Cyclovirobuxinum D suppresses lipopolysaccharide-induced inflammatory responses in murine macrophages in vitro by blocking JAK-STAT signaling pathway

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Aim: Cyclovirobuxinum D (CVB-D), an alkaloid isolated from the Chinese medicinal plant Buxus microphylla, has been found to be effective to treat cardiac insufficiency, arrhythmias and coronary heart disease. In the present study, we investigated the effects of CVB-D on the inflammatory responses in lipopolysaccharide (LPS)-stimulated murine macrophages in vitro and the underlying mechanisms.

Methods: Murine macrophage cell line RAW264.7 cells were incubated in the presence of LPS (0.1 μg/mL) for 24 h. The cell viability was measured using MTT assay. The release of NO and cytokines were detected using the Griess test and ELISA, respectively. The mRNA and protein levels were determined using RT-PCR and Western blot, respectively. Reporter gene assays were used to analyze the transcriptional activity of NF-κB.

Results: Treatment of RAW264.7 cells with CVB-D (25–300 μmol/L) did not affect the cell viability. Pretreatment with CVB-D (50, 100 and 200 μmol/L) concentration-dependently decreased NO release and iNOS expression in LPS-treated RAW264.7 cells (its IC50 value in inhibition of NO production was 144 μmol/L). CVB-D also concentration-dependently inhibited the secretion and mRNA expression of IL-1β and IL-6 in LPS-treated RAW264.7 cells. Furthermore, CVB-D remarkably inhibited the phosphorylation of STAT1 and STAT3, as well as JAK2 in LPS-treated RAW264.7 cells, but did not affect the activation of NF-κB and MAPKs pathways. Pretreatment with the JAK2 specific inhibitor AG490 (30 μmol/L) produced similar effects on NO release and iNOS expression in LPS-treated RAW264.7 cells.

Conclusion: CVB-D exerts anti-inflammatory effects in LPS-stimulated murine macrophages in vitro at least in part by blocking the JAK-STAT signaling pathway. The anti-inflammatory actions of CVB-D may contribute to its cardioprotection.

Keywords: cyclovirobuxinum D; macrophage; inflammation; nitric oxide; cytokine; JAK; STAT; myocardial infarction; cardioprotection

Introduction

Macrophages play an important role in the pathogenesis of inflammation by secreting various inflammatory mediators, such as NO, prostaglandin E2 (PGE2), tumor necrosis factor alpha (TNF-α), IL-1, and IL-6[4-3]. Overproduction of these mediators may lead to tissue damage and cause inflammatory-related diseases[4-3]. A growing body of evidence suggests that accentuation of the post-infarction inflammatory response results in poorer remodeling and dysfunction following myocardial infarction[6-7].

CVB-D, (3β, 5α, 16a, 205)-4, 4,14-trimethyl-3,20-bis(methylamino)-9,19-cyclopregnan-16-ol, is an alkaloid extracted from the Chinese traditional plant Buxus microphylla. It has been widely used in the treatment of heart-related conditions, such as arrhythmias and coronary heart disease in China[8-10]. More recent studies have shown that CVB-D can ameliorate heart failure induced by myocardial infarction[11, 12]. However, the exact mechanism behind its therapeutic basis is not clear. Clearance of necrotic debris by neutrophils, monocytes, and macrophages is a critical component of infarct healing. Nevertheless, tight control and timely repression of this inflammatory response are important to prevent excessive tissue degradation and adverse cardiac remodeling, which would lead to infarct expansion and heart failure. Therapies block-
ing pro-inflammatory pathways have been found to improve wound healing and to reduce the risk of heart failure in acute myocardial infarction\cite{13,14}.

Anti-inflammatory mechanisms and therapeutic opportunities in myocardial infarction and heart failure encouraged us to evaluate the anti-inflammatory effects and cardioprotective mechanism of CVB-D in myocardial ischemia. In the present study, we used the model of LPS-evoked murine macrophage-like cells RAW264.7 to investigate the anti-inflammatory action of this compound. LPS activates a set of intracellular signaling cascades in stimulated macrophages, including the nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPK) and Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways. These signaling pathways then regulate the expression of genes encoding various inflammatory mediators\cite{15–17}. The effects of CVB-D on the production of inflammatory mediators and related intracellular signaling pathways were evaluated in this study.

Materials and methods

Reagents
Cyclovirobuxinum D (purity: 98% by HPLC) was purchased from MUST BIO-TECHNOLOGY CO, LTD (Chengdu, China). Lipopolysaccharide (LPS), AG490 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St Louis, MO, USA). Two-step MMLV Platinum SYBR Green qPCR SuperMix-UDG kit, Novex ECL HRP Chemiluminescent substrate reagent kit and Lipofectamine 2000 were obtained from Invitrogen (San Diego, CA, USA). Griess reagent was purchased from Beyotime Institute of Biotechnology (Haimen, China). Antibodies against inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, p65, JAK2, p-JAK2 (Tyr1007/1008), STAT1, p-STAT1 (Tyr-701), STAT3, p-STAT3 (Tyr-705), and GAPDH were obtained from Bioword Technology, Inc (St Louis, MN, USA). Antibodies against IκB-α and PPAR and HRP-conjugated goat-anti-rabbit IgG antibody were obtained from Santa Cruz (Santa Cruz, CA, USA). The Luciferase Assay Kit was obtained from Promega (Madison, CA, USA). The pNF-kB-luciferase plasmid was kindly provided by the Anti-viral Research Center of Southern Medical University in China. The IL-1, IL-6, and TNF-α ELISA kits were purchased from eBiosciences (San Diego, CA, USA). CVB-D was dissolved in DMSO and diluted in culture media. The final concentration of DMSO never exceeded 0.1%.

Cell culture
The murine macrophage cell line RAW264.7 was obtained from the China Center for Type Culture Collection (Shanghai, China). Cells were grown in DMEM medium supplemented with 10% fetal bovine serum (Invitrogen), 100 IU/mL penicillin and 100 μg/mL streptomycin in a humidified incubator containing 5% CO₂ at 37°C.

Cell viability assay
Cell viability was measured using the MTT assay as described. Briefly, RAW264.7 cells at a density of 1×10⁴ cells/well were seeded in 96-well plates overnight and treated with various concentrations of CVB-D (25–300 μmol/L) for 24 h. Next, 20 μL of MTT solution (5 mg/mL) was added to each well, and the cells were cultured for another 4 h at 37°C. The supernatant was removed and DMSO was added for solubilization of formazan. The optical density at 570 nm was measured by a microplate reader (Benchmark plus Bio-Rad Laboratories, CA, USA).

Nitric oxide (NO) determination
NO levels were determined by measuring its stable oxidative metabolite nitrite levels in the culture media using the Griess reaction as previously described\cite{18}. Briefly, RAW 264.7 cells (2×10⁴ cells/well) were plated in 24-well plates followed by incubation with various concentrations of CVB-D (50–200 μmol/L) for 1 h. Then, LPS (0.1 μg/mL) was added into the culture medium for another 24 h of incubation. Next, 50 μL supernatant and 100 μL Griess reagent were reacted for 15 min and the nitrite content measured by absorbance at 540 nm. The NO concentration was calculated using a standard curve prepared with sodium nitrite.

Detection of cytokine production by enzyme-linked immunosorbent assay (ELISA)
RAW264.7 cells were pretreated with different concentrations of CVB-D (50–200 μmol/L) for 1 h and then treated with 0.1 μg/mL LPS for another 24 h. The levels of IL-1β, IL-6, and TNF-α in the culture media were measured using ELISA kits according to the manufacturer’s instructions.

Transient transfection and luciferase assay
RAW264.7 cells were seeded into 96-well plates (4×10⁴ cells/well) and incubated overnight. The pNF-kB-luciferase plasmid was transfected using lipofectamine 2000 according to the manufacturer’s instructions. After 1 h of pretreatment with CVB-D and 8 h of incubation with LPS and CVB-D, the cells were lysed and the luciferase activity was determined using the Luciferase Assay Kit with a luminometer (Genios Pro, TECAN, USA).

Western blot analysis
Western blots were performed to investigate the effect of CVB-D on the protein levels of iNOS, COX-2, NF-κB, IκB-α, and phosphorylated total STAT1, STAT3, JAK2, and MAPKs. After RAW264.7 cells were incubated with LPS in the presence or absence of CVB-D, whole cell extract, nuclear and cytoplasmic proteins were prepared according to the protocol of the Protein Extraction Kit (Beyotime). Protein concentrations were determined using the BCA protein assay system (Beyotime). Aliquots of the lysates (20–50 μg protein) were separated on 8%–10% SDS–PAGE and transferred onto polyvinylidene difluoride membranes (BIO-RAD, CA, USA). After blocked with 5% bovine serum albumin in TBST (0.1%),
the membranes were incubated overnight at 4 °C with primary antibodies and then incubated with the peroxidase-conjugated secondary antibodies for 60 min at room temperature. The membranes were incubated with an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA) and exposed to X-ray film. The protein signals were quantified by densitometry analysis using a FluorChem Q System (Alpha Innotech, CA, USA).

RNA isolation and real-time Polymerase Chain Reaction (PCR) analysis
RAW 264.7 cells were plated at 1×10^6 cells/well in 12-well plates, then pretreated with CVB-D (50–200 μmol/L) for 1 h and incubated with LPS (0.1 µg/mL) for another 6 h. Total RNA from RAW264.7 cells was prepared by Trizol reagent according to the manufacturer’s protocol.

Total RNA (1 μg) was reverse-transcribed with M-MuLV Reverse Transcriptase Kit to obtain cDNA. Real-time PCR was performed in an ABI7500 real-time PCR instrument (Applied Biosystems) with the SYBR Green qPCR SuperMix-UDG kit. The comparative cycle threshold (ΔΔC_t) method of relative quantification was used to determine the fold-change of expression. A melting curve analysis was carried out after amplification to verify the accuracy of the amplicon. Primer sequences for qPCR of TNF-α, IL-6, IL-1β, iNOS, COX-2, and GAPDH mRNA are shown in Table 1.

Statistical analysis
All data are expressed as the means±SD. One-way ANOVA and two-tailed Student’s t-test were used. P values <0.05 were considered statistically significant. Statistical analyses were conducted with SPSS 13.0 (SPSS Inc, Chicago, IL, USA).

Results
CVB-D inhibited NO production, as well as the mRNA and protein expression of iNOS, in LPS-stimulated RAW264.7 cells
NO levels in the medium of RAW264.7 cells increased approximately 6.5-fold after LPS treatment (Figure 1A). CVB-D inhibited NO production in LPS-stimulated RAW264.7 cells in a dose-dependent fashion, with an IC_{50} of 144 μmol/L. Because iNOS plays a vital role in NO production during inflammation, we performed Western blot and real-time PCR to determine whether the decreased level of NO was due to reduced iNOS expression. CVB-D was found to significantly inhibit the mRNA and protein expression of iNOS (Figure 1B and 1C). AG490, a specific JAK2 inhibitor, was found to have similar effects on NO release and iNOS mRNA expression (Figure 1A and 1C). PGE2, which is mainly produced through the COX-2 pathway, is another important inflammatory mediator in the initiation of inflammatory disorders to induce pain, fever and edema. In our study, CVB-D did not show any effects on the mRNA and protein levels of COX-2 in LPS-stimulated RAW264.7 cells (data not shown).

The inhibitory effect of CVB-D on the production of inflammatory mediators was not due to cytotoxicity because the compound, even at a dose of up to 300 μmol/L, did not significantly suppress the cell viability as measured by the MTT assay (Figure 1D).

CVB-D decreased LPS-induced IL-1β and IL-6 gene expression and protein secretion in RAW264.7 cells
CVB-D inhibited LPS-induced IL-1β and IL-6 gene expression and protein secretion in a dose-dependent manner (Figure 2A and 2B). Treatment with 200 μmol/L CVB-D led to a 99% inhibition of IL-1β secretion and a 48% inhibition of IL-6 secretion. Conversely, at the same concentration, CVB-D was unable to inhibit the secretion of TNF-α (Figure 2C). In addition, CVB-D markedly inhibited IL-1β and IL-6 mRNA expression (Figure 2D). CVB-D (200 μmol/L) inhibited IL-1β mRNA expression by 57% and inhibited IL-6 mRNA expression by 28% in LPS-stimulated RAW264.7 cells. However, no significant change was detected in TNF-α mRNA expression (Figure 2D). Similarly to CVB-D, AG490 also significantly inhibited IL-1β and IL-6 but not TNF-α release or gene expression.

CVB-D did not inhibit NF-κB nuclear translocation and IκB-α protein degradation stimulated by LPS in RAW264.7 cells
The NF-κB pathway has been reported to play important

| mRNA   | Primer   | Sequence (5′–3′)           | GeneBank accession # | Position | Size (bp) |
|--------|----------|---------------------------|----------------------|----------|-----------|
| IL-6   | Sense    | AGTTGCGCTTCTCTGGGACTGA     | J03783               | 81–100   | 191       |
|        | Antisense| CAGAATTGGCATTGCAACAC       |                      | 271–252  |           |
| TNF-α  | Sense    | GTGGAACCTGGCAGAAGAGGC      | NM_013693.2          | 184–203  | 122       |
|        | Antisense| AGACAGAAGAGCTGGTGGCC       |                      | 305–286  |           |
| iNOS   | Sense    | TCCATGACCTCCACGACA         | NM_010927.3          | 215–232  | 108       |
|        | Antisense| CCATCTCCTGACATTTCTCC       |                      | 322–303  |           |
| IL-1β  | Sense    | GCAACTGTTCCTGGGACTGA       | NM_008361.3          | 72–93    | 89        |
|        | Antisense| ATCTTTTGGGGTCCGCTCAACT     |                      | 140–160  |           |
| COX-2  | Sense    | GAAGTCTTTGGTCTGGTGGCTCG    | NM_011198.3          | 926–947  | 133       |
|        | Antisense| GTCGGTGTTTGCAAGATGTGCG     |                      | 1058–1036|           |
| GAPDH  | Sense    | GGTGAAGGTCGGTGTTGAGAGC     | GU214026.1           | 49–68    | 233       |
|        | Antisense| CTGCCTCTGGAAGATGCGG        |                      | 281–262  |           |
regulatory roles in the macrophage-mediated inflammatory response\textsuperscript{[19]}. NF-κB is normally present in the cytosol in an inactive form linked to inhibitor κB (IκB) protein. In response to an activation signal, the IκB protein is degraded. NF-κB is then released from the NF-κB/IκB complex and translocates into the nucleus, regulating the transcription of a large number of genes, including those that encode pro-inflammatory mediators. In our study, the protein levels of IκB-α and the p65 subunit of NF-κB in the cytoplasm significantly decreased after LPS challenge for 30 min, and the p65 protein level in the nucleus markedly increased, indicating that the p65 subunit was translocated into the nucleus (Figure 3A). Pretreatment with CVB-D (50–200 µmol/L) had no distinct effect on this degradation and translocation. In addition, 8 h of LPS treatment resulted in a 7-fold increase in NF-κB transcriptional activity, as determined by measuring the luciferase activity of the reporter plasmid, and CVB-D did not affect this response (Figure 3B).

CVB-D did not inhibit the phosphorylation of MAPKs in LPS-stimulated RAW264.7 cells
MAPK cascades are known to be critical for LPS-stimulated induction of inflammatory mediators in RAW264.7 cells\textsuperscript{[20]}. To determine whether the CVB-D-induced anti-inflammatory effect is mediated by MAPK pathways, we subsequently evaluated the effects of CVB-D on the phosphorylation of ERK1/2, p38, and JNK MAPKs in LPS-stimulated RAW 264.7 cells. As shown in Figure 4, LPS significantly promoted the phosphorylation of ERK1/2, JNK, and p38 MAPKs in RAW 264.7 cells. Pretreatment with CVB-D did not inhibit the phosphorylation of MAPKs. The total protein expression of ERK1/2, p38, and JNK MAPKs was unaffected by LPS or LPS in combination with CVB-D.

CVB-D inhibited JAK-STAT pathway activation in LPS-stimulated RAW264.7 cells
The inhibition of the JAK-STAT pathway has been reported
to result in suppression of LPS-induced expression of NO, IL-1β, and IL-6 but not TNF-α or COX-2 [21–24], which is consistent with the anti-inflammatory profile of CVB-D found in our study. The specific JAK2 inhibitor AG490 was also found to have similar effects in inhibiting gene expression and protein secretion of inflammatory mediators. These results encouraged us to investigate the effect of CVB-D on the activation of the JAK-STAT pathway.

As shown in Figure 5A, phosphorylation of STAT1 and STAT3 started at 1–2 h after LPS challenge and peaked at 6 h, whereas significant phosphorylation of JAK2 was detected at approximately 15 min after LPS stimulation. CVB-D significantly blocked the phosphorylation of JAK2, as well as the subsequent phosphorylation of STAT1 and STAT3, in a concentration-dependent manner. However, no distinct effect was observed on total JAK2, STAT1, and STAT3 protein level (Figure 5B).

**Discussion**

In the present study, we found that CVB-D suppressed the production of inflammatory mediators (NO, IL-1β, and IL-6) in a dose-dependent manner in LPS-stimulated RAW 264.7 cells. These results reveal, for the first time, that CVB-D isolated from *Buxus sinica* exhibits anti-inflammatory properties in vitro. The underlying mechanisms appear to be attributable to the blockade of the JAK-STAT pathway.

NO induced by iNOS is involved in a broad spectrum of pathophysiological processes, including atherosclerosis [25], diabetes [26] and septic shock [27]. NO and iNOS induction have also been reported to contribute to the process of cell injury in myocardial infarction and heart failure [28]. Blocking NO production by inhibiting iNOS activity may be a valid way of treating myocardial infarction. PGE2 generated by COX-2 is another important mediator contributing to vasodilation, pain and fever in inflammation. In our study, CVB-D effectively decreased NO production by suppressing the mRNA and protein expression of iNOS in LPS-stimulated RAW264.7 cells but did not affect COX-2 expression at either the protein or gene levels. These results indicate that the specific inhibition of iNOS protein and gene expression may be responsible for the anti-inflammatory activity of CVB-D, and this inhibitory effect may be involved in its cardioprotective role.

The cytokine-mediated inflammatory responses play important roles in cardiac remodeling and heart failure. Effects of antagonists against the pro-inflammatory cytokines, such as IL-1β, IL-6, or TNF-α, in patients and animal models of coro-
nary artery disease and acute myocardial infarction have been evaluated\[29–31\]. IL-1 blockade was found to be effective in limiting atherosclerosis and atherothrombosis and improves outcomes in acute myocardial infarction and ischemic stroke\[32, 33\].

In our study, CVB-D greatly inhibited the production and mRNA expression of IL-1β and, to a lesser extent, IL-6 in LPS-stimulated RAW264.7 cells. However, CVB-D showed no significant effect on LPS-induced TNF-α secretion and gene expression. These data suggest that CVB-D may ameliorate inflammation-related disorders, including myocardial infarction and heart failure, by modulating IL-1β and IL-6 expression.

To further explore the mechanism underlying the anti-inflammatory effect of CVB-D, our studies focused on the NF-κB, MAPK, and JAK-STAT inflammatory pathways. We determined that CVB-D showed no inhibitory effect on the LPS-activated MAPK pathways, including the phosphorylation of ERK1/2, p38, and JNK in RAW264.7 cells. CVB-D also had no impact on LPS-stimulated IκB-α degradation and the subsequent NF-κB transcriptional activity in RAW264.7 cells. Taken together, we show that CVB-D suppresses inflammatory mediator expression via a mechanism independent of the MAPK and NF-κB pathways in macrophages.

Previous studies have demonstrated that activation of the JAK2-STAT pathway results in LPS-mediated expression of NO, IL-1β, and IL-6 but not COX-2 and TNF-α. Our studies show that the effects exerted on the production of inflammatory mediators by CVB-D were similar to the effects of the specific JAK2 inhibitor AG490. Based on the above findings, we...
hypothesize that the main target of CVB-D is the JAK-STAT pathway. Mammalian JAKs are composed of four members, among which JAK2 is the dominant member in macrophages. JAK2 is activated after RAW264.7 cells are stimulated with LPS, leading to the activation of the downstream molecules STAT1 and STAT3, which translocate into the nucleus to regulate the transcription of target genes encoding inflammatory mediators. As expected, CVB-D markedly inhibited the phosphorylation of JAK2, STAT1, and STAT3 in LPS-stimulated RAW264.7 cells. We found that the activation of the JAK-STAT pathway caused by LPS occurred much later than the MAPK and NF-kB pathways in macrophages. A 15-min exposure to LPS resulted in a significant increase in the phosphorylation of JNK, p38, and ERK. Degradation of IκB-α and nuclear translocation of p65 NF-kB were distinct within 30 min after LPS stimulation. However, tyrosine phosphorylation of STAT1 and STAT3 was detected at 1–2 h, and 6 h was required to reach the maximum levels of phosphorylation after exposure to LPS (Figure 5A). This result implies that the expression of inflammatory mediators that respond early to LPS stimulation, such as TNF-α, may be more dependent on MAPK and NF-kB than on the JAK-STAT signaling pathways. Previous studies have shown that IL-1β, IL-6, and iNOS, but not TNF-α, contain STAT binding elements in their promoter regions and that JAK2-STATs play a critical role in the LPS-induced expression of IL-1β, IL-6, and iNOS, with little effect on TNF-α expression. All these results support our hypothesis that CVB-D suppresses IL-1β, IL-6 and iNOS expression by blocking the JAK-STAT pathway in LPS-stimulated macrophages.

The activation of the JAK-STAT pathway leads to the production of several inflammatory mediators. Meanwhile, some pro-inflammatory cytokines, including members of the IL-6 family, may activate this signal pathway, mediating diverse biological responses. Therefore, the inhibitory action of CVB-D on the JAK-STAT pathway results in its strong anti-inflammatory effects. Because unrestrained inflammation in the infarcted heart induces cardiomyocyte apoptosis and matrix degradation, down modulation of cytokine responses by blocking the JAK-STAT pathway may be an effective way.
to protect the myocardium from dilative remodeling and progressive dysfunction. Suppression of JAK-STAT pathway activation is reported to result in protection against myocardial injury in rat acute myocardial infarction and septic shock. CVB-D is widely used in treating cardiac insufficiency, arrhythmias, coronary heart disease and myocardial infarction in China. Our study first implies that the anti-inflammatory effect of CVB-D is mediated by suppressing the activation of the JAK-STAT pathway, a mechanism that may facilitate its role in cardioprotection.

In conclusion, the present study demonstrates that CVB-D inhibits the LPS-induced inflammatory response in RAW264.7 macrophages by blocking the JAK-STAT pathway, and this effect may be involved in its cardioprotection in myocardial infarction and heart failure. These findings provide new insight into the anti-inflammatory activity of CVB-D and its mechanism in heart disease. Further studies are needed to explore how the anti-inflammatory action of CVB-D affects its roles in myocardial infarction.

Acknowledgements
This study is supported by grants from the Guangdong Natural Science Foundation (Ng S201101003787, S2012010009056), Science and Technology Planning Project of Guangdong Province (Ng 2011B031800128), Science and Technology Bureau of Guangzhou (Ng 2011J4100085), and Science and Technology Bureau of Baiyun District of Guangzhou (Ng 2011-KZ-60).

Author contribution
Dan GUO performed most of the experiments, analyzed the data and drafted the manuscript; Jing-rong LI and Ying WANG performed some of the experiments and statistical analysis; Chuan-lin YU and Lin-sheng LEI assisted in the experiments; Na-na CHEN designed the study and reviewed the manuscript.

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