Glibenclamide Pretreatment Attenuates Acute Lung Injury by Inhibiting the Inflammatory Responses and Oxidative Stress in A Polymicrobial Sepsis Animal Model
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ABSTRACT

Background: Emerging evidence suggests that the NLRP3 inflammasome pathway and its downstream cytokines play key roles in the pathophysiology of inflammatory diseases. Glibenclamide is a widely used sulfonylurea drug for the treatment of type 2 diabetes, which has been reported to inhibit the activation of NLRP3 inflammasome. However, the role of glibenclamide on acute lung injury (ALI) is not known in a mouse model induced by cecal ligation and perforation (CLP).

Background: Thirty mice were equally assigned to the Sham group, CLP group, and CLP + glibenclamide groups (N=10). One hour before CLP or sham operation, mice received an intraperitoneal injection of 50 mg/kg glibenclamide or the same volume of normal saline. Interleukin (IL)-1β, IL-6, IL-18, tumor necrosis factor (TNF)-α, Toll-like receptor 4, inducible nitric oxide synthase, caspase-1, nitric oxide, wet-to-dry weight ratio, malondialdehyde, and superoxide dismutase in the lung were assessed at 24 hours after the operation. The 7-day survival rate was also recorded.

Results: Glibenclamide pretreatment alleviated ALI, as indicated by decreased neutrophil infiltration and wet-to-dry weight ratio, which was accompanied by the decreased levels of interleukin (IL)-1β, IL-6, Toll-like receptor 4, inducible nitric oxide synthase, caspase-1, nitric oxide, and malondialdehyde in the lung. Furthermore, glibenclamide pretreatment prevented the sepsis-induced hyperglycemia at 6 hours after CLP. However, no significant difference was detected in pulmonary levels of nuclear factor (NF)-κB p65, TNF-α, IL-18, and superoxide dismutase or the 7-day survival rate between the CLP group and the CLP + glibenclamide group.

Conclusions: Glibenclamide pretreatment attenuates the ALI by inhibiting the inflammatory responses and oxidative stress in a polymicrobial sepsis animal model.
Sepsis, a systemic inflammatory disease developing after an infectious insult, and its sequelae are among the most frequent causes of mortality and morbidity in intensive care units (1). Sepsis is the most frequent cause of acute lung injury (ALI), leading to increased permeability pulmonary edema, enhanced polymorphonuclear neutrophil sequestration and respiratory failure (1-3). Despite recent advances in the understanding of the pathogenesis of ALI, few studies have identified therapies that show a beneficial impact on the outcome of ALI, with the exception of the use of lung protective ventilatory strategies (4). Therefore, the development of new drugs for the treatment of ALI is urgently needed.

Emerging evidence suggests that the inflammasome pathway and its downstream cytokines play key roles in the pathophysiology of inflammatory diseases (5, 6). Inflammasomes are cytosolic sensors that detect pathogens and danger signals in the innate immune system (7). Among these, the nucleotide-binding oligomerization domain (Nod)-like receptor 3 (NLRP3) inflammasome is the best characterized inflammasome that consists of NLR family, NLRP3, apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC), and pro-caspase-1 (7-9). Active NLRP3 inflammasome processes pro-interleukin (IL)-1β and pro-IL-18 to produce mature IL-1β and IL-18 to exert its biological activities (8), indicating the importance of NLRP3 inflammasome in the innate immune responses, and strategy targeting at this pathway may be useful for the treatment of ALI.

Glibenclamide is a widely used sulfonylurea drug for the treatment of type 2 diabetes with excellent safety profiles, which blocks the sulfonylurea receptor 1, the regulatory subunit of the KATP, and the NCCa-ATP channels (10). Recent studies have indicated glibenclamide can also inhibit the activation of NLRP3 inflammasome (5, 11). Furthermore, it has been suggested glibenclamide possesses antioxidation properties (10). Therefore, the present study was aimed to evaluate whether glibenclamide attenuates the ALI in a mouse model induced by cecal ligation and perforation (CLP). In addition, the mechanism underlying the protective effects of glibenclamide on lung was also explored in the present study.

**MATERIALS AND METHODS**

**Animals and Ethics**
The present study was approved by the Ethics Committee of Jinling Hospital, Nanjing University, and was performed in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Adult male C57BL/6 mice weighing 25-32 g were purchased from the Animal Center of Jinling Hospital, Nanjing, China. Animals were housed in standard conditions and maintained in a 12/12-hour light-dark cycle with food and water ad libitum.

**ALI Model**
Polymicrobial sepsis was induced by CLP as previously described (12). Briefly, C57BL/6 mice were anesthetized with intraperitoneal injection of 2% sodium pentobarbital in saline (50 mg/kg; Sigma Chemical Co, St. Louis, MO). The cecum was isolated and then ligated with 4.0 silk below the ileocecal junction, approximately 1.2 cm from the distal end. The cecum was then punctured twice on the anti-mesenteric side with a sterile 22-gauge needle and was gently squeezed to extrude the fecal contents into the peritoneal cavity. Finally, the cecum was placed back into the abdomen and the incision was closed with sutures in two layers. For the mice served as controls, the operation was performed in the same manner as CLP except that the cecum was neither ligated nor punctured. All mice were then resuscitated with subcutaneous lactated Ringers 30 ml/kg immediately after surgery. The entire procedure was completed within 8 min.

**Experimental Protocols**
Mice were randomly allocated into one of the following three groups (N=10 for each group). (1) Sham group: animals received an intraperitoneal injection of saline (10 ml/kg) at 1 hour before operation. (2) CLP group: animals received an intraperitoneal injection of saline (10 ml/kg) at 1 hour before CLP. (3) CLP + glibenclamide (CLP + GLB) group: animals received an intraperitoneal injection of 50 mg/kg glibenclamide (Glibenclamide was first dissolved in dimethylsulphoxide and then diluted with 0.9% saline) at 1 hour be-
before CLP. The dose of glibenclamide was determined on the basis of a previous study (5) and our preliminary experiment demonstrating that this dose reduced inflammatory mediators in a polymicrobial sepsis mouse model. At the end of the 24-hour period, mice were euthanatized by an overdose of sodium pentobarbital of 80 mg/kg. Mice were then perfused with 10 ml of phosphate-buffered saline through the right ventricle to rinse the pulmonary circulation of blood. Thereafter, lung tissues were harvested for further tissue analyses.

**Blood Glucose Measurement**

Blood was obtained from eyes by puncture, and blood glucose levels were measured at 6, 12, 18, and 24 hours after sham operation or CLP (Life Scan, Inc, Inc. Milpitas, CA 95035 USA).

**Histological Analysis**

An isolated central lobe in the right lung was excised and immediately immersed into 4% formalin. The samples were sectioned and stained with hematoxylin and eosin for light microscopy. The severity of microscopic injury was graded from 0 (normal) to 4 (severe) based on the following categories: neutrophil infiltration, interstitial edema, hemorrhage, hyaline membrane. The sum of all scores was combined to calculate a composite score as described previously (12).

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Pulmonary levels of inflammatory cytokines were quantified using specific ELISA kits for rats according to the manufacturers’ instructions [tumor necrosis factor (TNF)-α from Diacclone Research, Besanson Cedex, France; IL-1β and IL-18 from R&D Systems, Minneapolis, MN, USA; IL-6 from Biosource Europe SA, Nivelles].

**Western Blotting Analysis**

Western blotting was performed as previously described (12) using the following primary antibodies: The primary antibodies used to detect caspase-1, IL-1β, Toll-like receptor 4 (TLR4), nuclear factor (NF)-κB p65, and iNOS were from Santa Cruz Biotechnology. The antibody for β-actin was from Cell Signaling Technology. We used the NIH Image J software (National Institutes of Health, Bethesda, MD, USA) to quantitate protein band concentrations.

**Myeloperoxidase (MPO) Activity and Nitric Oxide (NO) Concentration**

Myeloperoxidase (MPO) activity, a marker for polymorphonuclear neutrophil infiltration into the lung, was determined using a MPO (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) assay kit according to the manufacturer's protocol. NO concentration was measured using a technique as previously described (12).

**Malondialdehyde (MDA) and Superoxide Dismutase (SOD) Assay**

MDA concentrations were determined by the thiobarbituric acid method and SOD activities were evaluated by the xanthine oxidase method as previously described (12). The absorbance was measured at 532 and 550 nm for MDA and SOD, respectively. MDA concentrations are expressed as nanomoles per milligram of protein and SOD activities are expressed as units per milligram of protein.

**Wet-to-Dry Weight (W/D) Ratio**

The left lower lobe of the lung was harvested, weighed, and dried for 48 hours in a heated stove. Lung wet to dry (W/D) weight ratio was calculated by dividing wet by the dry lung weight.

**Survival Rate**

In additional three groups of animals: Sham group
(N=12), CLP group (N=20), and glibenclamide + CLP group (N=20). Mice were then allowed to food and water ad libitum, animals were frequently observed by a researcher blinded to the group assignment to determine the 7-day survival rate.

Statistical Analysis
Data are expressed as the mean ± standard error of the mean (S.E.M.). Normal distribution for the continuous variable was tested using Kolmogorov-Smirnov test and all variables were normally distributed. Statistical significance was determined by one-way, two-way, or repeated-measures of analysis of variance (ANOVA) followed by a Bonferroni test, as appropriate. The survival rate was estimated by Kaplan-Meier method and compared by the log-rank test. A P value <0.05 was regarded as statistically significant difference.

RESULTS

Glucose Concentration Changes Over Time
In the sham group, blood glucose levels were not altered during the experimental period. CLP induced biphasic changes in blood glucose, with hyperglycemia at 6 hours after CLP followed by hypoglycemia from 12 hours onwards. Pretreatment of CLP mice with glibenclamide prevented sepsis-induced hyperglycemia at 6 hours after CLP as compared with CLP group (P=0.003) (Figure 1).

Lung Histopathologic Changes
The lung tissues of mice subjected to CLP illustrated an increase in alveolar wall thickness, increased neutrophil infiltration, and alveolar congestion/collapse (Figure 2). However, the injury of the lung was significantly attenuated by glibenclamide pretreatment (P=0.034) (Figure 2).

TNF-α, IL-6, IL-1β and IL-18 Levels in the Lung
The concentrations of IL-6 and IL-1β in the lung were significantly increased after CLP, which were attenuated by glibenclamide pretreatment (P=0.033 and P=0.027, respectively). No difference was observed in TNF-α and IL-18 levels between the CLP group and glibenclamide + CLP group (P=0.765 and P=0.578, respectively) (Figure 3).

MDA, SOD, MPO Activity, W/D, and Nitride Oxide (NO) Levels in the Lung
Glibenclamide pretreatment significantly decreased pulmonary MDA and NO levels compared with the CLP group (P=0.037 and P=0.015, respectively). However, no difference was observed in SOD levels between the CLP group and glibenclamide + CLP group (P=0.643) (Figure 4). MPO activity and W/D ratio in the lung were significantly increased after CLP, whereas glibenclamide pretreatment significantly decreased the MPO activity and W/D ratio (P=0.023 and P=0.012, respectively) (Figure 5).

TLR4, NF-κBp65, and iNOS Expression in the Lung
TLR4 and iNOS expression in the lung was significantly increased after CLP, whereas glibenclamide pretreatment significantly inhibited the TLR4 and iNOS expression (P=0.012 and P=0.003, respectively). No difference was observed in NF-κBp65 expression between the CLP group and glibenclamide + CLP group (P=0.865) (Figure 6).

Caspase-1 and IL-1β Expression in the Lung
Caspase-1 and IL-1β expression in the lung was significantly increased after CLP, whereas glibenclamide pretreatment significantly inhibited the caspase-1 and IL-1β expression (P=0.007 and P=0.037, respectively) (Figure 7).

Survival Rate
No animal died in the sham group. The 7-day survival rate in the CLP group and glibenclamide + CLP group was 55% and 65%, respectively (P=0.354) (Figure 8).

DISCUSSION
In the present study, although no benefit in the 7-day survival rate was observed, our data showed that pretreatment of mice subjected to severe sepsis with glibenclamide attenuated ALI, as indicated by decreased neutrophil infiltration, pulmonary edema, and lung injury. Moreover, we suggest that the protective effects may result from its ability to inhibit inflammatory response and oxidative stress.
**Figure 2.** Histological Scores (Hematoxylin-Eosin × 400) in Lung Tissue Specimens at 24 Hours after Operation (N=3).

*P < 0.05 vs. Sham group; #P < 0.05 vs. CLP+GLB group.

**Figure 3.** Changes in Pulmonary Levels of TNF-α, IL-6, IL-1β, and IL-18 in the Sham, CLP, Glibenclamide+CLP Groups 24 Hours after Operation (N=6).

*P < 0.05 vs. Sham group; #P < 0.05 vs. CLP+GLB group.
Figure 4. Malondialdehyde (MDA) and Superoxide Dismutase (SOD) Activity in the Lung (N=6).

*P<0.05 vs. Sham group; #P<0.05 vs. CLP + GLB group.

Figure 5. Myeloperoxidase (MPO) Activity, Wet-to-Dry (W/D) Ratio, and Nitrite Oxide (NO) Levels in the Lung (N=3-6).

*P<0.05 vs. Sham group; #P < 0.05 vs. CLP + GLB group.

Figure 6. Representative Western Blot and Quantitative Analysis of Toll-Like Receptor 4 (TLR4), Nuclear Factor (NF)-κB p65, and iNOS in the Lung (N=3).

*P<0.05 vs. Sham group; #P<0.05 vs. CLP + GLB group.

Figure 7. Representative Western Blot and Quantitative Analysis of Caspase-1 and IL-1β in the Lung (N=3).

*P<0.05 vs. Sham group; #P<0.05 vs. CLP + GLB group.
CLP model is usually used to investigate the settings of sepsis and related organ damage including ALI because it closely mimics the pathophysiology of clinical sepsis, which has been well established in our previous studies (12). ALI results from the activation of innate immune cells and endothelial cells by endotoxins, leading to systemic inflammation through overproduction of proinflammatory molecules such as cytokines, NO, and reactive oxygen species (12). Accumulating evidence has demonstrated that inflammasomes mediated cytokines release such as IL-1β and IL-18 play key roles in ALI development in animal models of systemic inflammation (5, 6). A two-step stimulation process is necessary to release IL-1β. The first step requires NF-κB dependent production of pro IL-1β by TLR-signaling and the second step involves the activation of NLRP3 by pathogen-associated molecular patterns. On the other hand, IL-1β binding to its signaling receptor results in the activation of NF-κB, an important factor perpetuating the inflammatory responses. We confirmed previous findings that increased IL-1β levels were detected during ALI, whereas glibenclamide pretreatment decreased IL-1β levels. Surprisingly, we did not detect the decreased IL-18 levels after glibenclamide pretreatment, a phenomenon we cannot explain. Furthermore, TNF-α level was not affected despite glibenclamide pretreatment decreased TLR4 expression. One of the main reasons could be that TNF-α levels peak shortly after sepsis development and return to baseline levels more shortly compared with other cytokines. This characteristic kinetics of TNF-α may explain why we did not find such difference.

The release of the proinflammatory IL-1β and IL-18 is controlled by a dual pathway. Pathogen-recognition receptors such as TLRs control synthesis of pro-IL-1β and pro-IL-18, while NLRP3 inflammasome activation leads to IL-1β and IL-18 maturation and secretion (7-9). The NLRP3 in inflammasome senses pathogens and danger signals such as bacteria, viruses, fungi, components of dying cells, and uric acid in the innate immune system (7-9). Although inflammasome activation is critical for pathogen clearance and the induction of an adaptive immune response (7), dysregulation of NLRP3 inflammasome has been related to a number of human diseases, including type II diabetes (13), Crohn’s disease (14), and pulmonary disease (15-17). In support with the previous findings that NLRP3 inflammasome/caspase-1/IL-1β pathway activation is detrimental (5, 6), our data suggested pulmonary levels of caspase-1 and IL-1β were increased after sepsis insult. However, glibenclamide pretreatment attenuated lung lesions, as indicated by decreased leukocyte infiltration on histological examination and lung edema.

The anti-diabetic effects of glibenclamide raise the possibility that its treatment may result in hypoglycemia. Indeed, one recent report suggested that glibenclamide at 1 or 20 mg/kg failed to improve organ dysfunction, a mechanism might be attributed to worsening the hypoglycemia in experimental sepsis (18). However, our results found that glucose concentration was not significantly affected by glibenclamide pre-treatment even at the dosage of 50 mg/kg. The reason maybe that glibenclamide was administrated as a pretreatment strategy in our study, while hypoglycemia was reported when glibenclamide was used as a post treatment in other study (18). On the other hand, it has been demonstrated that glibenclamide inhibit NLRP3 inflammasome activation and subsequent cytokines release (5, 11). Likewise, an ex vivo study suggests that glibenclamide reduced shock induced overproduction of proinflammatory cyto-
kines only at high concentrations (19). In support with this finding, recent in vivo studies indicate that glibenclamide also exerts anti-inflammatory effects both in animal and human studies (11, 20). Together, these data suggested the anti-inflammatory profile of glibenclamide is partially dependent on NLRP3 activation. In addition, previous studies suggest that glibenclamide possesses anti-oxidant profiles (10, 21). It has been reported that glibenclamide may improve neurological outcome in animal models of stroke (21, 22). During sepsis, it is often associated with exacerbated production of free radicals (12), which was also observed in the present study. Once again, glibenclamide pretreatment reduced the MDA levels and thus suppressed the oxidative injury.

Although blood pressure is not significantly affected by the glibenclamide pretreatment in animal model of ventilator-induced lung injury (5), it has been demonstrated that glibenclamide can reverse endotoxin-induced hypotension, vascular hyporeactivity, and shock (23, 24). However, we did not monitor blood pressure due to technical reasons. Therefore, it is unknown whether the beneficial effects of glibenclamide are due to its hemodynamic beneficial effects or is, instead, the result of its direct anti-inflammatory effects. Furthermore, the dose response effects of glibenclamide were not performed in the present study. Finally, a potassium channel blocker such as tetraethylammonium is needed to confirm the beneficial effects of glibenclamide are dependent on potassium channel blocker other than inhibition of hyperglycemia. Therefore, further studies are required to address these important issues.

In conclusion, glibenclamide pretreatment attenuates ALI in an animal model of polymicrobial sepsis. This beneficial effects seems to be, at least in part, due to the amelioration of inflammation and oxidative stress.

Declaration of Interests

All authors have no financial support and potential conflicts of interest for this work.

References