Indirubin improves antioxidant and anti-inflammatory functions in lipopolysaccharide-challenged mice

Tianjie Qi1,*, Haitao Li1,*, Shuai Li1

1Department of Respiratory Medicine, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei 050000, P.R. China

*These authors contributed equally to this work and are co-first authors

Correspondence to: Shuai Li, email: shuaili322@sina.com

Keywords: indirubin, acute lung injury, oxidative stress, inflammation, NF-κB

ABSTRACT

Indirubin, a traditional Chinese medicine formulation from the Muricidae family, has been reported to exhibit abroad anti-cancer and anti-inflammation activities and mediate nuclear factor-κB (NF-κB) signal. Thus, this study aimed to investigate the protective effects of indirubin on LPS-induced acute lung injury and the potential mechanism in mice. The results showed that LPS treatment caused oxidative stress and inflammation in mice. Indirubin alleviated LPS-caused oxidative stress and inflammation via reducing MDA abundance and IL-1β and TNF-α expressions in mice. Meanwhile, indirubin improved lung NO production and inhibited NF-κB activation caused by LPS exposure. In conclusion, indirubin alleviated LPS-induced acute lung injury via improving antioxidant and anti-inflammatory functions, which might be associated with the NO and NF-κB signals.

INTRODUCTION

Indirubin, a traditional Chinese medicine formulation from the Muricidae family, has been demonstrated to affect physiological and pathophysiological processes, such as cell proliferation and death [1, 2]. Currently, indirubin has been considered to be a strong promise for clinical anticancer activity and also be useful in other diseases, such as Alzheimer’s disease and diabetes [3]. Anti-inflammatory function and immune mediation of indirubin have been identified in various models. For example, indirubin ameliorates dextran sulfate sodium-induced ulcerative colitis in mice through the inhibition of inflammatory response and the induction of regulatory T cells [4]. In lipopolysaccharide (LPS)-induced mastitis mouse model, indirubin improves inflammation via inhibiting the production of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) [5].

Nuclear factor-κB (NF-κB), a transcription factor of inflammatory cytokines, has been widely demonstrated to involve in cellular responses to various stress, such as oxidative stress and infection [6, 7]. While various reports suggest that indirubin also can influence NF-κB signal [5, 8], which further mediates inflammatory response. Thus, indirubin can be served as a potential anti-inflammatory agent to treat inflammation-associated diseases. Acute lung injury is a major causes of acute respiratory failure characterized by oxidative stress, inflammatory response, and immune suppression [9, 10]. While the mechanism of acute respiratory failure and protection strategies are not fully investigated. Thus, in this study, we used LPS-induced acute lung injury to investigate the protective role of indirubin in lung inflammation and the potential mechanism.

RESULTS

Effects of indirubin on LPS-induced lung wet/dry weight ratio in mice

As shown at Table 1, LPS treatment markedly increased lung wet/dry weight ratio (p < 0.05). Indirubin tended to reduce lung wet/dry weight ratio, but the difference was insignificant (p > 0.05).

Antioxidant function

In LPS-induced acute lung injury, glutathione peroxidase (GSH-Px) activity was markedly inhibited and
Malondialdehyde (MDA) abundance was significantly higher compared with the control group (Table 2), suggesting that LPS treatment caused lung oxidative stress ($p < 0.05$). Although indirubin failed to alleviate LPS-inhibited GSH-Px activity, indirubin markedly reduced MDA generation compared with the LPS group ($p < 0.05$).

**Immunoglobulins (Igs)**

Lung Igs (IgA, IgG, and IgM) were determined in this study and the results showed that LPS increased IgM and IgG levels ($p < 0.05$), while indirubin injection markedly reduced IgM abundance in the lung ($p < 0.05$) (Table 3).

**Nitric oxide synthase (NOS) activity**

NOS activity was inhibited after exposure to LPS in mice ($p < 0.05$) (Table 4). Similarly, LPS also reduced nitric oxide (NO) generation and indirubin treatment improved NO generation ($p < 0.05$), suggesting that NOS/NO involved in the protective mechanism of LPS-induced acute lung injury.

**Inflammatory response**

In this study, LPS treatment markedly induced inflammatory response in the lung evidenced by the upregulation of IL-1β, IL-6, and TNF-α ($p < 0.05$) (Table 5). Indirubin injection alleviated LPS-induced inflammation via decreasing IL-1β and TNF-α mRNA abundances in the lung ($p < 0.05$).

**NF-κB**

NF-κB widely involves in oxidative stress and inflammation. In this study, we found that NF-κB was markedly activated after LPS exposure in the lung ($p < 0.05$) (Table 6). As the upstream signal of NF-κB [11, 12], TLR4 and Myd88 were also determined and the results showed that LPS upregulated expression of TLR4 and Myd88 in the lung ($p < 0.05$). Meanwhile, indirubin markedly inhibited TLR4 and NF-κB signals, which might further mediate the oxidative stress and inflammation in the LPS-induced acute lung injury.

**DISCUSSION**

The goal of this study was to determine the protective effects of indirubin on LPS-induced acute lung injury and the potential mechanism in mice. The clinical index showed that LPS caused pulmonary edema evidenced by the increased lung wet/dry weight ratio. Indirubin tended to alleviate LPS-induced pulmonary edema in mice. Meanwhile, indirubin alleviated LPS-induced acute lung injury via improving antioxidant and anti-inflammatory functions.

Excess generation of free radical species and oxidative stress have been suggested to involve in the development of acute respiratory failure [13, 14]. To maintain cellular oxidative balance, antioxidant enzymes (i.e. GSH-Px, SOD, and CAT) are produced to reduce free radical species [15]. In this study, we found that indirubin failed to enhance the antioxidant function but significantly alleviated LPS-induced MDA production, a major lipid oxidative maker. These results indicated that indirubin alleviated lung oxidative stress in LPS-induced acute lung injury in mice. The antioxidant function of indirubin might contribute to the beneficial mechanism in the LPS-induced acute lung injury, as several reports have shown that oxidative injury occurs in LPS-induced acute lung injury and antioxidant therapy plays a beneficial role in LPS-induced acute lung injury [16, 17].
Dysfunction of NOS/NO exists in various pathological conditions, including inflammation and oxidative stress [18, 19]. In LPS-induced acute lung injury, NOS expression and NO production have altered in response to inflammatory response [20, 21]. In this study, indirubin improved lung NO production. NO is released into the blood circulation during sepsis, stimulating inflammatory cell recruitment and activation [22]. Meanwhile, NOS/NO also can involve in the activation of NF-κB signal [23], which further mediates inflammation in LPS-induced acute lung injury.

Inflammatory response and immune suppression have been confirmed to involve in the progression and development of acute lung injury [17]. The present results exhibited that LPS induced inflammation and immune suppression in the lung via influencing IgM, IgG, IL-1β, IL-6, and TNF-α, while indirubin markedly reduced IgM abundances and IL-1β and TNF-α mRNA abundances in LPS-induced acute lung injury. Similarly, Kim et al. also found that indirubin alleviated serum IgE production in 1-chloro-2,4-dinitrobenzene-induced skin inflammation [24]. Meanwhile, the anti-inflammatory function of indirubin has been widely identified in various models. In LPS-induced inflammatory response, indirubin inhibited inflammatory cytokines production via mediating NF-κB signaling pathway [5]. In addition, indirubin analogue (indirubin-3-monoxime) also exhibited anti-inflammatory effect via inhibiting the release of pro-inflammatory cytokines (IL-1β and IL-6) induced by LPS in RAW264.7 cells [25].

NF-κB mediates cytokines expression and involves in various inflammatory diseases, including LPS-induced acute lung injury. The present results exhibited that LPS induced inflammation and immune suppression in the lung via influencing IgM, IgG, IL-1β,

### Table 3: Effect of indirubin on Immunoglobulins (Igs) in LPS-induced acute lung injury (U/mL)

| Item  | Control          | LPS            | LPS+ indirubin |
|-------|------------------|----------------|---------------|
| IgA   | 0.15 ± 0.02      | 0.12 ± 0.01    | 0.13 ± 0.02   |
| IgM   | 0.57 ± 0.16      | 0.76 ± 0.08    | 0.69 ± 0.05   |
| IgG   | 0.99 ± 0.13      | 1.31 ± 0.12    | 1.06 ± 0.13   |

Data are expressed as the mean ± standard error of the mean. Values in the same row with different superscripts are significant ($P < 0.05$).

### Table 4: Effect of indirubin on NOS activity and NO in LPS-induced acute lung injury

| Item             | Control          | LPS            | LPS+ indirubin |
|------------------|------------------|----------------|---------------|
| NOS (U/ml)       | 10.15 ± 0.72     | 8.12 ± 0.51    | 9.13 ± 0.52    |
| NO (nmol/ml)     | 0.57 ± 0.16      | 0.36 ± 0.08    | 0.49 ± 0.05    |

Data are expressed as the mean ± standard error of the mean. Values in the same row with different superscripts are significant ($P < 0.05$).

### Table 5: Effect of indirubin on response in the lung in LPS-induced acute lung injury

| Genes | Control          | LPS            | LPS+ indirubin |
|-------|------------------|----------------|---------------|
| IL-1β | 1.00 ± 0.09      | 1.71 ± 0.27    | 1.32 ± 0.16    |
| IL-6  | 1.00 ± 0.12      | 1.27 ± 0.12    | 1.36 ± 0.09    |
| IL-10 | 1.00 ± 0.19      | 0.97 ± 0.19    | 1.23 ± 0.18    |
| TNF-α | 1.00± 0.17       | 1.50 ± 0.27    | 1.17 ± 0.15    |

Data are expressed as the mean ± standard error of the mean. Values in the same row with different superscripts are significant ($P < 0.05$).

### Table 6: Effects of matrine on expression of NF-κB

| Item   | Control          | LPS            | LPS+ indirubin |
|--------|------------------|----------------|---------------|
| TLR4   | 1.00 ± 0.11      | 1.55 ± 0.17    | 1.14 ± 0.17    |
| Myd88  | 1.00 ± 0.14      | 1.47 ± 0.13    | 1.29 ± 0.15    |
| NF-κB  | 1.00 ± 0.17      | 1.51 ± 0.09    | 1.20 ± 0.13    |

Data are expressed as the mean ± standard error of the mean. Values in the same row with different superscripts are significant ($P < 0.05$).
In this study, LPS upregulated expression of NF-κB and its upstream proteins (TLR4 and Myd88), while indirubin markedly alleviated NF-κB activation, which might serve as the protective mechanism in LPS-induced acute lung injury.

MATERIALS AND METHODS

Animal model and groups

Kunming mice (36 females) were purchased at 6–8 weeks of age and randomly assigned into 3 groups (n = 10): a control group, a LPS-challenged group, and a group in which mice given both indirubin and LPS. LPS (Sigma, St. Louis, MO, USA) was used to induce acute lung injury by i.p. injection of 15 mg/kg LPS according to previous report. Indirubin (Shanghai Yuan Ye Biological Technology Co., Ltd, Shanghai, China), dissolved in PBS (10:1), was given by i.p. injection of at dose levels of 0.2 mL/20 g 1 hour before LPS treatment. All mice were sacrificed after 24 h and lung samples were harvested. This study was approved by the animal welfare committee of the Second Hospital of Hebei Medical University.

Wet-to-dry lung weight ratio (W/D ratio)

The right lungs were obtained immediately weighed to get the wet weight. Then the lungs were placed at 80°C for 48 h to obtain the dry weight. The ratio of wet lung to dry lung was calculated to assess tissue edema.

Oxidative stress

Lung samples were weighed and then homogenized in phosphate buffer (w/v: 1/9) on crushed ice using a tissue grinder. After centrifugation at 3500 g for 10 min at 4°C, the supernatant was collected for future use. GSH-Px, SOD, and CAT activity and MDA level in the lung homogenate were measured using spectrophotometric kits (Nanjing Jiangcheng Biotechnology Institute, China).

NOS activity and NO determination

Lung NOS activity was detected using an ELISA kit according to the manufacturer’s instructions (Shanghai Meilian Bio. Tech., China). Nitric oxide NO concentration were measured as released NO metabolites (nitrates and nitrites) using assay kits in accordance with the manufacturer’s instructions (Biovision Inc., USA).

Immunoglobulins (Igs)

Lung Igs (IgA, IgG, and IgM) were determined by spectrophotometric kits (Nanjing Jiangcheng Biotechnology Institute, China).

Real-time PCR

One piece of lung were harvested and stored at −80°C. Total RNA of these tissues was isolated with TRIZOL regent (Invitrogen, USA) and reverse transcribed into the first strand (cDNA) using DNase I, oligo (dT) 20 and Superscript II reverse transcriptase (Invitrogen, USA). The reverse transcription was conducted at 37°C.
for 15 min, 95°C 5 sec. Primers were designed with Primer 5.0 according to the gene sequence of mouse to produce an amplification product (Table 7). β-actin was chosen as the house-keeping gene to normalize target gene levels. The PCR cycling condition was 36 cycles at 94°C for 40 sec, 60°C for 30 sec and 72°C for 35 sec. The relative expression was expressed as a ratio of the target gene to the control gene using the formula 2\(^{(∆∆Ct)}\), where △△Ct = (Ct \_target - Ct \_β-actin/treatment) - (Ct \_target - Ct \_β-actin/control). Relative expression was normalized and expressed as a ratio to the expression in the control group.

### Statistical analysis

All data were analyzed by SPSS 17.0 software. Difference was tested by Ducañ’s multiple comparison test. Data are expressed as the mean ± SEN. Values in the same row with different superscripts are significant (P < 0.05).

### CONFLICTS OF INTEREST

The authors have declared that no competing interests exist.

### REFERENCES

1. Blazevic T, Heiss EH, Atanasov AG, Breuss JM, Dirsch VM, Uhrin P. Indirubin and Indirubin Derivatives for Countering Proliferative Diseases. Evid Based Complement Alternat Med. 2015; 2015:654098.
2. Gaboriaud-Kolar N, Vougogiannopoulou K, Skaltsounis AL. Indirubin derivatives: a patent review (2010 – present). Expert opinion on therapeutic patents. 2015; 25:583–593.
3. Eisenbrand G, Hippe F, Jakobs S, Muehlbeyer S. Molecular mechanisms of indirubin and its derivatives: novel anticancer molecules with their origin in traditional Chinese phytomedicine. J Cancer Res Clin Oncol. 2004; 130:627–635.
4. Gao W, Guo Y, Wang C, Lin Y, Yu L, Sheng T, Wu Z, Gorg Y. Indirubin ameliorates dextran sulfate sodium-induced ulcerative colitis in mice through the inhibition of inflammation and the induction of Foxp3-expressing regulatory T cells. Acta histochemica. 2016; 118:606–614.
5. Lai JL, Liu YH, Liu C, Qi MP, Liu RN, Zhu XF, Zhou QG, Chen YY, Guo AZ, Hu CM. Indirubin Inhibits LPS-Induced Inflammation via TLR4 Abrogation Mediated by the NF-kB and MAPK Signaling Pathways. Inflammation. 2017; 40:1–12.
6. Luettig J, Rosenthal R, Lee IM, Krug SM, Schulzke JD. The ginger component 6-shogaol prevents TNF-alpha-induced barrier loss via inhibition of PI3K/Akt and NF-kappaB signaling. Mol Nutr Food Res. 2016; 60:2576–2586.
7. Fukumitsu S, Villareal MO, Fujitsuka T, Aida K, Isoda H. Anti-inflammatory and anti-arthritis effects of pentacyclic triterpenoids maslinic acid through NF-B inactivation. Molecular Nutrition & Food Research. 2016; 60:399–409.
8. Kim MH, Choi YJ, Yang G, Cho H, Nam D, Yang WM. Indirubin, a purple 3,2- bisindole, inhibited allergic contact dermatitis via regulating T helper (Th)-mediated immune system in DNBC-induced model. J Ethnopharmacol. 2013; 145:214–219.
9. Butt Y, Kudowska A, Allen TC. Acute Lung Injury: A Clinical and Molecular Review. Archives of pathology & laboratory medicine. 2016; 140:345–350.
10. Mokra D, Kosutova P. Biomarkers in acute lung injury. Respir Physiol Neurobiol. 2015; 209:52–58.
11. Sahasrabudhe NM, Dokter-Fokkens J, de Vos P. Particulate beta-glucans synergistically activate TLR4 and Dectin-1 in human dendritic cells. Molecular Nutrition & Food Research. 2016; 60:2514–2522.
12. Zhang YJ, Chen FP, Chen JD, Huang SQ, Chen JB, Huang J, Li N, Sun SX, Chu XW, Zha LY. Soyasaponin Bb inhibits the recruitment of toll-like receptor 4 (TLR4) into lipid rafts and its signaling pathway by suppressing the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent generation of reactive oxygen species. Molecular Nutrition & Food Research. 2016; 60:1532–1543.
13. Rashti Z, Koohsari H. Antibacterial effects of supernatant of lactic acid bacteria isolated from different Dough’s in Gorgan city in north of Iran. Integr Food Nutr Metab. 2015; 2:193–196.
14. Zhu L, Sun Y, Zhang G, Yu P, Wang Y, Zhang Z. Radical-Scavenging And Anti-Oxidative Activities Of TBN In Cell-Free System And Murine H9c2 Cardiomyoblast Cells. Journal of antioxidant activity. 2015; 1:55–68.
15. Rodrigo S, Rodriguez L, Otero P, Panadero MI, Garcia A, Barbas C, Roglans N, Ramos S, Goya L, Laguna JC, Alvarez-Millan JJ, Bocos C. Fructose during pregnancy provokes fetal oxidative stress: The key role of the placental heme oxygenase-1. Mol Nutr Food Res. 2016; 60:2700–2711.
16. Su ZQ, Mo ZZ, Liao JB, Feng XX, Liang YZ, Zhang X, Liu YH, Chen XY, Chen ZW, Su ZR, Lai XP. Usnic acid protects LPS-induced acute lung injury in mice through attenuating inflammatory responses and oxidative stress. Int Immunopharmacol. 2014; 22:371–378.
17. Jiang W, Luo F, Lu Q, Liu J, Li P, Wang X, Fu Y, Hao K, Yan T, Ding X. The protective effect of Trillin LPS-induced acute lung injury by the regulations of inflammation and oxidative state. Chem Biol Interact. 2016; 243:127–134.
18. Tang LF, Wang HL, Ziolo MT. Targeting NOS as a therapeutic approach for heart failure. Pharmacol Therapeut. 2014; 142:306–315.
19. McNally B, Griffin JL, Roberts LD. Dietary inorganic nitrate: From villain to hero in metabolic disease? Mol Nutr Food Res. 2016; 60:67–78.
20. Chen J, Liu XJ, Shuo QL, Li SQ, Luo FM. Gherlin attenuates lipopolysaccharide-induced acute lung injury through no pathway. Med Sci Monitor. 2008; 14:Br141–Br146.
21. Patruno A, Franceschelli S, Pesce M, Maccallini C, Fantacuzzi M, Speranza L, Ferrone A, De Lutiis MA, Ricciotti E, Amoroso R, Felaco M. Novel aminobenzylacetamidine derivative modulate the differential regulation of NOSs in LPS induced inflammatory response: Role of PI3K/Akt pathway. Biochim Biophys Acta. 2012; 1820:2095–2104.

22. Li WC, Zou ZJ, Zhou MG, Chen L, Zhou L, Zheng YK, He ZJ. Effects of simvastatin on the expression of inducible NOS in acute lung injury in septic rats. Int J Clin Exp Patho. 2015; 8:15106–15111.

23. Napolitano M, Zei D, Centonze D, Palermo R, Bernardi G, Vacca A, Calabresi P, Gulino A. NF-kB/NOS cross-talk induced by mitochondrial complex II inhibition: Implications for Huntington’s disease. Neuroscience Letters. 2008; 434:241–246.

24. Kim MH, Choi YY, Yang G, Cho IH, Nam D, Yang WM. Indirubin, a purple 3,2-bisindole, inhibited allergic contact dermatitis via regulating T helper (Th)-mediated immune system in DNBC-induced model. Journal of Ethnopharmacology. 2013; 145:214–219.

25. Kim JK, Park GM. Indirubin-3-monoxime exhibits anti-inflammatory properties by down-regulating NF-kappaB and JNK signaling pathways in lipopolysaccharide-treated RAW264.7 cells. Inflamm Res. 2012; 61:319–325.

26. Wang J, Liu YT, Xiao L, Zhu L, Wang Q, Yan T. Anti-inflammatory effects of apigenin in lipopolysaccharide-induced inflammatory in acute lung injury by suppressing COX-2 and NF-kB pathway. Inflammation. 2014; 37:2085–2090.

27. Lin MH, Chen MC, Chen TH, Chang HY, Chou TC. Magnolol ameliorates lipopolysaccharide-induced acute lung injury in rats through PPAR-gamma-dependent inhibition of NF-kB activation. Int Immunopharmacol. 2015; 28:270–278.