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Original Research Article

Floroindole confers protection against cecal ligation and puncture-induced sepsis via inhibition of NF-kB p65 phosphorylation

Zhiwen Zhou[1], Xiang Ren[1], Aiping Li[1], Wensheng Zhou[1], Li Huang[2]*
[1]Department of Neurology, Hunan Provincial People's Hospital, The First Affiliated Hospital of Hunan Normal University, Changsha, Hunan 410016, [2]Department of Critical Care Medicine, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China

For correspondence: **Email: lesueer@yahoo.com; **Tel:** 0086-0731-89753999*

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Abstract

Purpose: *To investigate the protective effect of floroindole against cecal ligation and puncture (CLP)-induced sepsis, as well as the underlying mechanism of action.*
Methods: *Thirty-five 10–week-old male Wistar rats weighing 190 - 210 g (mean: 200.00 ± 10.10 g) were used for this study. The rats were randomly assigned to seven groups of five rats each, viz, normal control group, and six CLP groups. The CLP groups were those subjected to cecal ligation and puncture (CLP). The first 5 CLP groups received 2, 4, 6, 8 or 10 mg/kg floroindole, respectively, 1 h after CLP, via intraperitoneal route (i.p.) while the 6th CLP group served as untreated control. Western blotting, enzyme-linked immunosorbent assay (ELISA) and real-time quantitative polymerase chain reaction (qRT-PCR) were used for the assessment of the expression levels of tumor necrosis factor-α (TNF- α), interleukn-6 (IL-6), nucleotide-binding oligomerization domain 2 (NOD2) and p-NF-κB p65.*
Results: *Cecal ligation and puncture (CLP) significantly and time-dependently upregulated the expressions of TNF-α, IL-6 and NOD2 in intestinal tissues of rats ($p < 0.05$). However, treatment with floroindole significantly, and dose-dependently down-regulated CLP-induced expressions of these proteins ($p < 0.05$). Treatment of rats with floroindole also significantly and dose-dependently inhibited CLP-induced phosphorylation of NF-κB p65 in rat ileum ($p < 0.05$).*
Conclusion: *The results obtained in this study demonstrate that floroindole confers some degree of protection against CLP-induced sepsis via inhibition of NF-κB p65 phosphorylation.*

Keywords: *Sepsis, Cecal ligation, Cytokines, Expression, Phosphorylation*

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INTRODUCTION

Sepsis is a complicated clinical syndrome caused by inflammatory reactions to microbial invasion [1]. This condition is characterized by high mortality [1]. Sepsis alone accounted for about 9 % of total deaths in the United States in 2000 [2,3]. Although several studies have attempted to unravel the mechanism underlying the pathogenesis of sepsis, no effective treatment has been developed till date. Thus, the development of new drugs that can effectively

treat sepsis has become necessary. The up-regulation of cytokine expression enhances membrane permeability, alters cellular metabolic pathways and induces coagulopathy [4]. These changes are associated with the development of multiple organ failure syndrome [4]. In humans, pro-inflammatory cytokines, i.e., TNF-α, IL-6 and interleukin-8 (IL-8) are released during severe pathogen infections [5,6]. In patients with sepsis, the network of cytokines is regulated through the release of TNF- α and IL-6 [5,6].

Indole, also known as benzopyrrole, is a bicyclic heteroaromatic compound consisting of six-membered benzene ring fused with five membered nitrogen-bearing pyrrole [7]. It is a structural component of several natural products such as vinca alkaloids which are metabolites isolated from fungal materials [7]. Compounds synthesized from indole have been shown to possess varied pharmacological properties, including anti-inflammatory effect. The compound [2- (3-oxo-3, 4-dihydro-2H-benzo[1,4]oxazin-6-carbonyl)-1H-indol-3yl]acetic acid is an indole derivative which exerts anti-inflammatory effect through the inhibition of cyclooxygenase-2 (COX-2) [8,9]. Several other derivatives of indole such as 2-(*p*-chlorophenyl)-1-[4-(2-(p-chorophenyl)-4-oxo-thiazolidin-3-yl]-5-mercapto[1,2,4,]-trizole-3-yl-methyl]-3[4,6-dibromo-2-carboxyphenyliminomethyl]-5-methoxyindole and 2-(1H-indol-3-yl)-6-methoxy-4-pentylpyridine-3,5-dicarbonitrile, have also been reported to possess significant anti-inflammatory properties [10,11]. The present study investigated the protective effect of floroindole against CLP-induced sepsis, and the underlying mechanism.

EXPERIMENTAL

Materials

Wistar rats were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (China). *Phosphate buffered saline* (PBS) was obtained from Bio-Diagnostics Company (Egypt). Rabbit polyclonal anti-p-NF-κB p65 was a product of Cell Signalling Technology (USA); mouse monoclonal anti-IL-6 was purchased from Sigma-Aldrich (USA), and horseradish peroxidase-conjugated secondary antibody was obtained from Santa Cruz Biotechnology, Inc. (USA). Phenylmethanesulfonyl fluoride was a product of Sigma (USA), while TNF-α and NOD2 ELISA kits were obtained from Diaclone (France). Interleukin-6 ELISA kit was purchased from BioSource (Belgium), and Trizol reagent was obtained from Invitrogen Co., Ltd. (USA). Rotor-Gene 6000 RT-PCR machine was a product of Corbett Research (Australia), while SYBR Premix Ex Taq was obtained from Thermo Fisher Scientific (USA).

Experimental rats

Thirty-five 10-week-old male Wistar rats weighing 190 - 210 g (mean weight = 200.00 ± 10.10 g) were used for this study. The rats were housed in plastic cages under standard laboratory conditions: 12 h light/dark cycles, 25 ˚C and 50 – 60 % humidity. They had free access to feed and clean drinking water. The study protocol was approved by the Ethics Committee for Care and Use of Laboratory Animal, Shanghai University (approval no. SUWR/16/102). The Guidelines of "Principles of Laboratory Animal Care" (revised NIH publication 85-23 were followed to provide humane care to all the rats [12].

Treatment regimen and grouping

The rats were randomly assigned to seven groups of five rats each, viz, normal control group and six treatment groups. The treatment groups were subjected to CLP. Five of the treatment groups received 2, 4, 6, 8 or 10 mg/kg floroindole 1 h after CLP via intraperitoneal route (i.p.). The sixth CLP group served as untreated control. The normal and untreated controls received equivalent volumes of physiological saline in place of floroindole. Two rats from each group were sacrificed at different time points: 2, 4, 6, 8 and 10 h of CLP after isoflurane anesthesia, and the ileal tissues excised and kept at -78 ^{o}C prior to use.

Establishment of sepsis model

The rat model of sepsis was established using CLP procedure. The rats were subjected to isoflurane anaesthesia and a small incision (2 cm) was carefully made on the ventral side along the ventral abdominal midline. The cecum of the rat was exposed and then immediately ligated next to ileocecal valve using 3-0 silk suture to avoid obstruction in the intestine. An 18-gauge needle was used for making puncture in the cecum between ligation and the cecum. The punctured cecum was put back into the peritoneal cavity and the incision was stitched using thin 6-0 silk suture under sterile conditions. Rats in the sham group were subjected to same procedure, except CLP.

Western blotting

The ileum of each rat treated with floroindole was excised under isoflurane anesthesia and washed twice with phosphate-buffered saline (PBS). A tissue homogenate was prepared on ice in

HEPES buffer (10 mM, pH 8.0) which comprised potassium chloride (10 mM), magnesium chloride (2 mM), EDTA (0.1 mM), dithiothreitol (1.0 mM) and phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$, 0.5 mM). The tissue homogenate was centrifuged at 4,000 rpm for 25 min at 4 ^oC, and the protein concentration in the supernatant was determined using bicinchoninic acid (BCA) assay kit. Protein separation was achieved on 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min. Non-fat milk powder (3 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 ^oC and incubated to block non-specific binding of the blot.

The blot was incubated overnight (at 4 ◦C) with primary antibodies of rabbit polyclonal anti-p-NF-κB p65, mouse polyclonal anti-TNF-α, mouse monoclonal anti-IL-6, NOD2 and β-actin at a dilution of 1 to 1000. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Enhanced chemiluminescence (ECL). The respective protein expression levels were normalized to that of β-actin which served as control.

Enzyme-linked immunosorbent assay (ELISA)

Excised rat ileum was washed twice in PBS, blotted dry in filter paper and weighed. The ileal tissues were homogenized on ice in HEPES buffer (10 mM, pH 8.0) which consisted of potassium chloride (10 mM), magnesium chloride (2 mM), EDTA (0.1 mM), dithiothreitol (1.0 mM) and $PhCH_2SO_2F$ (0.5 mM). The tissue homogenates were centrifuged at 4,000 rpm for 25 min at 4 ^oC to obtain supernatant, which was refrigerated at -78 ^oC till required. The levels of TNF-α and IL-6 were determined in the supernatant using their respective ELISA kits.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from homogenized ileal tissues of rats was isolated using Trizol reagent. The synthesis of cDNA from RNA was performed using Reverse Transcriptase M-MLV (RNase H) kit. The reaction conditions were: 1 cycle of 5 min at 93 ˚C, 10 cycles of 30 s at 92 ˚C, 30 s at 67 ˚C, and 30 s at 70 ˚C, followed by 27 cycles of 30 s at 92˚C, 30 s at 54 ˚C, and 30 s at 70 ˚C, 10 min at 70 ˚C for 1 cycle. Electrophoresis was performed on 2 % agarose gel for separation of PCR products and normalization of the mRNA expression level was done with GAPDH. Rotor-Gene 6000 RT-PCR machine connected to the SYBR Premix Ex Taq was used for running the qRT-PCR. Analysis of the data was performed in accordance with the $^{2-}\Delta\Delta Ct$ method. The primer sequences used are shown in Table 1.

Table 1: Primer sequences used for NOD2 and β-actin qRT-PCR

Variable	Forward	Backward
NOD2	ATC CCT CGG TTA CTA TGT TG	GCT TCC TGA ATA CTC CTC CT
β-Actin	CCC ATC TAT GAG GGT TAC GC	TTA ATG TCA CGC ACG ATT TC

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using SPSS (version 15.0). Groups were compared using Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

CLP-induced expressions of TNF- α and IL-6

As shown in Figure 1, CLP significantly and time-dependently upregulated the expressions of TNF-α and IL-6 in intestinal tissues of rats ($p < 0.05$).

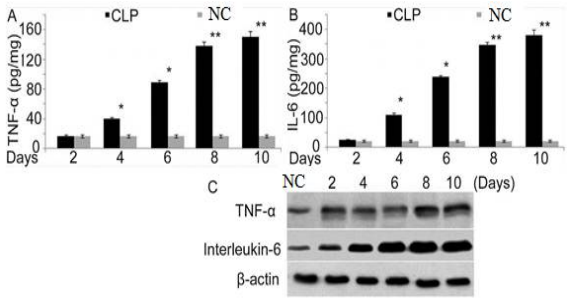

Figure 1: Effect of CLP on the expressions of TNF- α and IL-6. **(A)** Expressions of TNF-α and IL-6 as determined using ELISA; and **(B)** Expressions of TNF-α and IL-6 measured using Western blotting; $^*p < 0.05$ & $^{**}p < 0.01$, when compared with normal control group

Effect of floroindole on CLP-induced expressions of TNF- α and IL-6

Floroindole significantly, and dose-dependently down-regulated CLP-induced expressions of TNF-α and IL-6 ($p < 0.05$; Figure 2).

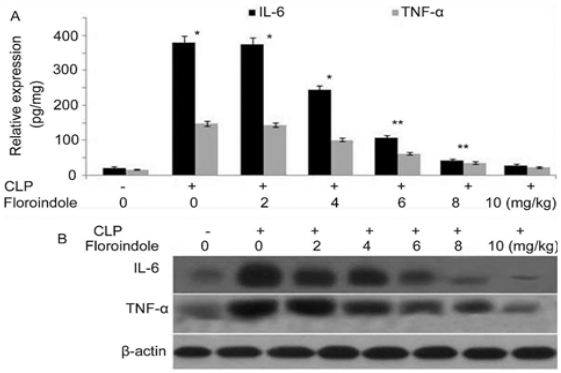

Figure 2: Effect of floroindole on the expressions of CLP-induced TNF-α and IL-6. **(A)** Expressions of TNF-α and IL-6 as measured using ELISA; and (B): Expressions of TNF-α and IL-6 as measured using Western blotting; $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared with normal control group

CLP-induced expression of NOD2 in rat intestinal tissues

As shown in Figure 3, CLP significantly and time-dependently increased the expression of NOD2 mRNA in intestinal tissues of rats ($p < 0.05$).

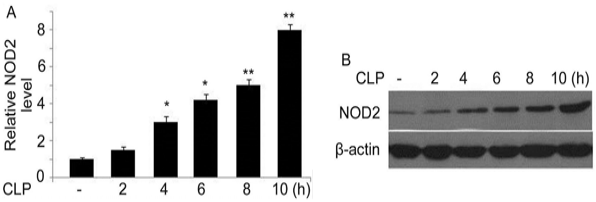

Figure 3: Effect of CLP on expression of NOD2 mRNA. **(A)** Expression of NOD2 as measured using ELISA; and **(B)** Expression of NOD2 measured using Western blotting; $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared with normal control group

Effect of floroindole on CLP-induced NOD2 expression

Treatment of rats with floroindole significantly and dose-dependently inhibited CLP-induced expression of NOD2 in rat intestine ($p < 0.05$; Figure 4).

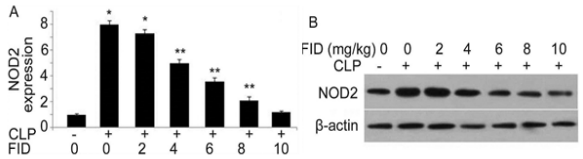

Figure 4: Effect of floroindole on CLP-induced overexpression of NOD2. **(A)** Expression of NOD2 mRNA, as determined using ELISA; and **(B)** expression of NOD2 mRNA, determined using Western blotting. $^{*}p < 0.05$ & $^{**}p < 0.01$, when compared with normal control group

CLP-induced phosphorylation of NF-κB p65

The results of Western blotting and qRT-PCR showed that CLP significantly and time-dependently promoted NF-κB p65 phosphorylation in rat ileum relative to normal control group ($p < 0.05$; Figure 5).

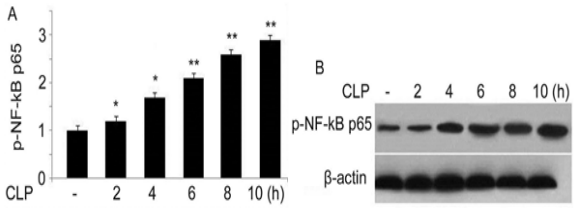

Figure 5: Effect of CLP on phosphorylation of NF-κB p65. **(A)** Expression of p-NF-κB p65, as measured using qRT-PCR; and **(B)** Expression of p-NF-κB p65, as measured using Western blotting; $^{*}p < 0.05$ & $^{**}p < 0.01$, when compared with normal control group

Effect of floroindole on CLP-induced phosphorylation of NF-κB p65

Treatment of the rats with floroindole significantly and dose-dependently inhibited CLP-induced NF-κB p65 phosphorylation in rat ileum ($p < 0.05$). These results are shown in Figure 6.

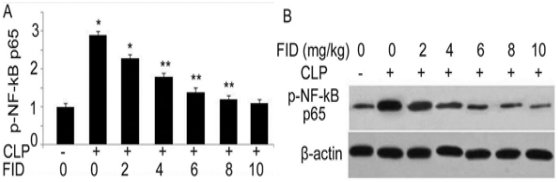

Figure 6: Effect of floroindole on CLP-induced NF-κB p65 phosphorylation. **(A)** Expression of p-NF-κB p65 as determined using qRT-PCR; and **(B)** Expression of p-NF-κB p65 as determined using Western blotting; $^{*}p < 0.05$ & $^{**}p < 0.01$, when compared with normal control group

DISCUSSION

Sepsis is a potentially life-threatening condition caused by physiological response to infection. The human body normally releases antibodies and complement proteins into the systemic circulation to fight infections. Sepsis occurs when these antibodies and complement proteins are overwhelmed, thereby triggering changes that damage multiple organs [1]. The present study investigated the protective effect of floroindole against CLP-induced sepsis, and the underlying mechanism. The results showed that floroindole significantly and dose-dependently suppressed CLP-induced expressions of TNF- α, IL-6 and NOD2, and NF-κB p65 phosphorylation in rat intestine. These results suggest that floroindole

may inhibit inflammatory reactions during sepsis. Sepsis is caused by the invasion of infectious particles leading to inflammatory reactions and ultimately cell injury [1,13]. In some cases, the inflammatory reactions lead to multi-organ failure [1,13]. At the initial stage, sepsis induces release of pro-inflammatory cytokines from intestinal tissues, which not only damage the intestine itself, but also affect the functioning of distant organs [14]. There is a strong association between the expression of pro-inflammatory cytokines and sepsis.

Nucleotide-binding oligomerization domain 2 (NOD2) exists as a cytosolic receptor for providing immunity to cells against various infectious agents [15]. It recognises the peptidoglycan portion of bacterial cell wall and then generates appropriate signals for the activation of receptor-interacting protein 2 (RIP2) [16,17]. The activated RIP2 in turn induces pro-inflammatory reactions and anti-infective responses leading to onset of infection inhibitory loop [16-18].

The peptidoglycan recognition capability of NOD2 is inhibited by polymorphism in the gene which encodes NOD2 protein [19]. The expression of NOD2 plays a key role in the phosphorylation of NF-κB [19]. In the present study, there were marked increases in the expressions of NOD2 mRNA and protein in rat intestinal tissues subjected to CLP. However, these increases were significantly and dose-dependently inhibited by floroindole treatment.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) regulates genes which encode inflammatory cytokines such as TNF-α and IL-6 [1, 20]. The phosphorylation of NF-κB increases the incidence of mortality in patients with sepsis [21]. In animal models of sepsis, inhibition of NF-κB phosphorylation has been found to elicit some therapeutic effects [22,23].

CONCLUSION

The results obtained in this study indicate that floroindole confers some degree of protection against CLP-induced sepsis via inhibition of NF-κB phosphorylation.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

Zhiwen Zhou, Xiang Ren, Aiping Li and Wensheng Zhou performed the experimental work. Zhiwen Zhou carried out the literature study and compiled the data. Li Huang designed the study and wrote the manuscript. All the authors wrote the paper before communication.

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