Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Nrf2 activation protects against intratracheal LPS induced mouse/murine acute respiratory distress syndrome by regulating macrophage polarization

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ARTICLE INFO

Article history:
Received 3 April 2018
Accepted 19 April 2018
Available online xxx

Keywords:
Acute respiratory distress syndrome
Nuclear factor E2-related factor 2
Macrophage polarization
Lipopolysaccharide
Inflammatory response

ABSTRACT

The transcription factor nuclear factor E2-related factor 2 (Nrf2) is known to control the expression of antioxidant response elements and cytoprotective genes and modulate inflammatory response, helping to ameliorate damage in many diseases. Exactly how Nrf2 regulates innate inflammatory homeostasis remains unclear. In this study, we provide *in vitro* and *in vivo* evidence that Nrf2 plays a crucial role in macrophage polarization and acute respiratory distress syndrome (ARDS). We conducted *in vitro* experiments using a mouse alveolar macrophage cell line as well as primary cultures of macrophages in which cells were exposed to lipopolysaccharide (LPS) or interferon-γ in order to mimic ARDS, in the presence or absence of the Nrf2 activator tert-butylhydroquinone (tBHQ). Using siRNA-mediated Nrf2 knockdown, we showed that Nrf2 inhibited the inflammatory response by promoting M2 macrophage polarization and inhibiting M1 macrophage polarization. At the same time, tBHQ activated Nrf2-mediated inhibition of the p65 nuclear factor-κB pathway and activation of peroxisome proliferator-activated receptor-γ, which play important roles in regulating macrophage polarization. We also conducted *in vivo* experiments in which mice were given tBHQ with or without intratracheal LPS, then their survival was monitored, lung injury was assessed using histology, and levels of pro- and anti-inflammatory cytokines were assayed in the lungs and serum. Activation of Nrf2 with tBHQ dramatically reduced LPS-induced mortality and lung injury, down-regulated pro-inflammatory mediators and up-regulated anti-inflammatory mediators. These results suggest that Nrf2 can help prevent ARDS progression by promoting M2 polarization of macrophages. Interfering with Nrf2 may be an effective strategy for reprogramming macrophage polarization in order to treat ARDS.

1. Introduction

Many critically ill patients suffer local infection of the lungs or systemic inflammation/sepsis that leads to acute respiratory distress syndrome (ARDS), a form of diffuse alveolar injury [1]. ARDS is associated with >40% mortality [2,3]. The disease appears to reflect the effects of oxidative stress and exaggerated inflammatory response [4,5]. Dysregulated inflammation within the lungs leads to disruption of the alveolar endothelial and epithelial barrier [6–8], which in turn results in the excessive influx of neutrophils, macrophages and other inflammatory cells, release of inflammatory and cytotoxic mediators, alveolar flooding with protein-rich fluid, impaired surfactant synthesis and metabolism, and a local pro-coagulant state.

Macrophages can help protect against these damaging processes in ARDS by modulating the progression of inflammation, promoting the resolution of inflammation, and initiating lung repair [8,9]. Whether macrophage populations tend to promote or reduce inflammation depends on the relative abundance of M1-or M2-polarized macrophages. M1 macrophages, which are generated in response to microbial products or interferon (IFN)-γ, efficiently present antigens and produce proinflammatory cytokines, including interleukin (IL)-6, IL-12 and tumor necrosis factor (TNF)-α

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https://doi.org/10.1016/j.bbrc.2018.04.161
0006-291X/

[10,11]. In this way, M1 macrophages promote a polarized type I immune response, inhibit cell proliferation and cause tissue damage. M2 macrophages, in contrast, are induced by IL-4/IL-13. These cells produce the anti-inflammatory cytokines resisitin like alpha, chitinase-like 3, IL-10 and Arginase1; they also promote tissue remodelling and wound healing [10–12].

The dynamic equilibrium between M1 and M2 macrophage polarization is regulated by a variety of transcriptional regulators. M1 polarization is stimulated by NF-κB and signal transducer and activator of transcription (STAT) 1; M2 polarization is stimulated by STAT6 and peroxisome proliferator-activated receptor (PPAR)-γ [13,14]. Recent studies have suggested that the transcription factor Nrf2 can modulate M1/M2 polarization by affecting expression of regulators such as PPARγ. Nrf2 may induce expression of PPARγ, leading to M2 macrophage polarization [15] and thereby protecting the lungs from oxidant injury [16]. Conversely, deleting the PPARγ gene from lung macrophages can induce pulmonary inflammation [17]. In fact, several studies have shown that Nrf2 controls the expression of antioxidant response elements and cytoprotective genes [18,19], allowing it to regulate the innate immune response [20,21] and protect against lung injury [18,22].

Whether Nrf2 also modulates macrophage polarization in ARDS is unclear. The present study examined this potential modulation *in vitro* and *in vivo*. The results identify Nrf2 as a potential therapeutic target for treating ARDS.

2. Materials and methods

2.1. Experimental design

All animal experiments were approved by the Animal Experiment Administration Committee of the Shanghai Pulmonary Hospital and carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publications NO.8023, revised 1978). See Supplementary Fig. 1 for details.

2.2. Animal experiments

ARDS was induced in adult male C57BL/6 J mice weighing 19–21 g (Bi Kai Experimental Animals, Shanghai, China) by treating them with lipolysaccharide (LPS; L4391, Sigma) intratracheally [23] (5, 10, or 20 mg/kg) using a MicroSprayer syringe assembly (MSA-250-M, Penn Century, USA) [24] under anesthesia with 0.75% intraperitoneal pentobarbital (75 μg/g). The selective Nrf2 activator tert-butylhydroquinone (tBHQ; HY-100489, MedChem Express, NJ, USA) were injected at a dose of 50 mg/kg (selected based on previous work [25]). Animal experiments were detailed in the supplementary materials.

2.3. Cell culture experiments

MH-S mouse alveolar macrophage cultures (CRL-2019, ATCC, USA) and primary cultures of macrophages (prepared as described in the next section) were exposed to LPS (100 ng/ml) or recombinant murine IFN-γ (40 mg/ml; C600059, Bio Basic, Markham, ON, Canada) in the presence or absence of tBHQ (100 μM). Macrophage polarization was determined 3 h later. Similar experiments were conducted in parallel using MLE-12 mouse lung epithelial cells to assess macrophage-specific effects. In some cultures, Nrf2 expression was knocked down using short interfering (si) RNA. The interfering sequences were listed in Supplementary Table 1. The cultural conditions and interfering manipulation were detailed in the supplementary materials.

2.4. Primary macrophage cultures

Primary mouse macrophages were isolated and cultured as described [26]. See supplementary materials for details.

2.5. Real-time quantitative PCR

Total RNA was extracted from the lungs using an RNA isolation kit (Feijie, Shanghai, China) and reverse-transcribed at 37 °C for 15 min using a commercial kit from Takara; the reverse transcriptase was inactivated at 85 °C for 5 s, and the reaction was stored at 4 °C. Then expression levels of target genes were determined using the SYBR Premix Ex Taq™ II (RR820A, Takara, Japan) with an ABI 7500 PCR system (Life Technologies, USA). Primers used for reverse transcription and PCR are listed in Supplementary Table 2. Expression levels were calculated using the ΔΔCt method relative to levels of the β-actin gene.

2.6. Western blot analysis

Whole-cell and nuclear extracts were prepared as described in Ref. [27]. Proteins were extracted using a commercial kit (Thermo Fisher Scientific) and assayed using a BCA method. The proteins were separated on 10% SDS-PAGE, transferred onto a PVDF membrane and incubated overnight at 4 °C with primary rabbit polyclonal antibodies against p-P65 (1:2000; ab86299, Abcam), P65 (1:1000; 8242S, CST), Nrf2 (1:1000, 12721S, CST), p-STAT6 (1:1000; ab188080, Abcam), STAT6 (1:1000; ab32520, Abcam), or PPARγ (1:1000; ab191407, Abcam); or with primary mouse monoclonal antibodies against β-actin (1:1000; sc-58673, Santa Cruz) or lamin A/C (1:1000; sc-376248, Santa Cruz). Membranes were washed extensively, incubated with a horseradish peroxidase-conjugated secondary antibody, and imaged using enhanced chemiluminescence.

2.7. Assay of IL-10, IL-6, and TNF-α

Serum concentrations of IL-10, IL-6, and TNF-α were assayed using cytometric bead arrays [28]. Bead position and phycoerythrin (PE) signal were detected using a flow cytometer (Accuri C6, Becton-Dickinson, Franklin Lake, New Jersey, USA) equipped with lasers at 488 nm (blue) and 640 nm (red). Antibodies in serum were quantified based on median PE fluorescence intensity values, which were converted to concentration (pg/ml), and a five-parameter logistic regression equation was used to generate a standard curve for each antigen using FCAP version 3 software (Becton–Dickinson).

2.8. Statistical analysis

Results are presented as mean ± SEM of three independent experiments. Differences between treatments were assessed for significance using one-way ANOVA, followed by Bonferroni's posthoc test for pairwise comparison. Survival of treatment groups was compared using the log-rank test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Effects of tBHQ on LPS-induced mortality

LPS delivered at a dose of 10 mg/kg killed 65% of animals within 7 days (Fig. 1A). Administering tBHQ at a dose of 50 mg/kg either simultaneously with LPS or 24 h beforehand led to mortality rates of, respectively, 50% and 30%, which did not differ significantly from

Please cite this article in press as: J. Wei, et al., Nrf2 activation protects against intratracheal LPS induced mouse/murine acute respiratory distress syndrome by regulating macrophage polarization, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.04.161