## Original Article
# Ligustrazine attenuates LPS-induced endothelial dysfunction by inhibiting NF-κB pathways

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Abstract: Ligustrazine, an alkaloid extracted from chuanxiong, has been demonstrated to exert immune regulation effects in endothelial cells. However, the molecular mechanisms underlying these actions have not been elucidated clearly. The aim of the current research was to investigate the possible pharmacological mechanisms of ligustrazine on lipopolysaccharide (LPS)-induced endothelial dysfunction. In this study, human umbilical vein endothelial cell (HUVECs) were stimulated with LPS. There were three experimental groups: the control group, LPS group, and LPS with ligustrazine group. The activity of cells was measured by CCK-8. Expression of the phosphorylation of NF-κB, p65 was detected by Western blotting and mRNA levels of NF-κB, p65 were detected by qRT-PCR. Results showed that LPS (8 μg/mL) could increase the phosphorylation of NF-κB, p65 and levels of NF-κB, p65 mRNA. Ligustrazine ($1 \times 10^{-6}$ mmol/L) attenuated the abnormal rising in endothelial cells challenged by LPS. In conclusion, it was found that Ligustrazine can effectively protect endothelial cell dysfunction challenged by LPS. Its protective efforts may be mediated by suppressing NF-κB activation through inhibiting the phosphorylation of NF-κB p65 and reducing expression of NF-κB, p65 mRNA.

Keywords: Ligustrazine, LPS, NF-κB, pathways

## Introduction

The endothelium is not only a single layer of cells covering all the inner surface of blood vessels, but it also maintains vascular homeostasis. It plays a critical role in many physiological functions, including the control of blood cell trafficking, vessel permeability, vasomotor tone, and homeostatic balance. However, when coronary risk factors are present, endothelial dysfunction may occur, characterized by a procoagulation, proinflammatory, and proliferative milieu that is involved in all stages of parthenogenesis [1]. Endothelial cells, as immunocompetent cells, have been known to play a pivotal role in inflammatory and immunity response by secreting a variety of proinflammatory cytokines. Its dysfunction has been proven to be a cornerstone of atherosclerosis occurrence, development, and progression [2-4]. Increasing evidence suggests that immune response play a key role in the pathogenesis of atherosclerosis and its complications [5-7]. In addition, nuclear factor-κB (NF-κB), a ubiquitous transcription factor, participates in immune and inflammatory response in chronic inflammatory diseases [8].

NF-κB, an important nuclear transcription factor, consisting of p65 and p50 subunits generally, mediates innate and adaptive immune response [9]. NF-κB is required for LPS-induced responsiveness. It can induce transcription and expression of multiple cellular cytokines by sequence, specifically binding to their promoter or enhancer region, promoting inflammation and immunological response [10, 11]. A growing body of evidence has indicated that drugs targeting NF-κB, the pivotal inflammatory mediator, might be useful for therapy of diseases involving inflammation and immune response [12-15].

Ligustrazine, also known as tetramethylpyrazine (TMP), is one of the effective ingredients of chuanxiong, a Traditional Chinese Medicine.

It is mainly used to treat a variety of vascular diseases, notably coronary vascular diseases, such as angina pectoris and myocardial infarction. Many studies have been demonstrated that it could reduce inflammatory response [16], inhibit oxidative stress [17, 18], block calcium channels, increase fibrinolytic activity, and attenuate platelet aggregation [19, 20], providing vascular regulation. However, the related pharmacological mechanisms remain largely unknown.

According to previous research, Ligustrazine suppressed TNF-α-induced HUVECs dysfunction via increasing the production of NO and inhibiting expression of HSP60 and ICAM-1. To further explore the molecular mechanisms of Ligustrazine action, a cell model of inflammation (human umbilical vein endothelial cells treated with lipopolysaccharide) was used to investigate the protective effects on endothelial dysfunction. Moreover, p65, a critical downstream activator in NF-κB signal pathways, was selected to address possible pharmacological mechanisms.

## Materials and methods

### Cell culture and experimental groups

HUVECs were purchased from Cell Applications Inc. (U.S.A). They were kept in RPMI-1640 (1640) or Ham's F12 (F12) medium. The medium was complemented with 1% antibiotics (100 mg/mL streptomycin sulfate and 100 U/mL penicillin) and 10% fetal bovine serum (FBS, Hyclone, USA). Cells were cultivated at 37°C in 95% air and humanized atmosphere of 5% $CO_2$. Additionally, dimethyl sulfoxide (DMSO) was used to prepare the stock solutions, while additional dilutions were done using a fresh culture medium. The DMSO concentration was 1% in the final culture medium. All experiments were performed with cells of passage 3. There were three experimental groups in this study: (1) Control group-HUVECs were incubated in medium without any drugs; (2) LPS group-cells were incubated with 8 μg/mL LPS for 24 hours; and (3) LPS and ligustrazine group-cells were stimulated with Ligustrazine for 24 hours, then 8 μg/mL LPS was added for 24 hours.

### Treatment with LPS and ligustrazine and measurement of activity of cells

After HUVECs reached confluence in 96-well plates, RPMI-1640 medium, containing various concentrations of LPS, was added. After 24

hours of culturing, absorbance was measured at 450 nm using an ELISA reader (BIORAD), within 4 hours, according to manufacturer protocol. All readings were repeated at least six times. A dose of 8 μg/mL LPS was chosen as an *in vitro* endothelial dysfunction model. After HUVECs reached confluence in 96-well plates, RPMI-1640 medium, containing various concentrations of Ligustrazine, was added. After 24 hours, the medium was removed and RPMI-1640 medium containing 8 μg/mL LPS was added to each well. Twenty-four hours later, the medium was removed in all groups, including the control group, and 110 μL RPMI-1640 medium containing 10 μL cck-8 was added. Absorbance was measured at 450 nm using an ELISA reader (BIORAD), within 4 hours. All readings were repeated at least six times. A concentration of 1×10$^{-6}$ mmol/L Ligustrazine was chosen as the treatment dose.

### Western blotting

Confluent HUVECs in 6-well plates were treated with Ligustrazine and with LPS, as previously described. After washing three times in ice-cold PBS, they were placed in 1 mL of RIPA Lysate buffer with PMSF (Bootee Biotechnology, China) for homogenization. The homogenized tissues were removed into a new EP tube (1.5 mL), kept at 4°C in the refrigerator for 30 minutes, and centrifuged at 12,000 g/min for 15 minutes. Total protein concentrations were measured using the protein assay, based on manufacturer instructions, in supernatant liquid. Fifty micrograms of total proteins per sample were resolved in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were electrically transferred onto nitrocellulose membranes. The membranes were incubated with blocking solution (1×TBS; 0.1% Tween 20; 5% non-fat milk) at room temperature (25oC) for 2 hours, then incubated overnight with the primary antibodies (1:2000 in dilution) of anti-phospho-NF-κB p65 (dilution 1:1000) and NF-κB p65 (dilution 1:1000) or with the primary antibody (1:1000 in dilution) of β-actin (AC004, Abclonal) in 5% milk in TBST. After incubation with corresponding secondary antibodies conjugated with peroxidase (Beyotime Biotechnology, China), these proteins were visualized using an enhanced chemiluminescence ECL Plus immunoblotting detection system (Climx Science Instruments Co. Ltd, China). Bands correspond-

**Table 1.** qRT-PCR primer information

| Genes | Primer sequence |
|---|---|
| P65 | Sense primer 5'-ACAACCCCTTCCAAGTTCCT-3' |
| | Antisense primer 5'-TGGTCCCGTGAAATACACCT-3' |
| β-actin | Sense primer 5'-GTGGGGCGCCCCAGGCACCA-3' |
| | Antisense primer 5'-CTCCTTAATGTCACGCACGATTTC-3' |

ing to the expected size were selected on the computerized scanner. The pixel density within each band was determined by this computer after the background correction for relative quantization. Optical densities of each band, relative to measured values of β-actin bands, were determined using Image-J software.

*Quantitative RT-PCR for validation of mRNA*

To determine mRNA levels, confluent HUVECs grown in 6-well plates were treated, as previously described. Total RNA was extracted using TRIzol, according to manufacturer instructions. All glassware and plastic ware were RNase-free. The operation was performed carefully to prevent RNase contamination. Isolated RNA samples were checked using electrophoresis on agarose gels and A260/A280 ratios read on a spectrophotometer were over 1.80. Complementary DNA (cDNA) samples were generated and amplified using a TaKaRa RNA PCR Kit (AMV) Ver.3.0, following manufacturer protocol. cDNAs were synthesized for mRNA expression assays from total RNA in 10 μl volume with 2 μl PrimeScript™ RT Master Mix (TaKaRa Biotechnology Co. Ltd. China), 1 μl total RNA, and 7 μl ddH$_2$O. qRT-PCR was conducted using Biosystems QuantStudio 7 Flex (Life Technologies, USA). Each reaction was carried in the total volume of 20 μl, including 1 μl cDNA, 10 μl SYBR Premix Ex Taq™ II (TaKaRa Biotechnology Co. Ltd. China), 0.5 μl/primer, and 9 μl ddH$_2$O. The program was set to 95°C for 5 minutes for pre-incubation, 40 cycles at 95°C in 5 seconds, and at 60°C in 20 seconds for annealing and amplification, as well as the addition dissociation curve. Relative expression levels of mRNAs were normalized to internal reference gene β-actin. All qRT-PCR runs were repeated three times. Results were calculated with the $2^{-\Delta\Delta Ct}$ method. Primer sequences used are listed in **Table 1**.

*Statistical analysis*

Data are expressed as mean ± S.E.M. Intergroup comparisons were carried out using one-way analysis of variance (ANOVA) for multiple comparisons, followed by Fischer's Protected Least Significant Difference (PLSD). The criterion for statistical significance was set at *P<0.05*.

**Results**

*Effects of Ligustrazine on activity of HUVECs stimulated with LPS*

LPS, with five different concentrations, was used to treat HUVEC cells for 24 hours. Results showed that 8 μg/mL LPS significantly reduced the amount of HUVECs (**Figure 1A**), indicating that endothelial dysfunction had occurred. Furthermore, a concentration of $1 \times 10^{-6}$ mmol/L Ligustrazine (**Figure 1B**) was chosen after carrying out dose-response experiments.

*Ligustrazine significantly downregulated expression of P65 in HUVECs stimulated with LPS*

The NF-κB family of transcription factors is comprised of five proteins in mammals, p65/RelA, c-Rel, RelB, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). P65 phosphorylation regulates activation, nuclear localization, protein-protein interactions, and transcriptional activity. Therefore, whether Ligustrazine can inhibit the phosphorylation of p65 on HUVECs was investigated. Treatment with Ligustrazine for 24 hours, following 24 hours of incubation with LPS, caused the P-P65/P65 ratio in endothelial cells to decrease, compared with the experimental group treated with LPS only (**Figure 2**).

*Effects of Ligustrazine on p65 mRNA expression in HUVECs stimulated with LPS*

P65 mRNA expression of HUVECs stimulated with LPS was demonstrated in each experiment using RT-PCR. Expression of p65 mRNA (**Figure 3**) was increased in the LPS-stimulated group but decreased after incubation with Ligustrazine.

**Discussion**

Vascular inflammatory response plays a critical role in the initiation and progression of atherosclerosis. Endothelial dysfunction represents a cardiovascular risk factor promoting atherosclerosis, the leading cause of death in Western populations. Ross revealed the relationship
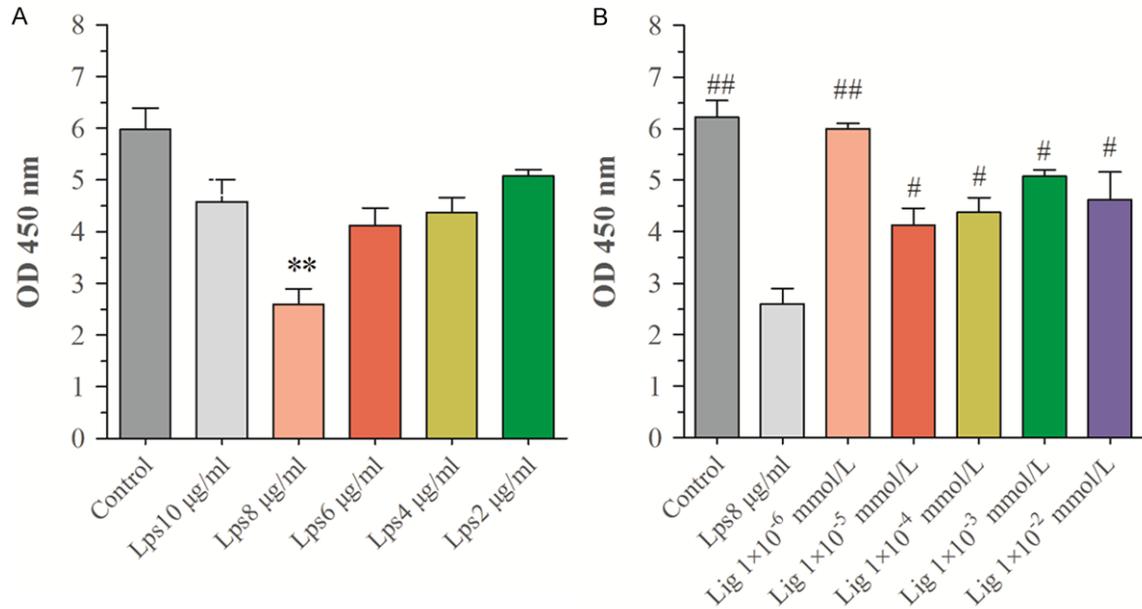
**Figure 1.** Effects of Ligustrazine on activity of HUVECs stimulated with LPS. (A) shows LPS with five different concentrations, was used to treat HUVEC cells for 24 hours (10, 8, 6, 4, 2 µg/mL). (B) ELISA reader was used to determine the capability of Ligustrazine on activity of HUVECs stimulated with LPS. Data are expressed as the mean ± standard deviation from at least three experiments. **$P<0.01$ vs. normal control, #$P<0.05$, ##$P<0.01$ vs. Lps. Lig, Ligustrazine; Lps, lipopolysaccharide.
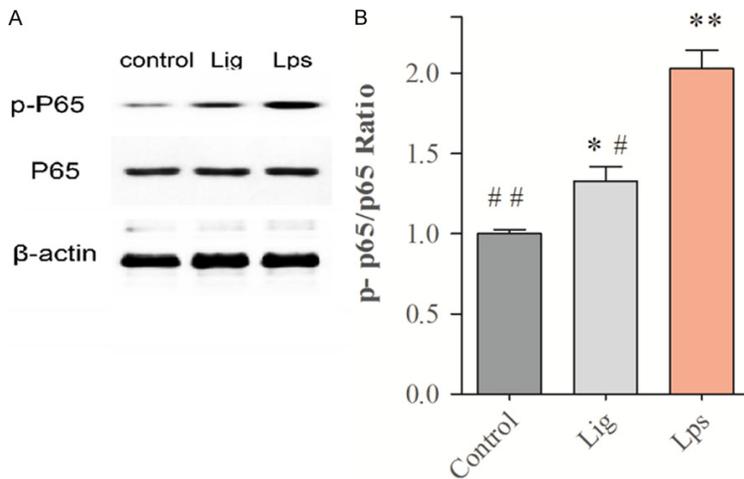


**Figure 2.** Ligustrazine significantly downregulated expression of P65 in HUVECs stimulated with LPS. (A, B) Expression and relative quantity of proteins p-P65 and P65 was studied by Western-blot. (A) Shows expression of p-P65 and P65 from HUVECs, where internal control was done with β-actin. (B) Illustrates the normalized ratios of proteins p-P65 to P65 from HUVECs. *$P<0.05$, **$P<0.05$ vs. normal control, #p, ##p vs. Lps. Lig, Ligustrazine; Lps, lipopolysaccharide.

the earliest indicator of development of cardiovascular disease (CVD), preceding the appearance of at herosclerotic plaque. Endothelial dysfunction is a key factor in the genesis, development, and progression of atherosclerosis. Atherosclerosis is a chronic inflammatory disease in which vessel wall inflammation participates in the pathophysiology of atherosclerotic plaque [22], providing new insight into the prevention and treatment of atherosclerosis by counteracting inflammation. It has been reported that excessive activation of NF-κB and sustained releasing of proinflammatory cytokines could lead to endothelial dysfunction [23]. Therefore, abnormal reglation of NF-κB signals may be important in the formation of atherosclerosis [24].

between endothelial dysfunction and atherosclerosis and discovered that the earliest changes that precede the formation of atherosclerotic lesions take place in the endothelium [21, 22]. Therefore, endothelial dysfunction is

Compelling evidence has demonstrated that bacterial infections can induce endothelial cell
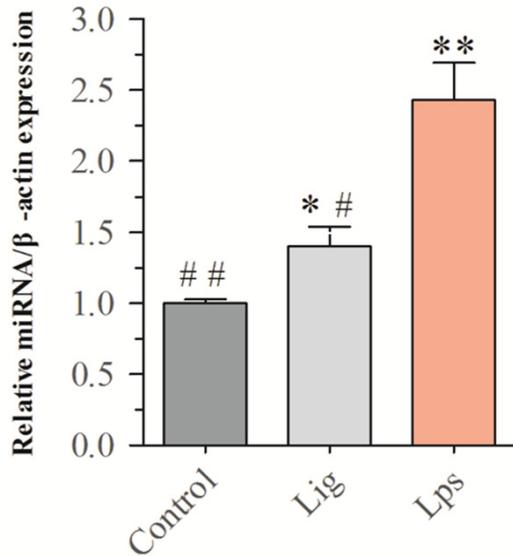
Figure 3. Effects of Ligustrazine on p65 mRNA expression in HUVECs stimulated with LPS. Relative values of mRNAs for *p65 mRNA* in HUVEC, which were done by qRT-PCR. β-actin was set as internal control. $^*P<0.05$, $^{**}P<0.01$ vs. normal control, $^#P<0.05$, $^{##}P<0.01$ vs. Lps. Lig, Ligustrazine; Lps, lipopolysaccharide.

dysfunction [25]. The importance of LPS-induced NF-κB activation cannot be ignored in bacterial infections. This process is initiated by toll-like receptor 4 (TLR4) signal pathways, following the activation of the proinflammatory transcription factor NF-κB [26], which regulates the inducible expression of a large range of/ various classes of proinflammatory mediators and plays an important role in the inflammatory response. Numerous reviews have provided detail descriptions of NF-κB signal transduction pathways [27, 28]. In non-stimulated cells, NF-κB, which is mainly a heterodimer of p65 and p50, is kept in an inactive state in the cytosol by its inhibitory proteins, such as NF-κB. This masks the nuclear localization signal. A variety of extracellular stimulus factors (LPS, proinflammatory cytokines, growth factor, and so forth) can lead to activation of the NF-κB signal path, which contains the activation of IκB-kinase(IKK) [29], degradation of IκB, and phosphorylation of P65. This allows liberated NF-κB to translocate into the nucleus where it can bind to specific DNA target sites to regulate transcription [30-32]. When binding to DNA, NF-κB induces expression of related cytokines, chemokines, or cell adhesion molecules, triggering inflammatory and immune response. P65 phosphorylation, a critical downstream activator of NF-κB signals, regulates activation,

nuclear localization, protein-protein interactions, and transcriptional activity [30]. It has been the focus of intense research over the last two decades [33-37].

Ligustrazine plays a key role in inhibiting oxidative stress NF–κB [17, 18], protection of endothelial cell [18], preventing platelet aggregation and thrombosis [38], relaxing blood vessels [39, 40], and improving coronary blood flow. Thus, it has been widely used in clinic for treatment of cardiovascular diseases. It has been reported that Ligustrazine can decrease cholesterol, triglycerides, and low-density lipoprotein levels, as well as attenuate endothelial injury and inhibit oxidative stress. These factors account for its protection in atherosclerotic plaque progression in animal research [17]. In addition, data suggests that Ligustrazine attenuates myocardial ischemic reperfusion damage and phosphorylation of endothelial nitric oxide syntheses (eNOS) was found to be a key effector contributing to protective action [41]. Furthermore, *in vitro* studies have proven that tetramethylpyrazine (TMP) can suppress LPS-induced IL-8 production, which is mediated by inhibiting ERK, p38 signals and blocking the translocation of NF-κB (p65) into the nucleus [23]. Recent studies have demonstrated that tetramethylpyrazine (TMP) suppressed IκB kinase phosphorylation, IκB degradation, and NF-κB translocation, inhibiting TNF-α-induced inducible NO syntheses (iNOS) expression in human umbilical vein endothelial cells [42].

In the present study, LPS was used to induce endothelial dysfunction. Significant protective effects on endothelial cells were observed at Ligustrazine concentrations of $1×10^{-6}$ mmol/L. Next, this study investigated the inhibiting effects of Ligustrazine on NF-κB activation in endothelial cells. LPS markedly increased the phosphorylation of P65 and levels of p65 mRNA expression, compared with non-stimulated cells. However, pretreatment with Ligustrazine effectively decreased phosphorylation of P65 and p65 mRNA expression, compared to LPS group cells. In summary, current results suggest that Ligustrazine shows a protective function on HUVECs stimulated with LPS. In addition, a previous study indicated that Ligustrazine increases production of NO in HUVECs and has immunomodulatory effects on HUVECs stimulated with TNF-α by downregulating expression of ICAM-1 and HSP60 [43]. Ligustrazine can improve endothelial function

by suppressing NF-κB pathway activation stimulated by LPS through decreasing the expression and phosphorylation of p65 in NF-κB pathways. These properties of Ligustrazine may contribute to its protective effects toward endothelial cells. The anti-inflammatory actions of Ligustrazine, found in the current study, were consistent with previous reports. However, the upstream regulation of NF-κB pathways and exact mechanisms responsible for the effects are incompletely understood. If there are some other regulatory mechanisms underlying these actions, further investigation is required. Additional studies are necessary to evaluate the implications of these findings in clinical trials.

## Conclusion

In summary, the present study found that Ligustrazine can effectively protect endothelial cell dysfunction challenged by LPS. Its protective efforts may be mediated by suppressing NF-κB activation through inhibiting the phosphorylation of NF-κB p65 and reducing expression of NF-κB p65 mRNA.

## Disclosure of conflict of interest

None.

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