

Lipopolysaccharide-induced lung injury does not require production of reactive oxygen species by NAD(P)H oxidase

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Abstract

We examined toll-like receptor 4 (TLR4) protein signaling in the innate immune response to the invasion of the body by gram negative bacteria. When humans are exposed to lipopolysaccharide (LPS or endotoxin), neutrophils attach to the endothelium and infiltrate the lung. This involves activation of TLR4 on the endothelium and on neutrophils and the subsequent activation of cell signaling pathways, such as NF- κ B and other transcription factors that stimulate reactive oxygen species (ROS) production, to promote the inflammation process. The activation of endothelial TLR4 produces a generalized inflammatory response that includes activation of adhesion molecules which results in the accumulation of neutrophils on the endothelial side of the inflamed tissue. To test the role of ROS production by NAD(P)H oxidase in this process, wild type (WT) mice and mice deficient in the p47^{phox} component of NAD(P)H oxidase enzyme (KO) were challenged with LPS and the degree of pulmonary injury was assessed. In both WT and KO mice maximal lung injury was observed 4 hours after challenge with LPS. In contrast to WT mice, lung injury in KO mice was more severe and persisted for 8 hours

Keywords

Sepsis: A generalized inflammatory response produced by invading organisms such as bacteria or their toxins; **Edema:** Abnormal accumulation of water-based fluid in the tissues or cavities of the body, often causing visible swelling; **Endotoxin:** the lipopolysaccharide coating of gram negative bacteria that is released only upon death of that cell and acts through toll-like 4 receptors to activate an innate immune response; **Innate immunity:** The early response of an organism to a foreign agent. It does not require previous exposure to the agent or the production of lymphocytes; **Neutropenia:** A hematological condition characterized by an abnormally low number of neutrophils, this makes affected patients more susceptible to bacterial infection; **Cytokine:** Proteins and peptides that signal a variety of immunological and inflammatory responses by activating other immune responsive cells.

Introduction

Sepsis is a systemic response to infection which is characterized by an intense state of widespread inflammation. A prime target of the generalized inflammatory response is the lung, and the inflammatory responses in the lung tissue can eventually lead to pulmonary failure (Hirsh et al. 2004). This

severe condition affects thousands of intensive care unit (ICU) patients every year, and there is a great need for efficient therapies that treat this condition. We conducted this research to elucidate the mechanisms underlying the septic shock-related inflammatory response. By understanding how the mediators involved in this pathway interact, researchers may be able to develop effective drugs and/or therapies that regulate the action of these mediators.

Toll-like receptor 4 (TLR4) is one in a family of receptors that provides a critical link between microbial recognition and the induction of immune and inflammatory responses (Medzhitov & Janeway 2000). Lipopolysaccharide (LPS) derived from gram-negative bacteria binds to TLR4 on neutrophils and this initiates an immune response. Bacteria or LPS in the blood can activate the inflammatory process, resulting in multiorgan dysfunction with the lung being a primary target. Cellular events that occur due to LPS infection include endothelial vascular leak and neutrophil attachment to the endothelium and subsequent infiltration into the lung. These immune responses are mediated by activation of NF- κ B as well as other cell signaling pathways. ROS, which are produced in a receptor-mediated fashion, are usually considered as a source of intracellular injury. However, recent studies have shown that NF- κ B activation can be controlled by the presence of reactive oxygen species (ROS) such as superoxide (Shah et al. 2001; Magder 2006). Moreover, ROS are also being recognized as important intracellular signaling molecules that can regulate various biological activities including host defense and metabolic conversions. Therefore, the importance of ROS may have been underestimated due to the fact that their detrimental reputation seems to take heed over their role in biological regulation.

The NAD(P)H oxidase enzyme has a signaling role in a variety of cells. In neutrophils, the complex mediates the oxidative burst that occurs during the inflammatory response. Its cytosolic components, p47^{phox}, p67^{phox} and RAC interact with membrane components, p22^{phox} and members of the Nox family to generate ROS. Nox proteins work by transferring electrons from NADPH to O₂, generating superoxide, a free radical, and reactive oxygen species (Park et al. 2004). P47^{phox} is the cytosolic component of NAD(P)H that helps regulate superoxide production. A loss of function in this component renders the NAD(P)H enzyme incapable of producing ROS (Nobuhisa et al. 2006). We hypothesized that LPS signaling through TLR4 requires ROS from NAD(P)H oxidase to activate an innate immune response by the endothelium.

Materials and Methods

Animal preparation

We were provided with P47^{phox} WT and KO mice (p47^{phox}^{-/-}) by

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Dr. S Holland (Laboratory of Host Defense, National Institutes of Health). We bred the mice, and the offspring were genotyped 21 days after birth. For this study, only homozygotes (WT or KO) were used. ROS production was impaired in some mice, though not in others; so the immunological effects of this duality could be determined. WT and KO mice were separated into 3 groups. Animals underwent three different experimental protocols to evaluate lung injury. Mice were injected with a final volume of 200µl of a 0.5mg/kg LPS solution or 200µl of a saline solution. After injections, the mice were kept under observation for 2, 4, or 8 hours and then were subject to a specific procedure that would assess the amount of lung injury they had experienced.

Wet-to-dry weight ratio

Mice were anesthetized with ketamine (200mg/kg) and xylazine (10mg/kg). The left lung was removed at 2, 4, or 8 hours following the LPS challenge, and immediately weighed (wet weight). The lungs were then placed in an oven set at 60°C for 24 hours and then re-weighed to obtain the dry weight. An increase in the wet-to-dry ratio compared to the values obtained with control experiments indicates edema formation in the lung and lung injury.

Histological Score for edema

Mice were anesthetized, and the organs of the thorax exposed. We removed all the blood from the lungs by injecting 5ml of Hank's bank salt solution (HBSS) into the right ventricle and used cardiac puncture to allow the blood to escape. The trachea was then cannulated with a PE 20 tube which was attached to a 2ml syringe filled with a polymer of optimal compound temperature (OCT). OCT was injected into the trachea to promote lung expansion. A single lobe of the left lung was excised, harvested and snap frozen. Lungs were sectioned with a microcycler, and the sections were mounted on glass slides. Sections were stained with Diff-Quik and analyzed using light microscopy. Edema was assessed by a histological score that scaled the thickness of the alveolar walls. Each lung section created was measured at 100 different regions of the lung and each measurement was classified as pertaining to levels 1 to 3 of edema. (0-6.5mm= level 1- no edema, 6.5-11.0= level 2- moderate edema, 11.0 and higher= level 3- maximal edema). A total score was then generated for each mouse. For example, a mouse with 85 measurements in level 3, 14 measurements in level 2 and 1 measurement in level 1 had a total score of $(85 \times 3 + 14 \times 2 + 1 \times 1) = 284$. Edema was confirmed as being observed in the lung when the total score was >130.

Pulmonary microvascular protein leak

Leakage of protein from the vascular lumen of lung tissue is a good indication that inflammation has occurred. Evans blue (EB) can be used as a sensitive marker of protein leak. For this experiment, all mice were infused with Evans blue dye (20mg/kg), 90 minutes after LPS or saline injection. After anesthesia, we flushed the pulmonary circulation with 10ml of PBS (pH 7.4, 20°C). Lungs were then excised, blotted dry, and snap frozen in liquid nitrogen. We then homogenized the frozen tissues with PBS (600µl) and formamide (1.2ml). After centrifugation (1500rpm, 5 minutes), supernatant absorbances at 620nm and 740nm were recorded, and tissue EB content was calculated as a ratio (µg of EB in plasma/µg of EB in lungs). EB content calculations were achieved by correcting

the absorbance at 620nm for the presence of heme pigments and comparing this value to a standard curve of EB in formamide/PBS (Razavi et al. 2004).

Statistical Analysis

All results are expressed as a mean from the data collected. Differences between groups were assessed by a two-way and three-way ANOVA to analyze variance. Pairwise multiple comparisons were performed with a Student-Newman-Keuls t test. Significance was accepted at $p < 0.05$.

Results

Lung injury was more dramatic in $P47^{phox-/-}$ mice. Lung sections for all control mice showed no inflammation of the alveolar walls (**Figure 1a, b**). After 2 hours, we observed minimal alveolar inflammation in both WT and KO mice (**Figure 1c, d**). After 4 hours, inflammation was present in both WT and KO mice but the injury was more severe in KO mice (**Figure 1e, f**). After 8 hours there was a decline in inflammation in WT but severe alveolar damage persisted in KO mice (**Figure 1g, h**). These data suggest that $p47^{phox}$ needs to be present to allow resolution of the inflammatory response induced by LPS. The $p47^{phox-/-}$ mice (KO) possess the other components of NAD(P)H oxidase, but its activity does not seem to increase without the $p47^{phox}$ regulatory component in response to LPS. Therefore the products of its activity (ROS) are never released and the

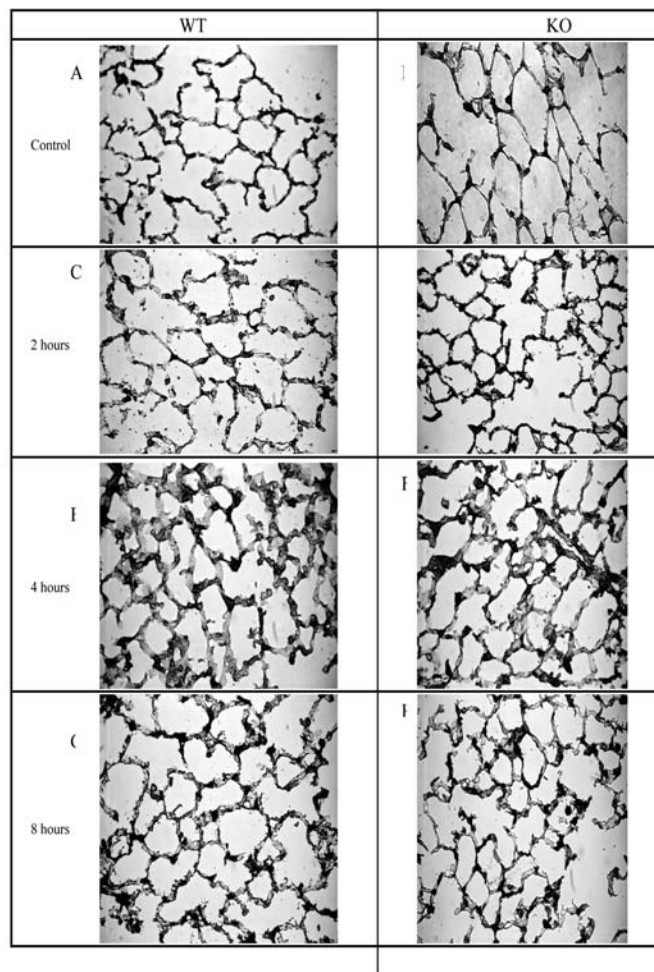


Figure 1. Histological analysis of lung sections, investigating pulmonary edema in WT and KO mice. Thick alveolar walls can be observed at 4 hours in both groups, and at 8 hours in KO mice. All lung sections were visualized via Diff-Quick differential staining; magnification: 40X.

immune response persists instead of resolving. Figure 2 shows a summary of the histological score.

Untreated mice had histological scores that ranged from 116-128 units. The average histological score for KO mice, after 4 hours, was 254.5 units and was significantly greater than WT mice after 4 hours. The difference was even more striking at 8 hours; the average scores were 116 and 254 respectively. These results further exemplify that the resolution of pulmonary edema that is evident in WT mice does not occur in KO mice.

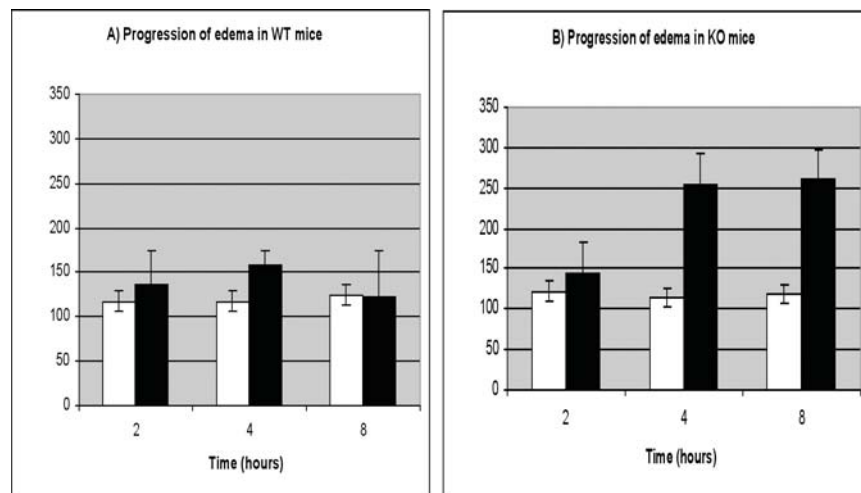


Figure 2. Graphical representation of the histological score for edema for WT (a) and KO (b) mice. The total score decreases after reaching its maximum in WT mice; however this does not occur in KO mice. The open bars represent the control group of mice; the closed bars represent LPS injected mice. Values for histological score were deduced in a blinded manner.

P47^{phox} mice had higher wet-to-dry ratio values.

The ratio of lung weight when wet and lung weight when dry is another measure of lung injury. We tabulated and statistically analyzed the results of this experiment to test their significance (**Table 1**). The ratio was <4.5 for control mice and these values were similar to those observed by others (Koay et al. 2001).

WT mice had an increase in the wet-to-dry ratio at 4 hours, whereas KO mice had higher ratios after 4 and 8 hours. This assessment further confirms that LPS-induced lung injury occurred in both WT and KO mice, but the injury persisted in KO mice.

P47^{phox} mice had more leakage of Evans blue into the lung vasculature than WT. Capillary protein leak is a significant aspect of syndromes related to lung injury (Green et al. 1988). Evans blue is a common marker used in assessing lung injury because its ability to accurately measure protein leak from the lung vasculature. This experiment was conducted 4 hours after LPS challenge. KO mice had a much higher level of Evans blue, an indication of capillary leak (Figure 3). This indicates that capillary permeability in the alveolar walls increased, and this finding can be linked to the fact that lung inflammation has occurred.

Discussion

Contrary to what we expected, p47^{phox} mice had greater lung injury with time than WT mice. Recent findings suggest that p47^{phox} may interact with TLR4 to downregulate the LPS-induced inflammatory response (Dusting et al. 2004). This could account for the lower degree of lung injury in WT mice compared to KO mice.

A previous study showed that NF-κB activation is signifi-

cantly attenuated in lungs of p47^{phox} mice. WT mice experience this same attenuation when neutropenia is induced beforehand (Fan et al. 2002). These findings led us to expect that KO mice would show less lung injury than WT mice, because they would not produce ROS to activate NF-κB. A potential difference between their study and ours is that they treated their mice with TNF γ , an inflammatory cytokine that acts through its own receptor and can directly activate the NF-κB pathway, whereas we used LPS, which acts through TLR4. Using TNF γ as the endotoxin may initially promote the inflammatory response, but with time, mediation and correction of the immune response will occur.

There are definite limitations to using mouse models to systematically study biochemical responses. Future experiments should analyze lung injury at a biochemical level to confirm exactly which signaling pathways are activated, and these analyses should also be obtained at different time points. Research with cytokines and signaling peptides could provide more information on what is occurring in the lung at a cellular level. Ultimately, it is the turning off of the inflammatory response that needs to be achieved for any stress-induced injury to ameliorate. Therefore, designating cellular factors that promote anti-inflammatory effects on the endothelium is important for the field to move in the direction of developing efficient therapies. One of these factors, the peroxisome proliferator activated receptor γ (PPAR γ), has already been identified as a repressor of pro-inflammatory genes. PPAR γ has been shown to be expressed in adipocytes, macrophages and lymphocytes and upon activation by lipophilic ligands; it appears to antagonize NF-κB action in macrophages (Rogler 2005). Future experiments where PPAR γ is overexpressed or where ligands specific to this receptor are tested may result in observed amelioration of experimental inflammation, leading to powerful therapeutic agents against the inflammatory response.

During 8 hours of LPS challenge, different mediators are released to activate many signaling pathways. The regulation of each pathway needs to be determined so that the immune response can be understood as a whole. How the mechanism of the immune response proceeds with time is still the main focus of our research, and we hope to soon elucidate this enigma.

Average values for wet/dry ratios		
	WT	KO
Control	4.2±0.09	4.3±0.09
2 hours	4.26±0.1	4.8±0.5
4 hours	4.77±0.07	5.5±0.2
8 hours	4.33±0.45	5.27±0.7

Table 1. Table summarizes the values obtained from the wet/dry ratio experiments. WT mice show lower ratios in general. KO mice have higher wet/dry ratios due to the higher level of pulmonary edema that these mice experience. Statistical significance was assessed using a three-way analysis of variance.

Conclusion

Pulmonary edema, a symptom of septic shock, is characterized by swelling of the lung due to fluid buildup in the lung

tissue and the infiltration of neutrophils in the parenchyma of the lung. LPS can induce this rapid and profound neutrophil infiltration in the lung. When the NAD(P)H enzyme is functioning normally, the neutrophils can produce ROS, which regulates the immune response and decreases the inflammation that occurs. The NF- κ B pathway plays an important role in immune responses and inflammation processes and may be activated by the redox status of the cell. Therefore, a regulated concentration of ROS is needed to activate the NF- κ B pathways so that the immune response can evolve instead of persisting at the inflammation stage. Our results suggest that when the production of ROS is impaired, the inflammatory response persists and may worsen. Investigators may be skeptical in believing that ROS, which promote oxidative injury, can also promote the regulation of the immune response. However, our data suggests that ROS are necessary factors that promote both the activation of other pathways and the release of key cytokines. In effect, the activated pathways and the key cytokines collectively function to regulate the immune response. This regulation will eventually lead to stabilization of the response and minimize lung injury.

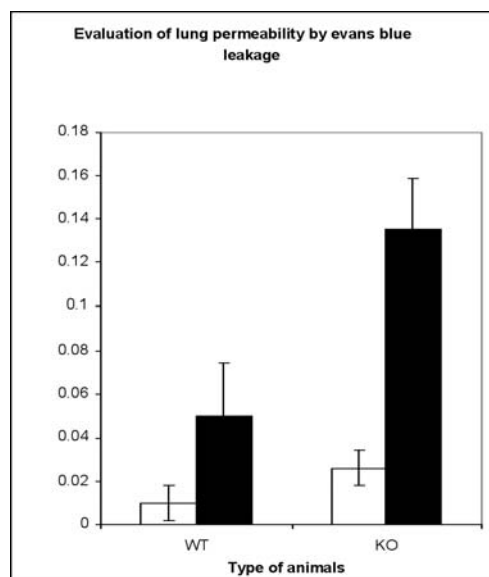


Figure 3. Protein leak was assessed using the Evan's blue marker. The open bars represent the control mice; the closed bars represent LPS injected mice. The KO mice showed a significantly higher level of protein leak due to excessive lung injury. Statistical significance was assessed using a two-way analysis of variance.

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