that STAZN reduces overall lung injury as measured by transpulmonary lung leak, leukocyte infiltration, and neutrophil sequestration in the lungs after shock/resuscitation and LPS. STAZN also reduces plasma levels of 8-isoprostane, a proxy measure of oxidative stress, and dampens the activation of the critical transcription factor NF-κB and the generation of the pro-inflammatory cytokine TNF-α.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (*Escherichia coli* O111:B4), sterile normal saline (NS), and sodium citrate were obtained from Sigma-Aldrich Co. (St. Louis, MO). Sodium citrate was prepared as 3.8% solution by using sterile water, stored at 4°C, and filtered before use in resuscitation. Endotoxin-free Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen Co. (Carlsbad, CA); phosphate-buffered saline (PBS) and EDTA were obtained from Gibco BRL (Burlington, Ontario, Canada); Ringer's lactate (RL), from Baxter Co (Mississauga, Ontario, Canada); and dimethylsulfoxide (DMSO), from Sigma-Aldrich Co. The anesthetic drugs were xylazine from Bayer, Inc. (Toronto, Ontario, Canada); and ketamine and pentobarbital, from Bimeda-MTC Pharmaceuticals (Cambridge, Ontario, Canada). STAZN was synthesized in the laboratory of Professor David Becker according to the published method of Becker *et al*. (1), and was dissolved in the lipophilic vehicle DMSO (0.2 mg/ml).

Animal model

Animals were cared for in accordance with the guidelines set forth by the Canadian Council on Animal Care. Male Sprague–Dawley rats weighing 300–350 g (Charles River, St. Constant, Quebec, Canada) were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (8 mg/kg). The right carotid artery was cannulated with a 22-gauge angiocath (Becton Dickinson, Franklin Lakes, NJ) for monitoring of mean arterial pressure (MAP), blood sampling, and resuscitation. Hemorrhagic shock was initiated by blood withdrawal, leading to a reduction of the MAP to 40 mm Hg within 15 min. This blood pressure was maintained by further blood withdrawal if the MAP was greater than 45 mm Hg, and by infusion of 0.5 ml of RL if the MAP was less than 35 mm Hg. Shed blood was collected into 0.1-ml Na citrate per milliliter of blood to prevent clotting. After a hypotensive period of 60 min, animals were resuscitated by transfusion of the shed blood plus an equal volume of RL. Total resuscitation time was standardized to 2 h. After resuscitation, a tracheostomy was performed with a 14-gauge angiocath and either LPS (300 μg/kg in 200 μl NS) or NS alone was administered intratracheally followed by 10 mechanically ventilated breaths by using a rodent ventilator. The animals received their first dose of STAZN (0.6mg/kg in 1 ml DMSO or the vehicle DMSO) by intraperitoneal injection at the beginning of resuscitation, and their second dose 2 h later, just before receiving LPS. The animals were assigned to one of the following groups: sham, shock, LPS, LPS/STAZN, shock/LPS, shock/LPS/STAZN, and shock/LPS/DMSO. Sham animals were instrumented but not bled, and NS alone was instilled intratracheally; shock animals were bled and resuscitated, but received only intratracheal NS; and LPS animals were instrumented but not bled, and received intratracheal LPS. Animals were killed by pentobarbital overdose at various time points depending on the specific study. For the animals undergoing histologic assessment, the right lung was fixed in 10% formalin, later stained with hematoxylin/eosin, and examined by using an optical microscope.

Bronchoalveolar lavage

Immediately after death, the lungs were perfused *via* the tracheostomy cannula with cold PBS (containing 0.1 m*M* EDTA) in 10-ml aliquots and gently withdrawn to a total volume of 50 ml (11). For cell counts and differential, BAL fluid was centrifuged at 300 *g* (1,200 rpm) for 10 min. After the supernatant was discarded, the pelleted cells were resuspended in serum-free DMEM. Total cell counts were determined on a grid hemocytometer, and differential cell counts were measured on a cytospin-prepared slide stained with Wright-Giemsa (Fisher

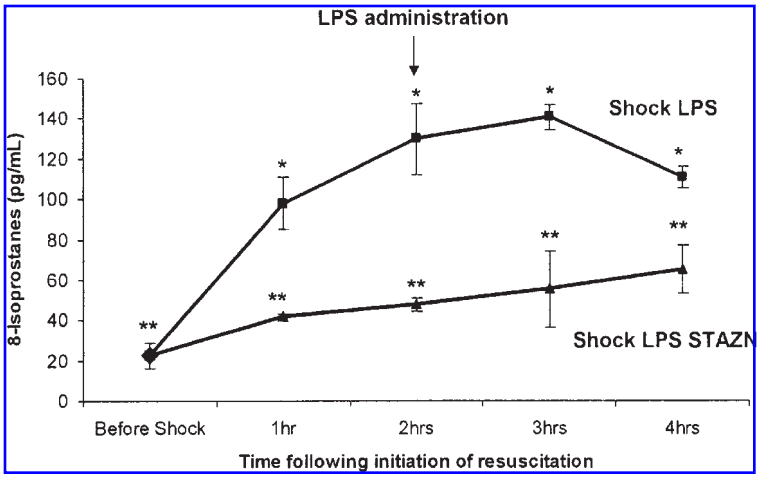

FIG. 1. The effect of STAZN on plasma 8-isoprostane levels. Rats were subjected to hemorrhagic shock, resuscitation, and intratracheal LPS, with or without intraperitoneal STAZN, as indicated. Plasma samples were withdrawn at various times, and 8-isoprostane ELISA measurements were performed as a proxy measure of oxidative stress. STAZN caused a significant reduction in plasma 8-isoprostane levels at all times. Data are represented as mean ± SEM of three animals per group. *Statistically significant at $p < 0.05$ compared with before-shock value and corresponding data marked **.

Scientific, Middleton, VA). A total of 300–500 cells was counted in cross section per sample, and the number of neutrophils and alveolar macrophages was calculated as the total cell count multiplied by the percentage of the respective cell type in the BAL fluid sample.

Transpulmonary albumin flux

Transpulmonary albumin flux was assessed by injecting 1 mCi of ^{125}I-albumin in a total volume of 0.2 ml NS into the tail vein immediately after intratracheal LPS or NS (4, 11). Six hours later, 1 ml of blood was withdrawn by cardiac puncture for measuring counts per minute (CPM). At the same time, the lungs were perfused with 10 ml of PBS (in a manner similar to the BAL described earlier). The perfused PBS was withdrawn gently, and 1 ml was used to calculate CPM. CPM was determined by using a gamma counter machine (Perkin Elmer Life Sciences), and the transpulmonary albumin flux was normalized to blood CPM as follows: transpulmonary albumin flux = BAL fluid CPM /blood CPM.

8-Isoprostane assay

8-Isoprostane levels in plasma were determined by using an eicosanoid immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). Then 500 μl of plasma sample was vortexed with 1 ml of 100% ethanol, allowed to stand at 4°C for 5 min, and then centrifuged at 1,500 *g* for 10 min. The supernatant containing 8-isoprostanes was then decanted, an equal volume of 15% KOH was added, and samples were incubated at 40°C for 1 h. The assay was performed as per the manufacturer's specifications, and the developed plate was read by a microplate reader (Diamed, Mississauga, ON, Canada) at 405 nm. The concentration was calculated by comparing with a standard curve.

Tissue collection and analysis of TNF-α and NF-κB

Lungs were retrieved at 1, 2, and 4 h after LPS, flash frozen in liquid nitrogen, and stored at −80°C. Lung tissue levels of the pro-inflammatory cytokine TNF-α and the activated nuclear transcription factor NF-κB were measured by ELISA analysis (Biosource, Camarillo, CA, and Active Motif, Carlsbad, CA, respectively) according to specifications. Aliquots of 200–500 mg of frozen lung samples were homogenized in 1 ml of the following buffer: 25 m*M* TRIS/HCl (adjusted to pH 7), 2 m*M* EGTA, 1 m*M* benzamidine, and 1 m*M* phenyl methyl sulfonyl fluoride (PMSF) added immediately before use. The homogenized lung tissue was centrifuged at 12,000 rpm for 20 min,

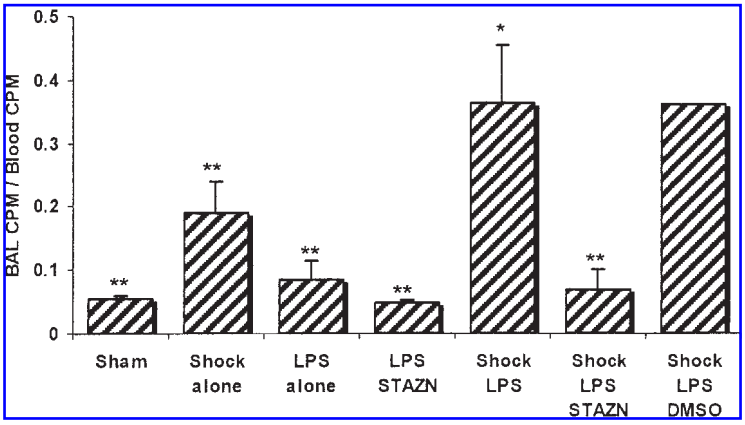

FIG. 2. The effect of STAZN on transpulmonary albumin flux. Rats were subjected to all or part of the two-hit model including hemorrhagic shock, resuscitation, and/or intratracheal LPS, with or without intraperitoneal STAZN, as indicated. Then 1 mCi of ^{125}I-albumin was injected in the rat tail vein immediately after LPS. BAL and blood samples were withdrawn for CPM analysis 6 h after LPS. Shock + LPS caused a significant increase in transpulmonary albumin flux, which was prevented by treatment with STAZN. The STAZN vehicle DMSO had no effect on its own. Data are represented as mean ± SEM of three animals per group. *Statistically significant at $p < 0.05$ compared with data marked **.

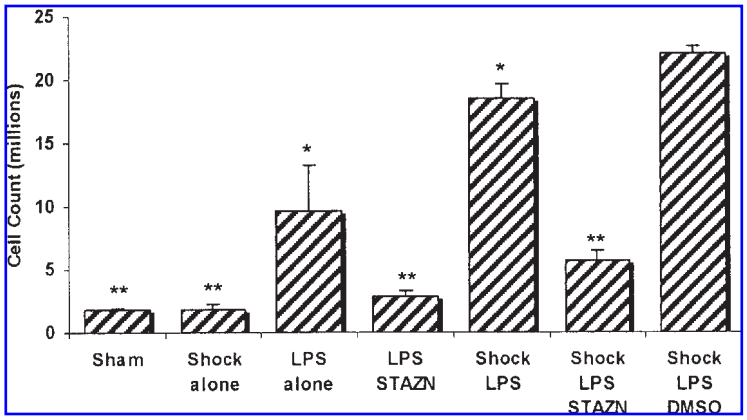

FIG. 3. The effect of STAZN on leukocyte lung infiltration. Rats were subjected to all or part of the two-hit model including hemorrhagic shock, resuscitation, and/or intratracheal LPS, with or without intraperitoneal STAZN, as indicated. Four hours after LPS, BAL was performed, and cell counts were determined. LPS alone and shock + LPS both caused a significant increase in leukocyte infiltration, which was prevented in both cases by treatment with STAZN. The STAZN vehicle DMSO had no effect on its own. Data are represented as mean ± SEM of three animals per group. *Statistically significant at $p < 0.05$ compared with data marked **.

and the supernatant (extracellular fluid) was used for TNF-α ELISA analysis. To standardize the samples, total protein concentration was determined by adding 1 μl of sample to diluted Protein Assay Dye Reagent (BioRad, Hercules, CA), and measured by spectrophotometry at 595 nm against a bovine serum albumin (BSA) standard curve. The leftover pellets were washed once in cold PBS and then sonicated for 10 sec after resuspension in 150 μl of the following buffer: 50 m*M* TRIS/HCL (adjusted to pH 7), 250 m*M* sucrose, and 1 m*M* PMSF added immediately before use. The sonicated product was centrifuged at 12,000 rpm for 10 min, and the supernatant (cytoplasm and nucleoplasm) was used for activated NF-κB analysis. The primary antibody used in this ELISA analysis is specific for an epitope of the p65 subunit that is accessible only when NK-κB is activated and bound to target DNA. To standardize the samples, protein concentration was determined as described earlier.

Statistical analysis

The data are presented as mean ± standard error of *n* determinations, as indicated in the figure legends. Data were analyzed by one-way analysis of variance, and post hoc testing was performed by using the Newman–Keuls Multiple Comparison Test. In the figures presented, significance is indicated with an asterisk (*) and signifies that the mean difference between indicated treatment groups is significant at the 0.05 level.

RESULTS

Effect of STAZN on oxidative stress after shock/resuscitation and LPS

STAZN has been shown to exhibit antioxidant properties *in vitro* and to prevent neurologic injury in a rat model of focal ischemia, an injury considered to be related to oxygen free radical generation. However, its ability to quench oxidative stress after shock/resuscitation and hence potentially prevent cellular priming in this scenario is unknown. 8-Isoprostane is a by-product of lipid peroxidation, a consequence of direct cell-membrane damage caused by ROS, and has been used as a measure of oxidative stress in the blood. As shown in Fig. 1, a steady increase in the level of 8-isoprostanes occurs during the 2-h resuscitation phase. Consistent with its antioxidant properties, treatment with STAZN significantly reduced plasma levels of 8-isoprostane at all time points. This effect persisted over the several hours after LPS administration.

Effect of STAZN on lung injury after shock/resuscitation and LPS

Animals undergoing shock/resuscitation followed by intratracheal injection of LPS sustained a significant increase in

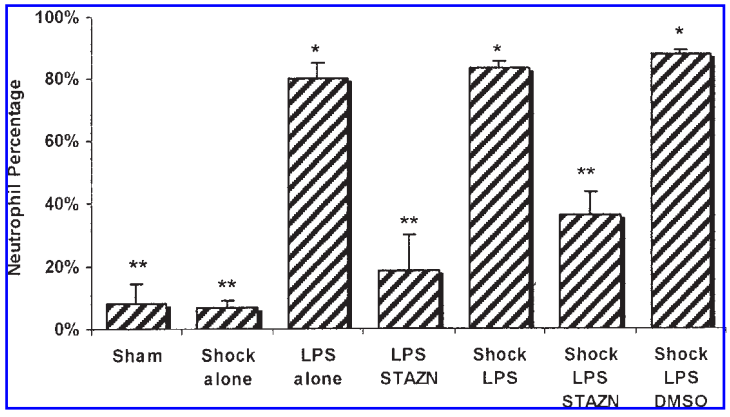

FIG. 4. The effect of STAZN on neutrophil percentage in the lung. Rats were subjected to all or part of the two-hit model including hemorrhagic shock, resuscitation, and/or intratracheal LPS, with or without intraperitoneal STAZN, as indicated. Four hours after LPS, BAL was performed, and cell counts were determined. Slides were further analyzed to determine the neutrophil percentage of the total BAL leukocyte population. LPS alone and shock + LPS both caused a significant increase in neutrophil percentage, which was prevented in both cases by treatment with STAZN. The STAZN vehicle DMSO had no effect on its own. Data are represented as mean ± SEM of three animals per group. *Statistically significant at $p < 0.05$ compared with data marked**.

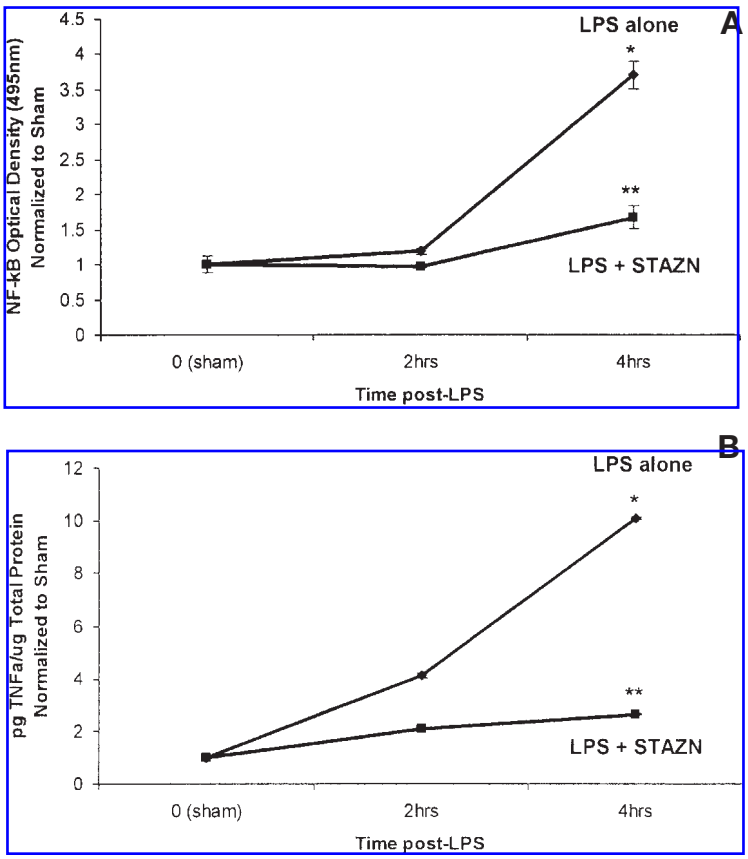

FIG. 5. The effect of STAZN on generation of activated NF-κB and TNF-α in animals treated with LPS. Rats were given intratracheal LPS, with or without intraperitoneal STAZN, as indicated. Two and 4 h after LPS, rat lungs were retrieved, and ELISA measurements of activated transcription factor NF-κB were made (**A**), and ELISA measurements of pro-inflammatory cytokine TNF-α were made (**B**). STAZN caused a significant reduction in the generation of activated NF-κB and TNF-α at 4 h after LPS. Data are represented as mean ± SEM of three animals per group. *Statistically significant at $p < 0.05$ compared with corresponding data marked **.

transpulmonary albumin leak, a measure of lung damage, whereas neither shock nor LPS alone exerted such an effect (Fig. 2). This is consistent with our previous data with this model (4). As shown, STAZN significantly reduced transpulmonary albumin leak in animals exposed to both shock and LPS. The diluent DMSO is known to have some antioxidant properties. To rule out a role of the diluent in the effect, we administered DMSO vehicle alone. DMSO had no effect on transpulmonary albumin flux.

Figure 3 illustrates the effect of STAZN on lung leukocyte infiltration, an alternate marker of lung inflammation. Animals receiving LPS alone had a significant increase in their BAL total leukocyte count at 4 h after LPS as compared with sham. This effect was accentuated when animals were subjected to antecedent shock/resuscitation. In both cases, STAZN treatment reduced leukocyte infiltration. In a separate group, the vehicle DMSO was again shown to have no effect (see Fig. 3).

As neutrophil sequestration represents one of the hallmarks of ARDS, we evaluated the proportion of cells in the BAL total leukocyte count that were neutrophils. We found that both LPS alone and shock/LPS caused a significant increase in the percentage of neutrophils to ~80% from a baseline sham value of less than 10%. Animals treated with STAZN experienced a significant reduction in neutrophil sequestration in both the LPS-alone and shock/LPS groups. The vehicle DMSO is again shown to have no effect (Fig. 4). Taken together, these data support the notion that the antioxidant STAZN reduces lung injury after shock/resuscitation and LPS.

Effect of STAZN on generation of pro-inflammatory markers NF-κB and TNF-α

A common target of antioxidant therapy on cell signaling is nuclear translocation of the transcription factor NF-κB. With an ELISA kit specific for the DNA-bound/active form of NF-κB, we first measured the concentration of active NF-κB in whole lungs from LPS-treated animals (Fig. 5A). A progressive increase was found in the level of activated NF-κB after intratracheal LPS, reaching statistical significance by 4 h after LPS injection. As shown, this increase was attenuated by treatment with STAZN. In parallel, STAZN was observed to prevent the increase in whole-lung TNF-α by 4 h after LPS treatment (Fig. 5B). These findings indicate that STAZN exerted antiinflammatory effects in nonprimed animals receiving LPS, suggesting a mechanism for the protective effects of STAZN in this model.

We also performed studies to discern whether comparable mechanisms were operating in LPS treatment of animals primed by antecedent shock/resuscitation. As anticipated, shock plus LPS increased both whole-lung NF-κB and TNF-α (Fig. 6A and B, respectively) compared with control animals. STAZN caused a modest, but not significant reduction in NF-κB at 4 h after LPS treatment and had no effect on whole-lung TNF-α levels.

DISCUSSION

Electron spin-resonance spectrometry with various nitrone spin traps is an established technique for measuring oxygen

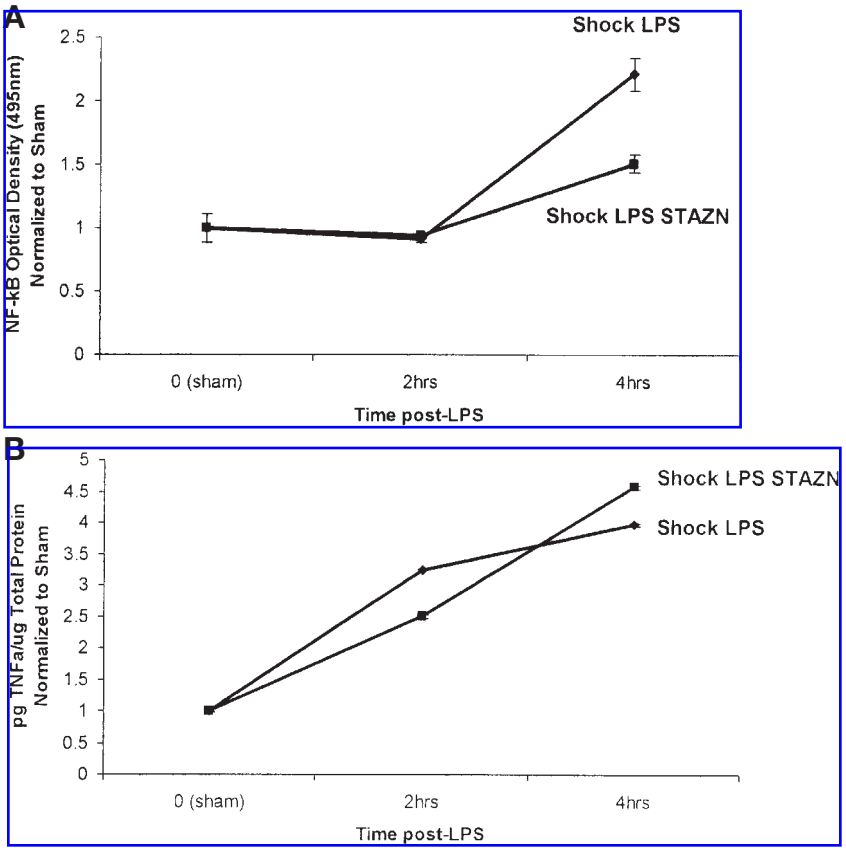

FIG. 6. The effect of STAZN on generation of activated NF-κB and TNF-α in animals exposed to shock/resuscitation followed by LPS. Rats underwent hemorrhagic shock, resuscitation, and intratracheal LPS, with or without intraperitoneal STAZN, as indicated. Two and 4 h after LPS, rat lungs were retrieved, and ELISA measurements of activated transcription factor NF-κB were made (**a**), and ELISA measurements of pro-inflammatory cytokine TNF-α were made (**b**). Although STAZN did not have a significant impact on activated NF-κB or TNF-α generation, a strong trend was seen toward reduction in activated NF-κB at 4 h after LPS. Data are represented as mean ± SEM of three animals per group.

free radical generation in both *in vitro* and *in vivo* settings. The ability of these agents to trap oxygen free radicals also led to their use as antioxidants in a number of experimental models, in which oxidative stress is known to cause injury. This is best studied in neurologic diseases such as ischemic stroke, in which these agents have been shown to mitigate neurologic injury. STAZN is a second-generation azulenyl nitrone with superior antioxidant properties compared with other nitrones. Prior work with this compound has demonstrated its ability to be neuroprotective in models of focal ischemic stroke and traumatic brain injury. The present studies are the first to demonstrate that an azulenyl nitrone compound such as STAZN is able to prevent lung inflammation in LPS-induced lung injury with or without antecedent shock/resuscitation. In addition, our investigations demonstrate the ability of this compound to prevent oxidative stress, as demonstrated by reduced 8-isoprostanes in the plasma, thereby confirming the antioxidant efficacy of this compound *in vivo* and suggesting a mechanism for its effect.

Several laboratories, including our own, have previously investigated antioxidant strategies in the treatment of lung inflammation induced by LPS administration in rodent models. The rationale for this approach has been twofold. First, because reactive oxygen species are known to contribute to tissue damage, quenching of these molecules by various compounds has been considered a reasonable strategy for tissue protection. Second, *in vitro* investigations have implicated intracellular induction of oxidative stress as a key mechanism leading to activation and nuclear translocation of the transcription factor NF-κB and downstream induction of pro-inflammatory genes (10, 17). The ability of antioxidants to prevent these events has been repeatedly demonstrated in cell systems. Interestingly, the efficacy of antioxidants as an antiinflammatory strategy *in vivo* has been quite variable, depending on the specific agent tested and also the model of lung injury. For example, *N*-acetylcysteine (NAC), a substrate for the synthesis of intracellular reduced glutathione, was shown to prevent lung neutrophil sequestration and permeability when LPS was given intraperitoneally (2), yet was without effect when LPS was administered intratracheally (4). By contrast, in the present work, STAZN was highly effective in protecting against lung neutrophil infiltration when LPS was given intratracheally. We attribute this difference to the high lipid solubility of STAZN and the potential that its effect may be more pronounced and prolonged than that of NAC. In this regard, P-*tert*-butyl nitrone (PBN) was shown to be effective in reducing shock-induced lipid peroxidation in the lung when PBN was given intraperitoneally (13), suggesting the ability of this class of compounds to penetrate into the lung. We did not examine oxidative stress in lung tissue *per se*. However, the finding that STAZN reduced whole-lung NF-κB translocation and TNF-α levels after LPS treatment is consistent with its ability to exert antioxidant properties in the lung. It also suggests that the effect is, at least in part, mediated by inhibition of pro-inflammatory signaling pathways.

The two-hit model of shock/resuscitation followed by LPS administration has been favored as one that recapitulates the events after trauma and leading to delayed lung inflammation. In this model, shock/resuscitation is presumed to prime for increased cellular responsiveness to a subsequent stimulus, leading to exaggerated inflammation and injury. These findings were

recapitulated in the present studies. The important role of oxidants as priming agents in this model has been demonstrated by several groups. In the present studies, we demonstrate that STAZN reduces oxidative stress in the systemic circulation, presumably generated by xanthine oxidase elaborated by the gastrointestinal tract after ischemia/reperfusion. Concomitantly, STAZN reduced both lung neutrophilia and lung albumin permeability back to baseline levels when administered *in vivo*. Fan and colleagues (4) implicated infiltrating neutrophils as the source of reactive oxygen species involved in priming alveolar macrophages, demonstrating that a membrane-permeant form of catalase prevented the effect of LPS on macrophage activation. Whereas STAZN was effective in reducing inflammation and injury in the shock/resuscitation-plus-LPS group, we were unable to attribute its effects to its ability to inhibit cell signaling, because neither NF-κB translocation nor TNF-α was reduced by STAZN treatment. These findings suggest that STAZN may be exerting its effect on some other signaling pathway. In this regard, we previously reported that oxidative stress reprograms LPS signaling in inflammatory cells *via* an Src-dependent pathway (7). Alternatively, STAZN may have exerted its effect through a direct antioxidant effect in the lung tissue as its primary action, as has been reported with P-*tert*-butyl nitrone.

In summary, the present studies are the first to show that STAZN, a novel second-generation azulenyl nitrone, is able to prevent lung injury in response to hemorrhagic shock and LPS. Future studies should investigate the optimal dosing and timing of this agent and its relative effectiveness compared with existing antioxidant compounds.

ACKNOWLEDGMENTS

This study was supported by an operating grant from the Canadian Institutes of Health Research and NIH grant 5 U01 NS 046295 (M.D.G.). Dr. P. S. Tawadros was also supported by a Johnson & Johnson Medical Products/Surgeon Scientist Fellowship, as well as a McLaughlin Center for Molecular Medicine Fellowship.

ABBREVIATIONS

LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-B; STAZN, stilbazulenyl nitrone; TNF-α, tumor necrosis factor-α.

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Date of first submission to ARS Central, May 30, 2007; date of acceptance, June 9, 2007.

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