Inhibition of NADPH oxidase prevents acute lung injury in obese rats following severe trauma

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First published January 10, 2014; doi:10.1152/ajpheart.00868.2013.—Lung capillary filtration coefficient (Kf) and impacts of oxidative stress have not been determined in the setting of severe trauma, especially in obese patients who exhibit increased lung injury. We hypothesized that severe trauma leads to a greater increase in lung Kf in obesity due to exacerbated production of and/or vulnerability to oxidative stress. Severe trauma was induced in lean and obese Zucker rats by muscle injury, fibula fracture, and bone component injection to both hindlimbs, with or without 24-h treatments of apocynin, a NADPH oxidase (NOX) inhibitor. Lung wet/dry weight ratios, lung vascular Kf, lung neutrophil counts, lung NOX and myeloperoxidase (MPO) activity, and plasma IL-6 levels were measured 24 h after trauma. In an additional study, lungs were isolated from nontrauma lean and obese rats to determine the acute effect of phenazime methosulfate, a superoxide donor, on pulmonary vascular Kf. After trauma, compared with lean rats, obese rats exhibited greater increases in lung capillary Kf, neutrophil accumulation, NOX and MPO activity, and plasma IL-6. The lung wet/dry weight ratio was increased in obese rats but not in lean rats. Apocynin treatment decreased lung Kf, neutrophil counts, NOX and MPO activities, wet/dry weight ratio, and plasma IL-6 in obese rats. Phenazime methosulfate treatment resulted in a greater increase in lung Kf in nontrauma obese rats compared with nontrauma lean rats. These results suggest that obese rats are susceptible to lung injury following severe trauma due to increased production of and responsiveness to pulmonary oxidative stress.

Methods

Animals. Male lean Zucker rats (LZ, 325 ± 6 g) and OZ (497 ± 12 g) (~12 wk) were purchased from Harlan Laboratories (Indianapolis, IN). The experimental protocols for this study were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and were carried out according to both the “Guide for the Care and Use of Laboratory Animals” from the National Institutes of Health and the guidelines of the Animal Welfare Act.

Severe trauma protocol. In brief, severe trauma was induced on both hindlimbs in LZ and OZ (~12 wk old) under anesthesia (5% isoflurane inhalation) as previously described (31). Soft tissue injury was induced by crushing all of the muscle groups adjacent to both the femur and fibula with an Angled-Kelly clamp (19 cm) at the first notch for 30 s. Bone components (1.5 ml/leg) were injected bilaterally into the region near the femur using a 15-gauge needle. During the insertion of the needle, the fibula was fractured in each leg. The femur and tibia bones used to make the bone component suspension to inject into LZ and OZ were harvested from previously killed LZ and OZ, respectively, crushed (along with marrow) in liquid nitrogen, and homogenized in PBS (2 g/5ml) (31, 40–42).

Following severe trauma, obese patients exhibit an increased incidence of acute lung injury (ALI) (4, 29), the earliest and most frequently occurring component of multiple organ failure, and also a major cause of mortality (3, 6, 8, 35). The majority of studies on ALI have been performed in the setting of sepsis, while little is known about the mechanisms for severe trauma-induced ALI and why it occurs more frequently in obesity. Understanding the mechanisms for increased ALI in obese patients with orthopedic trauma is important to improve outcomes and reduce medical costs associated with the treatments.

Sepsis-induced ALI is characterized by damage to the pulmonary microvasculature, causing increases in capillary filtration coefficient (Kf) and the resultant noncardiogenic pulmonary edema associated with exacerbated systemic inflammation, lung neutrophil retention, and oxidative stress (15, 38). The increase in oxidative stress is initiated by the accumulation and activation of neutrophils in the lung and the resultant production of NADPH oxidase (NOX)-dependent superoxide (respiratory burst) (15), which is converted by dismutase and myeloperoxidase (MPO) to more toxic free radicals. To our knowledge, the impacts of oxidative stress and antioxidant treatments targeting NOX activation on lung capillary Kf have not been determined following severe trauma, especially in a setting of obesity. In the current study, we induced severe trauma in obese Zucker rats (OZ), a widely used model of obesity with metabolic and cardiovascular dysfunction, which mimics the pathophysiological conditions in obese patients (3, 40, 43). We tested the hypothesis that, following severe trauma, OZ exhibit increased lung capillary Kf due to an elevated production of and/or vulnerability to oxidative stress, with these abnormalities improved by NOX inhibition.

Methods

Animals. Male lean Zucker rats (LZ, 325 ± 6 g) and OZ (497 ± 12 g) (~12 wk) were purchased from Harlan Laboratories (Indianapolis, IN). The experimental protocols for this study were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and were carried out according to both the “Guide for the Care and Use of Laboratory Animals” from the National Institutes of Health and the guidelines of the Animal Welfare Act.

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Our aim was to mimic severe trauma from a large long bone (femur) fracture, in which soft tissue injury and the release of bone components are present at the same time. We induced fibula fracture to simulate the stress of long bone fracture, but unlike a femur fracture, this manipulation does not necessitate fixation surgery that could interfere with outcomes (27, 30). Because the fibula is very small, we injected additional bone components to the injured area as...
The other lung lobes were fixed in formalin or liquid nitrogen immediately after isolation. After 24 h in formalin, the tissues were transferred to 70% alcohol until histological analysis of the neutrophil counts was performed. Liquid nitrogen-fixed tissues were stored in −80°C for later measurements of oxidase activities.

Neutrophil counts. Hematoxylin and eosin-stained lung tissue sections were used to count the number of neutrophils per high-power field (×150). For each slide, neutrophils were counted in a blinded manner on five random nonoverlapping fields of lung alveoli from each rat. The mean number of neutrophils per field was reported (7).

NOX and MPO activities. Lung tissue was homogenized and centrifuged at 4°C at 12,000 g for 20 min. The homogenates (supernatant) were incubated with lucigenin (final concentration: 5 μM) for chemiluminescence detection of NOX activity using a Berthold luminometer as previously described (39). The activity of MPO in homogenates was measured using the Invitrogen EnzChek Assay Kit (Life Technologies, Grand Island, NY). The enzyme activity was normalized by protein concentration.

Lung capillary filtration coefficient (Kf) and vascular resistance. In a separate set of experiments, control and trauma rats were anesthetized with pentobarbital (65 mg/kg ip), and lungs and hearts were removed surgically to measure the pulmonary vascular Kf, as previously described (42). Pulmonary arterial pressure (Pa) and venous pressure (Pv) were measured using a PowerLab system (model: ML 118, Colorado Springs, CO). The Pw was adjusted at 3.5 mmHg by the height of the perfusate reservoir. After a 15-min equilibration period, the vascular resistance was calculated by the following formula: (Pa − Pv)/flow (20 ml·kg⁻¹·min⁻¹). Baseline capillary pressure (Pc)

Previous studies have described (19, 24, 31). These manipulations cannot completely mimic the exact circumstance of a trauma involving a long bone fracture in humans, but that is not possible due to the highly heterogeneous nature of traumatic injury. Indeed, this trauma protocol focuses more on mimicking posttrauma outcomes rather than the injury components.

NOX inhibition following trauma. In a group of animals, LZ and OZ were treated with apocynin (50 mg/kg ip), a NOX inhibitor, immediately after the trauma, followed by apocynin treatment in the drinking water (2 mM) for 24 h. Apocynin was completely dissolved in the drinking water but not in the saline used for intraperitoneal injection. Before each injection, the solution was well vortexed to ensure the rats received the same concentration.

Body temperature and plasma interleukin 6 (IL-6) levels. The core body temperature was measured before and 24 h after trauma by inserting a thermometer probe (PhysiTemp, model: BAT-12) into the stomach immediately after isoflurane anesthesia. In a separate set of experiments, plasma was collected from control and trauma lean and obese rats after decapitation, and IL-6 levels were measured via ELISA (R&D Systems, Minneapolis, MN).

Lung edema, neutrophil counts, and oxidative stress. Following measurements of body temperature, rats were decapitated to collect lung lobes to minimize the blood volume trapped in the lung circulation. The lower left lung lobe was isolated and stored at room temperature for 3–4 wk until a stable dry weight was achieved. The wet/dry weight ratio was used as an index of pulmonary edema (32). The other lung lobes were fixed in formalin or liquid nitrogen.
Kf was calculated by using the following formula: Kf = (ΔW/ΔPc)/lung dry weight. ΔPc is the delta change of Pc from the basal to the 15th min after the elevation of Pv.

Lung capillary Kf and vascular resistance in nontrauma LZ and OZ treated with superoxide donor. Lungs were isolated from nontrauma LZ and OZ to determine the acute effects of the superoxide donor, phenazime methosulfate (PMS, 1 μM), on pulmonary vascular Kf and vascular resistance. PMS was added to the perfusate 15 min before the increase in Pv, yielding a final concentration of 1 μM of PMS for 15 min.

Resistance (mmHg/ml/min) was determined by occlusion on both the arterial and venous sides. The Pv was then increased by 5 mmHg for a period of 15 min, and the increase in lung weight (ΔW) between the 5th and 15th min was recorded. At the end of the 15-min period, the Pc was measured again. Kf was calculated by using the following formula: Kf = (ΔW/ΔPc)/lung dry weight. ΔPc is the delta change of Pc from the basal to the 15th min after the elevation of Pv.

RESULTS

Systemic inflammatory responses. Figure 1, A and B, presents the plasma IL-6 levels and core temperature 24 h after trauma, respectively. IL-6 and body temperature were significantly increased in both LZ and OZ after trauma. Compared with LZ, OZ exhibited larger increases in IL-6 levels after trauma. Apocynin treatment had no effect on the posttrauma IL-6 levels in LZ but significantly decreased the IL-6 levels in OZ to levels similar to those observed in LZ after trauma. Apocynin treatment blunted the increase in temperature in LZ, with no effect on the OZ.

Lung edema, neutrophil counts, and oxidative stress. The basal wet/dry weight ratio and histological neutrophil counts 24 h after trauma were similar between nontrauma LZ and OZ (Fig. 2, A and B, respectively). Twenty-four hours after trauma, only the OZ exhibited an increased wet/dry ratio, which was normalized by apocynin treatment. In addition, OZ exhibited a larger increase in pulmonary neutrophil numbers, which were decreased by apocynin treatment. The basal NOX and MPO activities in OZ compared with trauma.

Quantitative and statistical analyses. The DHE levels before and after PMS treatments were compared by t-test. All of the other data were compared by using two-way ANOVA. Where significant effects occurred, individual groups were compared using the Holm-Sidak method. All of the data are presented as means ± SE. A probability of P < 0.05 was accepted as statistically significant for all comparisons.

Fig. 3. NADPH oxidase (A) and myeloperoxidase (MPO) (B) activities in LZ and OZ before and 24 h after severe orthopedic trauma. Trauma results in significantly larger increases in both enzyme activities in OZ compared with LZ. *P < 0.05, trauma vs. control; +P < 0.05, OZ vs. LZ; #P < 0.05, trauma vs. trauma + apocynin. A: n = 5 for OZ trauma, n = 6 for all the other groups, n = 6 for apocynin-treated groups. B: n = 6 for LZ trauma, n = 6 for all the other groups, n = 6 for apocynin-treated groups.

Fig. 4. Vascular filtration coefficient (Kf; A) and vascular resistance (B) in isolated lungs 24 h after trauma. *P < 0.05, trauma vs. control; +P < 0.05, OZ vs. LZ within control or trauma; #P < 0.05, trauma vs. trauma + apocynin within OZ. n = 5 for LZ basal, n = 4 for apocynin-treated LZ, n = 6 for apocynin-treated OZ, n = 6 for all the other groups.
activities were not significantly different between LZ and OZ (Fig. 3, A and B, respectively). Twenty-four hours after trauma, the NOX and MPO activities were increased in both LZ and OZ, with OZ exhibiting larger increases in both enzyme activities compared with LZ. Apocynin treatments reduced NOX and MPO activities to similar levels in both LZ and OZ.

**Lung capillary Kf and vascular resistance.** Figure 4, A and B, presents the vascular Kf and vascular resistance, respectively, in isolated lungs 24 h after trauma. The isolated lungs from nontrauma OZ exhibited a higher basal Kf but a similar vascular resistance compared with the isolated lungs from nontrauma LZ. Twenty-four hours after trauma, the lung Kf was increased in both LZ and OZ, with OZ exhibiting a significantly larger increase. The vascular resistance was similarly elevated in LZ and OZ following trauma. Apocynin treatment normalized the increased Kf in OZ following severe trauma with no effect in the LZ. Additionally, apocynin treatment had no effect on the vascular resistance in LZ or OZ following severe trauma.

PMS increased the Kf in both LZ and OZ with no effect on vascular resistance (Fig. 5, A and B, respectively). Notably, the PMS-induced increase in Kf was significantly larger in the isolated lungs from OZ compared with LZ. Figure 5C shows that PMS treatment significantly increased the superoxide levels in the aortas of nontrauma LZ rats.

**DISCUSSION**

The major findings in the current study were 1) in OZ compared with LZ, severe orthopedic trauma in the OZ resulted in greater increases in systemic inflammation, lung neutrophils, lung NOX and MPO activities, and lung Kf, along with the development of lung edema; 2) these abnormalities were improved following apocynin treatments; and 3) treatment with the superoxide donor, PMS, induced a larger increase in capillary Kf in nontrauma OZ compared with nontrauma LZ.

**Severe trauma model and systemic inflammation.** To avoid posttrauma (fixation) surgery that could be a “second hit” that exacerbates systemic inflammation (27, 30), previous studies applied a moderate soft tissue injury adjacent to the femur bone followed by a bone component injection, mimicking a long bone fracture in rats and mice (19, 24). However, with this trauma model, these studies did not provide direct evidence of increased pulmonary Kf, a key characteristic of ALI (19, 24). Our previous study showed that following moderate trauma, isolated lung capillary Kf in the OZ was intact, despite a functional increase in pulmonary permeability and alveolar protein concentration triggered by a circulating inflammatory agonist(s) (42). The current study applied a more severe orthopedic trauma with fibula bone fracture (no fixation is required) and a greater soft tissue injury in both hindlimbs. We found increased lung capillary Kf and lung edema in OZ following severe trauma, suggesting development of ALI. In addition, our recent study demonstrated that, following this trauma protocol, OZ exhibit exacerbated inflammation and increased acute kidney injury (31). Therefore, the current trauma protocol may be used as a model to study the mechanisms for increased multiple organ failure observed in obese trauma patients (2).

Following severe trauma, OZ exhibited exacerbated systemic inflammation as demonstrated by the larger increases in body temperature and IL-6 levels, consistent with the findings in obese trauma patients (2, 14, 21). Circulating IL-6 is an early inflammatory marker, providing a strong correlation to systemic inflammatory response scores (10, 13). Circulating IL-6 levels have been shown to tightly correlate with the extent of trauma (10, 13, 37). In the current study, the IL-6 levels were significantly elevated in both LZ (~10 fold) and OZ (~20 fold) following severe trauma. In our previous study, a milder trauma increased IL-6 levels by only ~50% in the OZ with no effect in LZ (42).

![Figure 5](image-url)
Lung capillary Kf. Similar with clinical observations (4, 29), OZ exhibited increased ALI as indicated by lung edema and a 5-fold increase in pulmonary capillary Kf following severe trauma. The pulmonary vascular resistance was also increased in OZ following trauma, consistent with previous studies suggesting an elevated pulmonary vascular resistance during systemic inflammatory responses (9, 18). However, the increased vascular resistance is unlikely to be the major cause of lung edema since similar increases in lung vascular resistance were also observed in LZ. In addition, the slight increases in lung oxidative stress and capillary Kf in LZ following trauma did not lead to edema, suggesting functional protective factors such as increased alveolar fluid clearance. Notably, alveolar and distal airway epithelia are remarkably resistant to injury (11, 22, 26), and the lung fluid clearance is usually increased in the injured lung (34, 35). Lung injury accompanied by a decreased fluid clearance usually leads to extreme edema with the wet/dry weight ratio being larger than 6 (23, 34, 35). In the current study, the maximal wet/dry ratio after the development of edema was less than 5. Therefore, these results suggest that the lung edema in OZ following severe trauma is mainly due to increased capillary Kf rather than increased vascular resistance or impaired alveolar clearance.

Oxidative stress. Antioxidant trials for sepsis-induced ALI have resulted in controversial findings (15, 16). Thus, identifying the cellular source and targeting the particular pathway of oxidant generation for redox-based therapies have been emphasized in recent years. In sepsis-induced ALI, the increase in oxidative stress has been demonstrated to be initiated by the respiratory burst of infiltrated neutrophils (15, 38). NOX is the critical enzyme for the neutrophil respiratory burst to produce superoxide, which is then converted by dismutase and MPO to more toxic oxidants. MPO is abundant in neutrophil cytoplasmic granules and thus acts as a marker for neutrophils. Following trauma, OZ exhibited a larger number of neutrophils in the lung as assessed by histology associated with greater increases in NOX and MPO activities. Therefore, in the current study, our antioxidant treatment targeted NOX-mediated superoxide production, which is an essential step for neutrophil-derived reactive oxygen species.

In the current study, the beneficial effects of apocynin in OZ support an obligatory role of NOX-dependent superoxide in mediating trauma-induced acute lung injury. In addition, neutrophils contain very few mitochondria, and neutrophil depletion was shown to significantly decrease the degree of chest trauma- and sepsis-induced ALI (33). Thus we believe that NOX activation in neutrophils is the major mechanism for superoxide production during the development of acute lung injury following trauma. There may be other cellular sources of NOX-dependent superoxide in addition to neutrophils, but it would be difficult to quantify the NOX activity and superoxide production from each individual cell type.

Numerous studies have demonstrated that reactive oxygen species impair the regulation and integrity of endothelial cell junctions and thus increase endothelial Kf (1, 25, and 39). Compared with LZ with trauma, the larger increases in pulmonary NOX and MPO activities in OZ with trauma parallels a larger increase in pulmonary capillary Kf. Infusion of PMS, a superoxide donor (12), in the isolated lungs of nontrauma rats increased pulmonary capillary Kf without altering vascular resistance. These results provide evidence for an oxidant-induced increase in pulmonary capillary Kf. Treatment with the same amount of PMS resulted in a greater increase in lung Kf in OZ compared with LZ. Thus the larger increase in pulmonary capillary Kf in OZ following severe trauma could be due to an integrative effect of increased production and/or vulnerability to ROS. Indeed, apocynin only showed effects on the lung Kf, wet/dry ratio, and circulating IL-6 in the OZ after trauma, suggesting that the impact of oxidative stress following severe trauma is minor in LZ.

ROS play an important role in mediating innate immune responses, including activation of damage-associated molecular pattern molecules (44), neutrophil priming (17), stimulation of oxidant-related transcription factors and molecular events (15), and production of cytokines and chemokines (29). We found that apocynin treatment decreased lung neutrophil numbers, MPO activity, and plasma IL-6 levels in OZ following trauma, suggesting that exacerbated oxidative stress in the OZ facilitates or further stimulates immune responses. Thus the beneficial effects of apocynin on preventing ALI in OZ are likely due to both diminished pulmonary vasculature damage (mediated by oxidative stress) and inflammatory responses.

In conclusion, the current results suggest that OZ are susceptible to ALI following severe trauma due to increased production of and/or responsiveness to oxidative stress, including increased lung vasculature damage and exacerbated lung inflammation. Apocynin treatment decreased the exacerbated inflammation and the ROS-mediated damage to lung Kf and prevented the development of ALI in OZ following severe trauma. This study provides important insights into the area of redox-based therapeutics by using cytosolic NOX-targeting compounds during trauma-induced ALI in obesity. Future studies are needed to determine the mechanisms responsible for the exacerbated oxidative stress and its interaction with inflammatory responses in OZ following severe trauma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.X. conception and design of research; L.X., S.L., P.M., J.C., and R.L.H. approved final version of manuscript. R.L.H. interpreted results of experiments; L.X. and S.L. prepared figures; L.X. and P.M. performed experiments; L.X. and S.L. analyzed data; L.X., S.L., and P.M. manuscript; L.X., P.M., J.C., and R.L.H. edited and revised manuscript; L.X., S.L., P.M., J.C., and R.L.H. approved final version of manuscript.

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