Anti-inflammatory Effect of d-(+)-Cycloserine Through Inhibition of NF-κB and MAPK Signaling Pathways in LPS-Induced RAW 264.7 Macrophages

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Abstract
Recently, additional therapeutic potentials of classical antibiotics are gaining considerable attention. The discovery of penicillin in the 1920s had a major impact on the history of human health. Penicillin has been used for the treatment of fatal microbial infections in humans and has led to the discovery of several new antibiotics. d-(+)-Cycloserine (DCS) is an antibiotic isolated from Streptomyces orchidaceous and is used in conjunction with other drugs in the treatment of tuberculosis. However, there have been no studies on the anti-inflammatory effects of DCS in RAW 264.7 macrophage cell line. To investigate the anti-inflammatory effects of DCS, we examined the ability of DCS to inhibit the inflammatory responses in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages in this study. Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were pretreated with various concentrations (2, 4, and 6 mM) of DCS, then treated with 1 μg/mL LPS to detect its anti-inflammatory effects. d-(+)-Cycloserine inhibited the production of nitric oxide (NO) in a concentration-dependent manner, and to some extent, inhibited the production of prostaglandin E₂. Consistent with these findings, DCS suppressed the expression of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-6. However, it had no effect on the expression of tumor necrosis factor-α. Western blot analysis demonstrated that DCS inhibited inducible nitric oxide synthase and suppressed cyclooxygenase type-2 (COX-2) expression. In addition, investigation of its effects on nuclear factor kappa B signaling showed that DCS inhibited phosphorylation of inhibitory kappa B-α (IκB-α) and increased intracellular IκB-α in a concentration-dependent manner. Furthermore, DCS inhibited the phosphorylation of LPS-induced extracellular signal-regulated kinase, however it did not affect phosphorylation of c-jun N-terminal kinase and p38. Further studies confirmed that the inhibition of phosphorylation of IκB-α was mediated through the inhibition of phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway. To determine the applicability of DCS to the skin, cytotoxicity on HaCaT keratinocytes was measured following treatment with various concentrations (2, 4, 6, 8, and 10 mM) of DCS using MTT assay. These results suggest that DCS may be used as a potential drug for the treatment of inflammatory diseases.

Keywords
d-(+)-cycloserine, antibiotic, anti-inflammation, NF-κB, MAPK, phosphorylation, bioactivity

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Inflammation is a protective mechanism of host defense in response to invading pathogens or tissue damage.¹ When the body is infected by pathogens, the macrophages remove the pathogens through phagocytosis and produce various inflammatory mediators such as nitric oxide (NO), prostaglandin E₂ (PGE₂), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 to recruit other immune cells.²³ Although these inflammatory processes are important to maintain homeostasis in vivo, prolonged or excessive inflammation induces pathogenesis of diseases such as asthma, atopic dermatitis, atherosclerosis, and inflammatory bowel disease.⁴ Therefore, it is necessary to control the level of inflammation. Lipopolysaccharide (LPS) is a fundamental component of the outer membrane of gram-negative bacteria that acts as an endotoxin.⁵ Macrophages are activated by recognizing the pathogen-associated molecular pattern of LPS through pattern recognition receptors, leading to the production of pro-inflammatory cytokines and chemokines. Inhibition of these pathways may have potential therapeutic applications.

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Activated macrophages are characterized by the production of several inflammatory mediators such as NO and PGE$_{2}$, and pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6. The released pro-inflammatory molecules are regulated by inducible gene expression that is mediated by the activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathway. Nuclear factor kappa B is a transcription factor that exists in the form of homodimers or heterodimers. In the unstimulated state, NF-κB is located in the cytoplasm bound by the inhibitory protein kappa B (IκB). Inhibitory protein kappa B is phosphorylated by the MAPK and phosphoinositide 3-kinase/Akt (PI3K/Akt) pathways. Phosphorylation of IκB triggers its ubiquitination and subsequent degradation by the proteasome. The free NF-κB dimers rapidly translocate from the cytoplasm to nucleus and bind to the promoter of responsive genes and activate the transcription of inducible target genes including inducible nitric oxide synthase (iNOS), cyclooxygenase type-2 (COX-2), and pro-inflammatory mediators.

Mitogen-activated protein kinases are well-conserved serine/threonine kinases that play an important role in cellular activities such as gene expression, mitosis, differentiation, and cell survival/apoptosis. The MAPK family is composed of 3 protein kinases including extracellular signal-regulated kinase 1/2 (ERK 1/2), c-jun N-terminal kinase 1/2 (JNK 1/2), and p38. Mitogen-activated protein kinases are activated by phosphorylation and induce activation of NF-κB and activator protein-1 (AP-1) that modulate gene expression. Thus, NF-κB and MAPK signaling pathways are regarded as major targets for anti-inflammatory effect.

DCS suppresses inflammatory activities and studied the underlying molecular mechanism in RAW264.7 macrophages. In addition, we examined cytotoxicity of DCS using HaCaT keratinocytes to investigate whether DCS is applicable to the skin.

We first evaluated the cytotoxicity of DCS in RAW 264.7 macrophages. The cells were treated with various concentrations (2, 4, 6, 8, and 10 mM) of DCS for 24 hours (Figure 1). Cell viability was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) formazan crystals which are converted by tetrazolium salt via mitochondrial dehydrogenase in RAW264.7 cells. D-(+)-Cycloserine did not affect the viability of RAW264.7 cells at the tested concentrations 2 to 6 mM. Therefore, we used DCS at concentrations between 2 and 6 mM (% of cells survival ≥90) in the subsequent experiments.

NO is a molecular messenger that plays an important role in cellular signaling in vivo. NO molecules are produced by several types of nitric oxide synthases (NOSs), including neuronal NOS (nNOS), endothelial NOS (eNOS), cytokine-iNOS, and bacterial NOS (bNOS), and are involved in various physiological functions such as neurotransmission, vasodilation, oxidative stress, antibacterial activity, and immune defense against pathogens. In inflammatory responses to pathogenic stimuli, iNOS induces the overproduction of NO. In previous studies, it has been reported that the excessive NO production leads to inflammatory and autoimmune diseases. Therefore, downregulating NO production and iNOS expression may be an appropriate strategy for anti-inflammatory therapies. To investigate whether DCS could inhibit NO production in LPS-stimulated RAW264.7 cells, we measured NO production in the form of nitrite in the cell culture media using the Griess reagent. As shown in Figure 2A, the LPS-only treated group showed markedly increased level of NO production compared to the LPS nontreated group. 1-N6-(1-iminoethyl) lysine dihydrochloride (1-NIL), an iNOS specific inhibitor, was used as a positive control. D-(+)-Cycloserine effectively inhibited NO production in the LPS stimulated cells in a dose-dependent manner (27.5%, 51.8%, and 60.5%) compared to the DCS untreated LPS stimulated cells (Figure 2a). Western blot analysis was performed to investigate...
whether the inhibitory effect of NOS on DCS was caused by the regulation of iNOS protein. Western blot analysis revealed significantly increased production of iNOS in the LPS-only stimulated group. However, the groups stimulated with LPS following DCS treatment showed a decrease in the expression of iNOS protein in a concentration-dependent manner (Figure 2b). Thus, these results demonstrate that DCS inhibits NO production by inhibiting the iNOS protein.

Prostaglandin E2, synthesized by COX-2, is associated with a wide range of pathological conditions, and drugs that inhibit PGE₂ production are being studied for therapeutic purposes. To examine whether DCS could inhibit LPS-induced PGE₂ production in RAW264.7 macrophages, we evaluated the amount of PGE₂ in cell culture using enzyme-linked immunosorbent assay (ELISA). As shown in Figure 3A, N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide (NS-398), a specific inhibitor of COX-2 protein, was used as a positive control. Lipopolysaccharide induced an increase in PGE₂ production. The group treated with the COX-2 inhibitor showed a 59.5% reduction in PGE₂ production compared to

**Figure 2.** The effect of d-(-)-cycloserine on production of nitric oxide in lipopolysaccharide-induced RAW 264.7 cells. The cells were plated in 24-well plates (1.0 × 10⁵ cells/well) and incubated for 24 hours, and then pretreated with d-(-)-cycloserine (2, 4, and 6 mM) for 1 hour followed by lipopolysaccharide stimulation for 24 hours. (a) Cytotoxicity of d-(-)-cycloserine was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The amount of nitric oxide in the medium was measured using the Griess reagent. l-N⁶-(1-Iminoethyl)lysine dihydrochloride was used as a positive control. (b) Western blotting result and corresponding protein level of inducible nitric oxide synthase. The results are presented as the mean ± standard deviation from 3 independent experiments. #P < .001 vs unstimulated control group. ***P < .001 vs lipopolysaccharide alone. The results shown are representative of 3 independent experiments.
the group treated with LPS alone. d-(-)-Cycloserine inhibited PGE₂ production to a certain extent (4.3%, 7.7%, and 12.7%) at the concentrations tested (Figure 3B).

Western blot analysis was used to investigate whether the inhibitory effect on PGE₂ production was due to the regulation of COX-2 protein. The production of COX-2 was significantly increased in the group stimulated with LPS-only compared to the nontreated group. The expression of COX-2 protein reduced to some extent in the LPS-treated group in the presence of DCS. d-(-)-Cycloserine is therefore believed to regulate the production of PGE₂ through the inhibition of COX-2 protein expression.

Well-known pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are activated by pathogenic stimuli and mediate inflammatory responses that have been linked to a variety of diseases. To investigate the anti-inflammatory potential of DCS, the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 was measured in LPS-stimulated RAW264.7 cells using ELISA. Lipopolysaccharide stimulation induced TNF-α, IL-1β, and IL-6 production in the cell culture media. At the concentrations tested, DCS inhibited LPS-induced production of IL-1β (49.3%, 80.1%, and 86.6%) and that of IL-6 (41.6%, 71.9%, and 81.6%) in a
Figure 4. The effect of D- (+)-cycloserine on the lipopolysaccharide-induced production of interleukin-1β, interleukin-6, and tumor necrosis factor-α in RAW 264.7 cells. Cells were pretreated with D- (+)-cycloserine (2, 4, and 6 mM) for 1 hour and then stimulated for 20 hours with lipopolysaccharide. The production of (a) interleukin-1β, (b) interleukin-6, and (c) tumor necrosis factor-α were determined using ELISA. The results are presented as the mean ± standard deviation from 3 independent experiments. #P < .001 vs control group. ***P < .001 vs lipopolysaccharide alone. The results shown are representative of 3 independent experiments.
concentration-dependent manner (Figure 4). However, DCS did not influence the LPS-induced TNF-α production.

Nuclear factor kappa B is a regulator of genes including inflammation and immunity. Inactive NF-κB complex is controlled strictly by binding to IκB-α in the cytoplasm. Activation by LPS leads to ubiquitination and subsequent degradation of IκB-α through the proteasome. The released NF-κB complex then translocates from the cytoplasm to the nucleus and encodes genes which are involved in the expression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6. To confirm the mechanism whether DCS regulates NF-κB activation via phosphorylation of IκB-α, we evaluated the protein level of IκB-α and phosphorylated IκB-α in LPS-induced RAW264.7 using Western blot analysis. As shown in Figure 5, DCS inhibited the phosphorylation of IκB-α in a concentration-dependent manner. Consistent with these results, DCS suppressed the degradation of IκB-α in the cytoplasm in a concentration-dependent manner. In a previous study, it has been reported that NF-κB mediates the expression of pro-inflammatory mediators such as TNF-α, IL-1β, and IL-6, and induces the expression of inflammatory enzymes such as iNOS and COX-2, thereby enhancing the inflammatory response. This study showed that DCS inhibits the degradation of IκB-α by regulating phosphorylation of IκB-α, thereby inhibiting NF-κB activation.

We found that DCS inhibited the phosphorylation of IκB-α. Previous studies have reported that PI3K/Akt signaling pathways induce NF-κB activation by enhancing the degradation of IκB-α. To determine whether DCS regulates NF-κB activation through the PI3K/Akt pathway, we checked the phosphorylation of Akt using Western blot analysis. As shown in Figure 6, DCS inhibited the phosphorylation of Akt compared to LPS-only treated group.

Mitogen-activated protein kinases, the well-conserved serine/threonine protein kinases, play an important role in cellular activities. In particular, MAPKs are known as inflammation-associated signaling pathways. Lipopolysaccharide activates the phosphorylation of MAPKs. The phosphorylated MAPKs, in turn, induce the expression of iNOS and COX-2 to

**Figure 5.** The effect of D-(-)-cycloserine on the level of phospho-inhibitory kappa B-α and inhibitory kappa B-α in lipopolysaccharide-induced RAW 264.7 cells. Lysates were prepared from cells pretreated with D-(-)-cycloserine (2, 4, and 6 mM) for 1 hour and then treated with lipopolysaccharide (1 µg/mL) for 15 minutes. β-Actin was used as a loading control. Total cellular proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and detected using specific antibodies against phospho-inhibitory kappa B-α, inhibitory kappa B-α, and β-actin. The results are presented as the mean ± standard deviation from 3 independent experiments. *P < .001 vs control group. **P < .001 vs lipopolysaccharide alone. The results shown are representative of 3 independent experiments.
mediate overproduction of inflammatory mediators such as TNF-α, IL-1β, and IL-6 through NF-κB activation. Therefore, the phosphorylation of ERK, JNK, and p38 reflects the progression of inflammation. In the present study, to investigate whether DCS regulates the phosphorylation of MAPK, we examined the expression of phosphorylated MAPKs by Western blot analysis at various concentrations (2, 4, and 6 mM) of DCS in LPS-induced RAW264.7 macrophages. As shown in Figure 7, the LPS treatment group showed a marked increase in the phosphorylated ERK, JNK, and p38 compared to the LPS nontreated group. D-(+)-Cycloserine downregulated the phosphorylation of ERK, but not of JNK and p38 in the LPS-induced RAW264.7 cells. Thus, these data implicate the therapeutic potential of DCS via the ERK pathway in inflammation.

To further analyze the effect of DCS on the viability of HaCaT human keratinocytes, we evaluated the cytotoxicity of DCS using MTT assay. The cells were incubated in 24-well plates (5.0 × 10^4 cells/well) for 24 hours, and then treated with various concentrations of DCS (2, 4, 6, 8, and 10 mM) for an additional 24 hours. As shown in Figure 8, treatment with DCS at concentrations below 6 mM showed no cytotoxicity (% of cell survival ≥85%). However, cytotoxicity of DCS was detected at concentrations above 8 mM (31.7% and 11.9% at 8 and 10 mM DCS, respectively) in the HaCaT keratinocytes.

In summary, our findings demonstrate that DCS treatment inhibits the production of NO, PGE2, IL-1β, and IL-6 and the expression of iNOS and COX-2 in LPS-stimulated RAW264.7 cells. Furthermore, DCS suppresses NF-κB activation by blocking degradation of IκB-α through the inhibition of ERK and Akt phosphorylation. Based on these anti-inflammatory functions of DCS, we suggest that DCS could be considered for therapeutic uses in inflammatory diseases.

### Experimental

#### Chemicals and Reagents

D-(+)-Cycloserine, LPS from Escherichia coli, fetal bovine serum (FBS), MTT, dimethyl sulfoxide (DMSO), Griess reagent, sodium nitrite, and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, United States). Dulbecco’s Modified Eagle’s Medium (DMEM) and penicillin/streptomycin

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**Figure 6.** The effect of D-(+)-cycloserine on the level of phospho-Akt and Akt in lipopolysaccharide-induced RAW 264.7 cells. Lysates were prepared from cells pretreated with D-(+)-cycloserine (2, 4, and 6 mM) for 1 hour and then treated with lipopolysaccharide (1 µg/mL) for 10 minutes. β-Actin was used as a loading control. Total cellular proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and detected using specific antibodies against phospho-Akt, Akt, and β-actin. The results are presented as the mean ± standard deviation from 3 independent experiments. *P < .001 vs control group. ***P < .001 vs lipopolysaccharide alone. The results shown are representative of 3 independent experiments.
were obtained from Thermo Fisher Scientific (Waltham, MA, United States). The enhanced chemiluminescence (ECL) kit, radioimmunoprecipitation assay (RIPA) buffer, phosphate buffered saline (PBS), and tris-buffered saline (TBS) were purchased from Biosesang (Seongnam, Gyeonggi-do, Korea). L-N6-(1-iminoethyl) lysine dihydrochloride and N-[2-(cyclohexyloxy)-4-nitrophenoxy] methanesulfonamide (NS-398) were purchased from Cayman chemical company (Ann Arbor, MI, United States). The specific primary antibodies for p-ERK, ERK, p-JNK, JNK, p-p38, p38, p-Akt, Akt, p-iKB-α, iKB-α, β-actin, and the secondary antirabbit and antimouse antibodies were purchased from Cell signaling technology (Beverly, MA, United States). The anti-α-iNOS antibody was purchased from Millipore (Temecula, CA, United States). The anti-COX-2 antibody was purchased from BD Sciences (San Diego, CA, United States). The ELISA kits for PGE2, TNF-α, IL-1β, and IL-6 were purchased from R&D systems Inc. (St. Louis, MO, United States) and BD Biosciences (San Diego, CA, United States). All the other reagents used were of analytical grade.

Cell Culture and Sample Treatment

RAW264.7 macrophage cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were subcultured at 37°C using DMEM including 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified
atmosphere of 5% CO₂. The cells were subcultured every 2 days. For the DCS treatment, DCS was dissolved in distilled water and diluted with culture medium. The cells were pretreated at various concentrations (2, 4, and 6 mM) of DCS for 1 hour and then stimulated with LPS 1 µg/mL for the indicated durations of time.

**Measurement of Cell Viability**

Cell viability was determined using the MTT assay. Cells were plated at 1.0 × 10⁵ cells/well in 24-well plates in DMEM with 10% FBS and 1% penicillin/streptomycin. The cells were pretreated with various concentrations of DCS (2, 4, and 6 mM) for 1 hour and then treated with 1 µg/mL LPS for 24 hours. A total of 100 µL of MTT solution (2 mg/mL) was added after incubation and the cells were further incubated for 4 hours. The formazan crystals in each well were dissolved in 1 mL of DMSO for 30 minutes on a microplate shaker. Optical density (OD) was measured by using a Microplate reader at 540 nm. Cell viability in the wells with control medium in the absence of any treatment was set at 100%.

**NO Production**

The accumulated nitrite in the culture media, an indicator of NO production, was determined using the Griess reagent. Cells were plated at 1.0 × 10⁵ cells/well in 24-well plates in DMEM with 10% FBS and 1% penicillin/streptomycin. The cells were pretreated with various concentrations of DCS (2, 4, and 6 mM) for 1 hour and then treated with 1 µg/mL LPS for 24 hours. After incubation, 100 µL of the culture supernatant was mixed with 100 µL of Griess reagent for 10 minutes. The absorbance of the mixture was measured at 540 nm with a microplate reader. The nitrite concentration in the supernatants was calculated from a standard curve of nitrite solution.

**Measurement of Cytokines**

RAW264.7 cells were plated at 1.0 × 10⁵ cells/well in 24-well plates in DMEM with 10% FBS and 1% penicillin/streptomycin. The cells were pretreated with various concentration of DCS (2, 4, and 6 mM) for 1 hour and then treated with 1 µg/mL LPS for 24 hours. The supernatants were collected and the level of TNF-α, IL-1β, IL-6, and PGE₂ was determined using ELISA kits following the manufacturer’s protocols.

**Western Blot Analysis**

RAW264.7 cells were plated in 60-mm cell culture dishes (6.0 × 10⁵ cells/dish) for 40 hours. The cells were then pretreated with various concentrations of DCS (2, 4, and 6 mM) for 1 hour and then treated with 1 µg/mL LPS for the indicated durations of time. After treatment, the cells were washed with PBS. The cells were lysed on ice with RIPA buffer (50 mM Tris-HCl [pH 7.5], 2 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, and 1% protease inhibitor cocktail) for 20 minutes. The harvested cell lysates were centrifuged at 15,000 rpm for 15 minutes. The protein concentrations in the supernatant were determined using a BCA protein assay kit. Equal amounts of total protein (30 µg) were separated by SDS-polyacrylamide gel electrophoresis on a 8% or 10% gel and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were then blocked with 5% bovine serum albumin (BSA) and incubated for 2 hours. The membranes were washed with TBS
buffer containing 0.1% Tween 20 (TBST) and then incubated with specific primary antibodies at 4°C overnight. The membranes were washed with TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibodies in TBST buffer at room temperature for additional 2 hours. After washing the membranes, the proteins were detected using an ECL kit.

**Statistical Analysis**

All data are presented as the mean ± standard deviation from 3 independent experiments. Statistical analysis was conducted using the Student’s t-test. P-value of <.05 was considered to be statistically significant.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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