Increased Susceptibility to *Klebsiella* Pneumonia and Mortality in GSNOR-Deficient Mice

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Abstract

S-nitrosoglutathione reductase (GSNOR) is a key denitrosylase and critically important for protecting immune and other cells from nitrosative stress. Pharmacological inhibition of GSNOR is being actively pursued as a therapeutic approach to increase S-nitrosoglutathione levels for the treatment of asthma and cystic fibrosis. In the present study, we employed GSNOR-deficient (GSNOR$^{-/-}$) mice to investigate whether inactivation of GSNOR may increase susceptibility to pulmonary infection by *Klebsiella pneumoniae*, a common cause of nosocomial pneumonia. We found that compared to wild-type mice, bacterial colony forming units 48 hours after intranasal infection with *K. pneumoniae* were increased over four folds in lung and spleen and strikingly, over a thousand folds in blood of GSNOR$^{-/-}$ mice. Lung injury was comparable between infected wild-type and GSNOR$^{-/-}$ mice, but inflammation and injury was significantly elevated in spleen of GSNOR$^{-/-}$ mice. Whereas all wild-type mice survived 48 hours after infection, 10 of 23 GSNOR$^{-/-}$ mice died. Thus, GSNOR appears to play a crucial role in controlling pulmonary and systemic infection by *K. pneumoniae*. Our results suggest that patients treated in clinical trials with inhibitors of GSNOR should be carefully monitored for signs of infection.

Keywords

Nitric oxide; S-nitrosylation; S-nitrosoglutathione reductase; *Klebsiella pneumoniae*

1. Introduction

Nitric oxide (NO) affects the functions of a wide range of proteins through S-nitrosylation, the covalent modification of cysteine thiols [1]. Protein S-nitrosylation is increased by NO synthases (NOSs) but down-regulated by S-nitrosoglutathione reductase (GSNOR), a ubiquitous and highly conserved denitrosylase [2–4]. By preventing excessive protein S-nitrosylation, GSNOR plays an evolutionarily conserved, critical role in protecting against nitrosative stress [2, 3]. Studies of GSNOR-deficient (GSNOR$^{-/-}$) mice have shown that

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

GSNOR deficiency results in protection from asthma and myocardial infarction but also leads to increased susceptibility to septic shock, liver cancer, and lymphopenia [3–8].

Accumulating evidence in humans and animals suggest important roles of S-nitrosothiols (SNOs) in lung. S-nitrosoglutathione (GSNO) may represent a major source of bronchodilatory NO bioactivity [9] and it is reportedly depleted from airway lining fluid in human asthmatics [10, 11]. Reduction in airway GSNO has been reported to be associated with increased GSNOR activity [12]. Single nucleotide polymorphisms in the human GSNOR gene have been linked to increased risk of asthma and decreased responsiveness to β-agonist therapy in asthmatics [13–15]. GSNOR deficiency protects mice from airway hyper responsiveness in experimental asthma [5]. In addition, GSNO may increase expression and maturation of wild-type and F508 mutant cystic fibrosis transmembrane conductance regulatory protein [16, 17].

Given the involvement of dysregulated S-nitrosylation in multiple pathological conditions, a number of approaches to regulate S-nitrosylation therapeutically are being tried in multiple indications [18]. In the lung, GSNO releasing compounds and GSNOR inhibitors are being considered as potential therapeutic approaches for asthma [19] and cystic fibrosis [17]. However, since GSNOR$^{-/-}$ mice appear to be more susceptible to experimental peritoneal sepsis [3] as well as lung inflammation [4], we hypothesize that GSNOR deficiency may increase sensitivity to lung infection. *Klebsiella pneumoniae* is a common cause of nosocomial pneumonia. Recent increase in nosocomial infection by carbapenem-resistant enterobacteriaceae, which is associated with high mortality, is mostly observed in *K. pneumoniae* [20]. *K. pneumoniae* infection induces NOS activity and NO production in mouse and human alveolar macrophages [21, 22]. NO or related reactive nitrogen species (RNS) produced by the macrophages is indispensible for effective phagocytosis and killing of *K. pneumoniae* [21, 22]. Therefore, we have investigated whether GSNOR$^{-/-}$ mice are more susceptible to pulmonary infection by *K. pneumoniae*.

2. Materials and methods

2.1. Animals

Wild-type C57BL/6 and congenic GSNOR$^{-/-}$ mice were housed in ventilated filter-top cages with centralized water supplies and fed normal mouse chow (5058 PicoLab Mouse Diet 20) *ad libitum* in a specific pathogen-free facility at the University of California at San Francisco (UCSF). Six- to 12-week-old mice were used for all experiments. The experimental protocol was approved by the Institutional Animal Care and Use Committee of UCSF.

2.2. Pulmonary K. pneumoniae infection

K. pneumoniae (subsp. *pneumoniae* (Schroeter) Trevisan (ATCC® 43816™), serotype 2) from ATCC was grown in Luria Broth (LB) overnight at 37 °C in a shaking incubator. Fifty microliters of the overnight culture was used to inoculate 25 ml of LB and grown for 3–4 hours to achieve log phase. Bacterial colony forming units (CFUs) were estimated by OD$_{600}$ measurements and confirmed by culturing on LB agar plates. This culture was diluted, and anesthetized mice were inoculated intranasally with 10^4 CFU *K. pneumoniae* in 30 μl volume. Inoculated mice were observed until fully recovered from anesthesia. Mice were checked twice daily for mortality until they were euthanized at 48 hours for bacterial load assessment.

2.3. Bacterial load quantification

Lungs and spleens were aseptically harvested from euthanized mice and homogenized in 500 μl of sterile phosphate buffered saline (PBS). The homogenates were serially diluted in PBS and 10 μl of each dilution were plated on LB agar plates and incubated overnight at 37°C, after which bacterial colonies were enumerated. The left lung was used for bacterial quantification. Spleen bacterial loads are normalized to the weight of the spleen that was homogenized. , Whole blood was serially diluted in PBS and 10 μl of each dilution were immediately plated on LB agar plates for quantification of blood bacterial titers.

2.4. Histopathology

Lung and spleen samples were fixed with formalin and embedded in paraffin. Tissue sections were stained with Hematoxylin and Eosin. The extent of lung injury induced by bacterial infection was quantified by histologically scoring neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris in the airway, and alveolar septal thickening, according to the guidelines of the American Thoracic Society [23]. Histological analysis and scoring were performed in five random fields per section and in a blinded fashion. Spleen sections were scored for inflammation, necrosis/abscess formation, thrombus formation, and hyperemia essentially as outlined by Wiersinga et al. [24].

2.5. Statistical analysis

Survival was compared using the two-tailed Fisher's exact test. Histological scores were compared with the Mann-Whitney *U*-test. Other data were analyzed with a two-tailed, unpaired Student's *t*-test.

3. Results

3.1 K. pneumoniae is increased in lung of GSNOR$^{-/-}$ mice in a pneumonia model

To test whether GSNOR$^{-/-}$ mice are sensitive to lung infection, we employed a pulmonary infection model using *K. pneumoniae* [25]. We found that 48 hours after intranasal infection with *K. pneumoniae*, bacterial CFUs in the lungs of GSNOR$^{-/-}$ mice were three- to 19-fold greater than wild-type control in three independent experiments (Fig. 1A). Analyzed together as a group, the bacterial CFU in GSNOR$^{-/-}$ lung was significantly higher than that in wild-type mice (Fig. 1B; $P = 0.003$). Thus, GSNOR$^{-/-}$ mice are more susceptible to lung infection by *K. pneumoniae*.

3.2. Comparable levels of lung injury in K. pneumoniae-infected wild-type and GSNOR$^{-/-}$ mice

To determine whether elevated bacterial loads in GSNOR$^{-/-}$ mice are associated with increased lung injury, lung sections from *K. pneumoniae*-infected wild-type (n = 13) and GSNOR$^{-/-}$ (n = 11) mice were analyzed and scored for the extent of neutrophils in the alveolar space and the interstitial space, hyaline membranes, proteinaceous debris in the airway, and alveolar septal thickening [23]. There was not a clear difference in the individual parameter scores or overall histological score between wild-type and GSNOR$^{-/-}$ mice (Fig. 1C). These data suggest that despite increased bacterial load in the lungs of GSNOR$^{-/-}$ mice, both wild-type and GSNOR$^{-/-}$ mice exhibit similar levels of lung injury.

3.3. Highly elevated bacterial loads in blood of K. pneumoniae-infected GSNOR$^{-/-}$ mice

When bacterial titers in the whole blood of *K. pneumoniae*-infected mice were analyzed, we found a striking 1,000- to 4,450-fold greater bacterial load in GSNOR$^{-/-}$ mice than in wild-type controls in three independent experiments (Fig. 2A). When analyzed together, blood

bacterial titer was significantly higher in GSNOR$^{-/-}$ mice, increasing about 1,750 fold (P = 0.001; Fig. 2B). *K. pneumoniae* was not detected in two of 17 wild-type mice analyzed, whereas all of 13 GSNOR$^{-/-}$ mice had detectible levels of bacteria in their blood. Also, not included in the bacterial load analysis were several GSNOR$^{-/-}$ mice that died between 24 and 36 hours, presumably from high bacterial loads. Thus, pulmonary infection of *K. pneumoniae* leads to a much more severe systemic infection in GSNOR$^{-/-}$ mice.

3.4. Increased K. pneumoniae infection and injury in spleens of GSNOR$^{-/-}$ mice

We also assessed the bacterial load in the spleens of wild-type and GSNOR$^{-/-}$ mice after pulmonary *K. pneumoniae* infection. Bacterial loads in the spleen were similarly matched to that in the lung for each animal in both wild-type and GSNOR$^{-/-}$ strains (Fig. 3A). Histopathological examination revealed a substantial increase in pathology in spleen of *K. pneumoniae*-infected mice (Fig 3B and C). Notably, GSNOR$^{-/-}$ spleens exhibited elevated pathological scores in inflammation, thrombus formation, and hyperemia when compared with those of wild-type mice (Fig 3C). The levels of necrosis and abscess formation were not different between wild-type and GSNOR$^{-/-}$ mice. Thus, pulmonary infection of *K. pneumoniae* results in a significant increase in splenic infection and injury in GSNOR$^{-/-}$ mice.

3.5. Increased mortality in GSNOR$^{-/-}$ mice after K. pneumoniae infection

We monitored *K. pneumoniae*-infected wild-type and GSNOR$^{-/-}$ mice for survival twice daily for 48 hours following infection. All mice survived for the first 24 hours after infection with some GSNOR$^{-/-}$ mice exhibiting signs of illness. By 40 hours, wild-type mice mostly remained active while most GSNOR$^{-/-}$ mice became severely sick and four of 23 *K. pneumoniae*-infected GSNOR$^{-/-}$ mice died. Whereas all wild-type mice survived 48 hours after infection, 10 of 23 GSNOR$^{-/-}$ mice died (Fig. 4). These data suggest that GSNOR is important for protection against lethal infection by *K. pneumoniae* .

4. Discussion

In this study, we demonstrated that GSNOR$^{-/-}$ mice exhibited increased bacterial load and mortality in a pulmonary *K. pneumoniae* infection model. Bacterial loads 48 hours after infection were increased over four folds in lung and spleen and strikingly, over a thousand folds in blood of GSNOR$^{-/-}$ mice. Thus, GSNOR appears to play a crucial role in controlling pulmonary and systemic infection by *K. pneumoniae*.

The important contribution to host defense against *K. pneumoniae* by GSNOR may result from its protection of immune cells from nitrosative stress. *K. pneumoniae* infection induces NOS activity and NO production in macrophages [21, 22], which is indispensible for effective phagocytosis and killing of *K. pneumoniae* [21, 22]. NO production by immune cells can cause nitrosative stress, which in absence of GSNOR can cause extensive cell death [2, 4]. Thus during immune response to *K. pneumoniae* infection, GSNOR may be essential for protection against NO-induced damage and death of immune cells in lung and spleen. Splenic macrophages, responsible for recycling of hemoglobin-derived iron [26], might sustain increased formation of S-nitrosothiols from NO [27] and thus critically depend on disposal of S-nitrosothiols by GSNOR. Lack of this protection in GSNOR$^{-/-}$ mice may account for increased damage and inflammation in spleen. Injury of the spleens, including particularly splenic macrophages, may disrupt their important function in the trapping and clearance of blood-borne pathogens [26], resulting in the marked failure of systemic control of the bacterial infection in GSNOR$^{-/-}$ mice. In addition, GSNOR$^{-/-}$ mice suffer increased apoptosis from nitrosative stress in thymic development that results in decreased CD4 T cells. While mice lacking αβ-T cells showed no increased susceptibility to *K. pneumoniae* in

a pneumonia model, mice lacking γδ-T cells displayed unimpaired clearance of pulmonary bacterial but mildly increased peripheral blood dissemination and mortality [28]. It remains to be determined whether GSNOR$^{-/-}$ mice exhibit a loss or deficiency of γδ-T cells.

GSNOR may contribute to the control of *K. pneumoniae* infection by preventing S-nitrosylation and inactivation of surfactant protein D (SP-D) or other proteins important for host defense. SP-D is secreted by lung epithelial cells and binds pathogen-associated molecular patterns as part of innate immune response [29]. SP-D can bind *K. pneumoniae* LPS [30] and improve phagocytosis and killing [31]. The binding to *K. pneumoniae* LPS requires higher-order multimerization of SP-D [30], which is inhibited by S-nitrosylation [32]. Whether GSNOR deficiency increases S-nitrosylation of SP-D or other antibacterial proteins during *K. pneumoniae* infection remains to be determined.

Pharmacologic approaches of inhibiting GSNOR activity have reached the point of clinical development with the recent announcement of the first cystic fibrosis patients treated with the first-in-class GSNOR inhibitor, N6022 (N30 Pharmaceuticals). Clinical development is ongoing for other indications including asthma. Our results suggest that GSNOR deficiency can increase sensitivity to *K. pneumoniae*, a common cause of nosocomial infection. Thus, clinical trials using S-notrosylating agents or GSNOR inhibitors should closely monitor patients for signs of infection.

Acknowledgments

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Highlights

GSNOR$^{-/-}$ mice are hypersensitive to pulmonary infection by *Klebsiella pneumoniae*.

Bacterial loads were increased over 4-fold in GSNOR$^{-/-}$ lung and 1000-fold in blood.

GSNOR$^{-/-}$ mice suffered significantly more inflammation and injury in spleen.

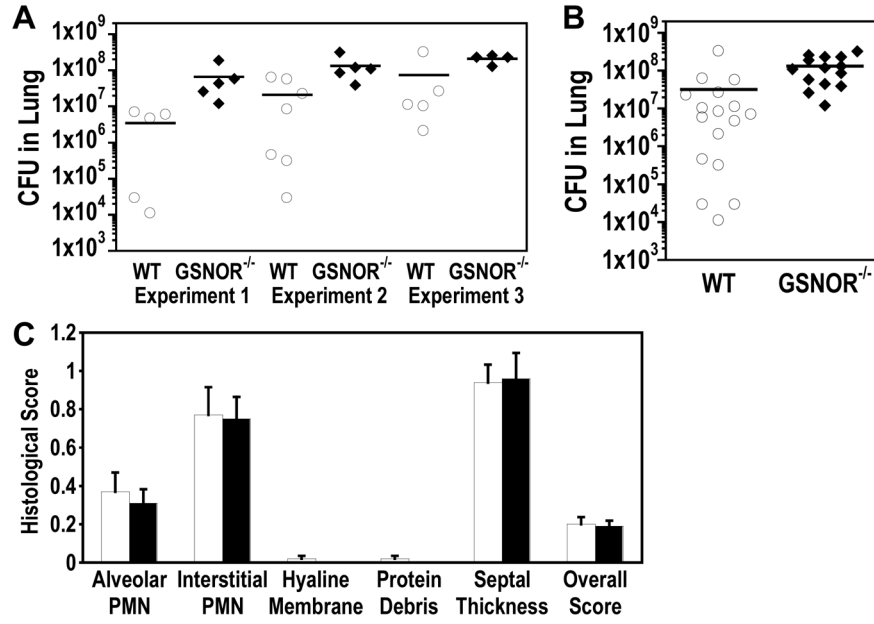All wild-type mice survived the infection, but 40% of GSNOR$^{-/-}$ mice died.

Fig. 1.
Increased *K. pneumoniae* infection in the lungs of GSNOR$^{-/-}$ mice in a pneumonia model.
(A) Bacterial loads in the left lung 48 hours after intranasal *K. pneumoniae* infection of
wild-type (WT, open circles) and GSNOR$^{-/-}$ (closed diamonds) mice were quantified by
colony forming units (CFU) for each of three independent experiments. (B) Aggregate lung
data from the three experiments shown in (A). Each data point represents a single mouse,
and horizontal lines represent the group mean. Bacterial loads in GSNOR$^{-/-}$ (n = 23) mice
are significantly higher than wild-type controls (n = 17; P = 0.003). (C) Comparable levels
of lung injury in K. pneumoniae-infected wild-type (open bars) and GSNOR$^{-/-}$ (closed bars)
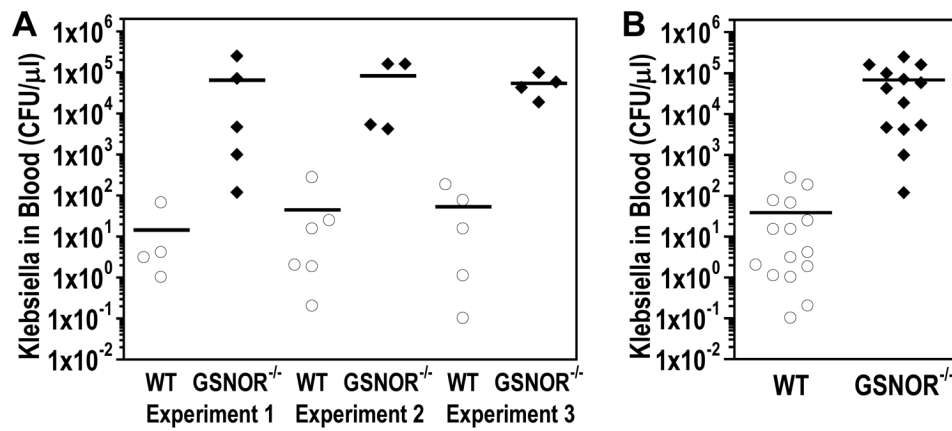mice. PMN, polymorphonuclear leukocyte.

Fig. 2.
Elevated systemic dissemination of *K. pneumoniae* in GSNOR$^{-/-}$ mice. Bacterial titers in whole blood 48 hours after intranasal *K. pneumoniae* infection of wild-type (WT, open circles) and GSNOR$^{-/-}$ (closed diamonds) mice in three independent experiments individually (A) and aggregately (B). Each data point represents a single mouse, and horizontal lines represent the group mean. Blood bacterial titers are over 1,700 fold higher in GSNOR$^{-/-}$ mice (n = 13) compared to wild-type controls (n = 17, two mice without detectible titer not shown), *P* = 0.001.
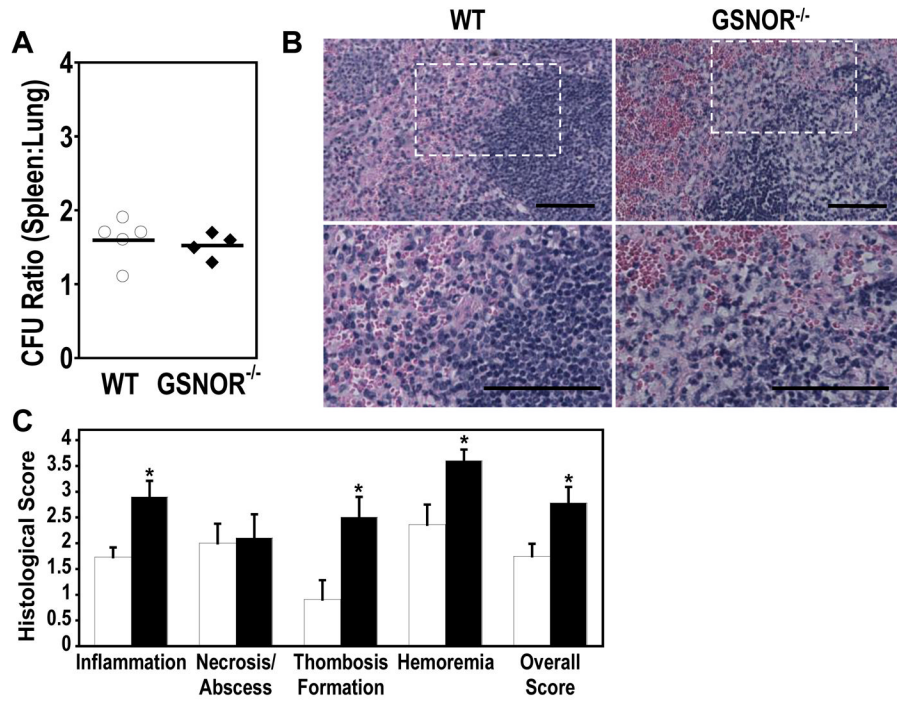
Fig. 3.
Increased *K. pneumoniae* infection and injury in spleens of GSNOR$^{-/-}$ mice. (A) Ratios of bacterial loads in the spleen (CFU/g) versus those in the lung (CFU/left lung) in wild-type (open circles, n = 5) and GSNOR$^{-/-}$ (closed diamonds, n = 4) mice. The ratios vary little within and between groups. (B) Representative spleen histology in *K. pneumoniae*-infected wild-type and GSNOR$^{-/-}$ mice. Bottom panels represent an expanded portion of the above sections outlined. Bar = 50 µm. (C) Histological scoring of infection-associated spleen injury revealed significant differences in wild-type (open; n = 11) and GSNOR$^{-/-}$ (closed; n = 10) mice. *, $P < 0.03$.
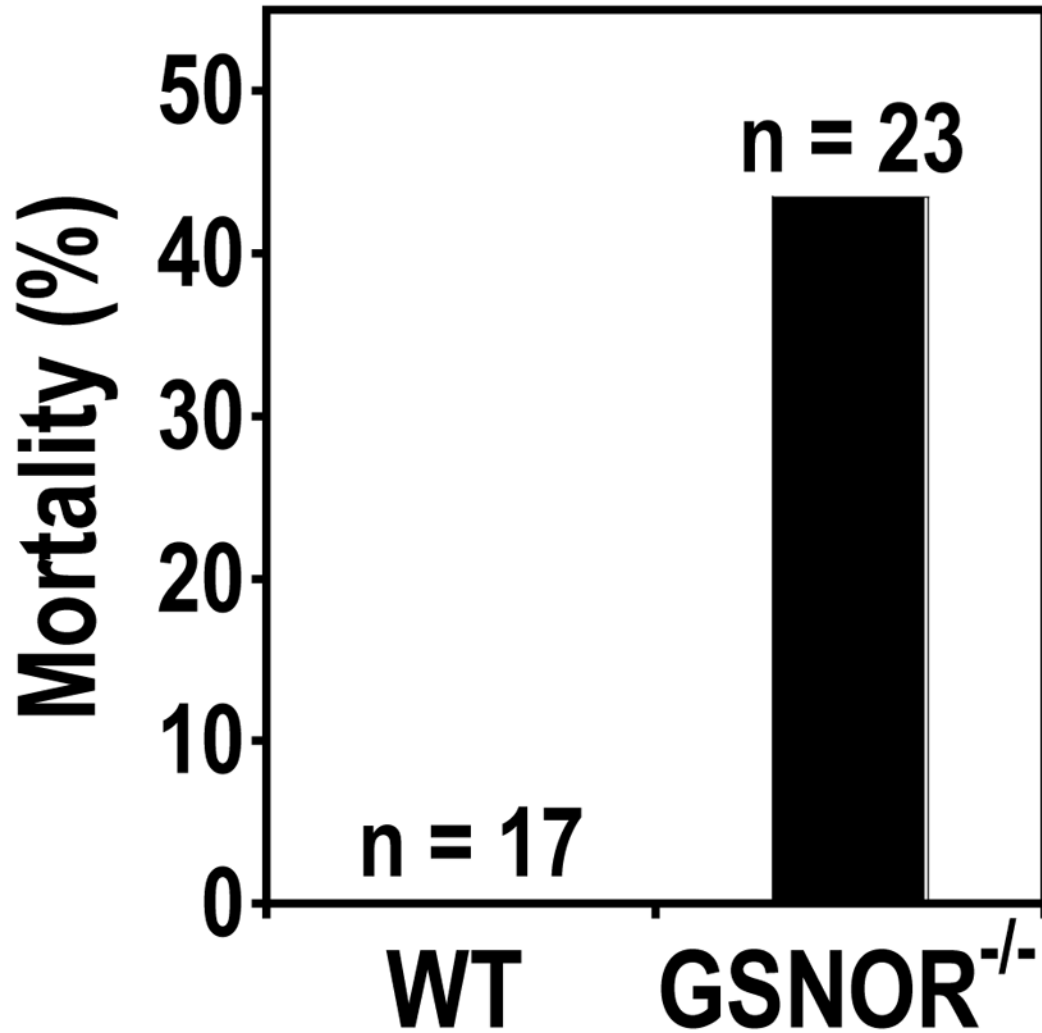
Fig. 4.
Increased mortality from *K. pneumoniae* infection in GSNOR$^{-/-}$ mice. Mortality of GSNOR$^{-/-}$ mice (Closed, n = 23) is significantly higher than WT control (n = 17; *P* = 0.002) 48 hours after intranasal infection with *K. pneumoniae*.