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Cimicifuga heracleifolia Extract Induces iNOS Expression via a Nuclear Factor-κB-dependent Pathway in Mouse Peritoneal Macrophages

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Abstract – *Cimicifuga heracleifolia* extract (CHE) was investigated for its effects on the release of nitric oxide (NO) and at the level of inducible nitric oxide synthase (iNOS) gene expression in mouse macrophages. We found that *C. heracleifolia* elicited a dose-dependent increase in NO production and the level of iNOS mRNA. Since, iNOS transcription has been shown to be under the control of the transcription factor NF-κB, the effects of CHE on NF-κB activation were examined. Transient expression assays with NF-κB binding sites linked to the luciferase gene revealed that the increased level of iNOS mRNA, induced by CHE, was mediated by the NF-κB transcription factor complex. By using DNA fragments containing the NF-κB binding sequence, CHE was shown to activate the protein/DNA binding of NF-κB to its cognate site, as measured by electrophoretic mobility shift assay. These results demonstrate that *C. heracleifolia* stimulates NO production and is able to up-regulate iNOS expression through NF-κB transactivation.
Keywords – *Cimicifuga heracleifolia,* macrophages, iNOS, NF-κB

Introduction

In immune-competent hosts, both innate and adaptive immune systems are relatively efficient at capturing and killing of microbial pathogens. Nitric oxide (NO) is a radical messenger molecule which is produced by the enzyme NO synthase (NOS).[1,2] Three NOS isoforms have been characterized: constitutively expressed neuronal NOS, endothelial NOS, and the inducible isoform of NOS (iNOS).[3] iNOS expression is significantly induced by lipopolysaccharide (LPS) or cytokines in a variety of immune cells, including macrophages.[4] Moreover, NO is the major effecter molecule that helps in the destruction of micro organisms and tumor cells by activated macrophages during the nonspecific host defense of the immune system.[5,6] NO has also been implicated as a mediator of tissue injury. NO also inhibits the proliferation of viruses, such as ectromelia virus, coxsackie virus B3 and hepatitis B virus and thus act as a host defense molecule.[7] Expression of genes encoding iNOS is coordinated by nuclear factor kB (NF-κB) in cooperation with other transcription factors in macrophages. Moreover, NF-κB plays a vital role in the activation of different immune cells by upregulating the expression of many cytokines which are essential for immune response.[8,9]

The rhizoma of *Cimicifuga* species, including *C. heracleifolia* and *C. dahurica* Max. has wide applications and has been used as an antidote, demulcent and as a folk medicine for generations in Asia and Europe. In addition, it has been as a flavoring and sweetening agent in many food products.[10-14] *C. heracleifolia* has also been used as a cosmetic component to treat hyperpigmentation and improve the appearance of wrinkles.[15] However, despite the many reports, the inhibitory activity of *C. heracleifolia* extract

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(CHE) on the macrophage activation signaling pathway is not completely understood.

Since, inducible production of NO by macrophages inhibits the growth of many pathogens, including bacteria, fungi, viruses, and parasites.[16] Thus, we hypothesized that CHE-derived NO production may mediates the anti-viral and anti-tumor activities. To test this hypothesis, we investigated the effects of CHE on NO production, and studied the molecular mechanisms underlying this effect.

Experimental

Sample Preparation of CHE – A standardized *C. heracleifolia* was supplied by Hansol Pharm. (Gwangju, Korea) in 2006. The dried rhizomes (3 kg) were exhaustively extracted three times with 80% methanol (6 L) at room temp. The resultant methanolic extract was concentrated at the reduced pressure to yield the CHE (330 g).

Chemicals – Chemicals and cell culture materials were obtained from the following sources: lipopolysaccharide (LPS) and Polymyxin B sulfate (Sigma Co., USA); MTT-based colorimetric assay kit (Roche Co., USA); LipofectAMINE Plus, RPMI 1640, fetal bovine serum, and penicillin-streptomycin solution (Life Technology, Inc., USA); pGL3-4κB-Luc and the luciferase assay system (Promega, USA); pCMV-β-gal (Clontech, USA); and AmpliTaq^R DNA polymerase (Perkin Elmer, USA), other chemicals were of the highest commercial grade available.

Animals – BALB/C mice (male, 8 weeks old) were obtained from Orient (South Korea). Animals were housed under normal laboratory conditions, i.e., at 21 - 24 ºC and 40 - 60% relative humidity under a 12 hr light/dark cycle with free access to standard rodent food and water.

Preparation of peritoneal macrophages and cell cultures – Peritoneal macrophages were isolated from mice and cultured as described previously.[17] RAW 264.7 cells, mouse macrophage cell line, were obtained from the American Type Culture Collection (Bethesda, MD), and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ºC in a 5% CO_2 humidified incubator. CHE was dissolved in dimethylsulfoxide and added directly to the culture media. Control cells were treated only with the vehicle; the final concentration of which never exceeded 0.1%, and this concentration did not show any effect on the assay systems.

Cell viability – Cell viability was assessed using a MTT-based colorimetric assay kit (Roche Co.), according to the manufacturer's instructions.

Nitrite assay – Peritoneal macrophages (2×10^5 cells/mL) or RAW 264.7 cells (5×10^5 cells/mL) were cultured in 48-well plates. After incubating for 24 hr, NO synthesis was determined by assaying the culture supernatants for nitrite, the stable reaction product of NO and molecular oxygen, using Griess reagent as described previously.[18]

Endotoxin Assay – An E-Toxate test (Limulus Amebocyte Lysate; Sigma Chemical Co.) was used to assay CR for the presence of gram-negative bacterial endotoxin (LPS), according to the manufacturer's instructions. Assay was performed in 10 × 75 mm glass culture tubes. Sample, water, and Endotoxin Standard Dilutions were added directly to the bottom of tubes along with E-TOXATE Reagent Working Solution. Tubes were incubated for 1 hour undisturbed at 37 ºC. After 1 hour incubation, tubes were gently removed one at a time and slowly invert 180º while observing for evidence of gelation.

RNA preparation and iNOS mRNA analysis by RT-PCR – RAW 264.7 cells were cultured with CHE at a density of 1×10^6 cells/mL for 6 hr. Total cellular RNA was isolated and cDNA synthesis, semiquantitative RT-PCR for iNOS and β-actin mRNA, and the analysis of results were performed as described previously.[16] DNA was synthesized from 2 μg of total RNA using an Omniscript RT-PCR kit as instructed. A cycle number was used that fell within the exponential range of response for iNOS (754 bp, 35 cycles) and β-actin (153 bp, 17 cycles). PCR reactions were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. Gel images were captured on a Gel Doc Image Analysis System (Kodak) and the yield of PCR products was normalized to β-actin after quantitative estimation using NIH Image software (Bethesda, MD). The relative expression levels were arbitrarily set at 1.0 in the control group.

Transfection and luciferase and β-galactosidase assays – RAW 264.7 cells (5×10^5 cells/mL) were plated in each well of a 12-well plate, and 12 hr later transiently co-transfected with the plasmids pGL3-4κB-Luc and pCMV-β-gal, using LipofectAMINE Plus according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.5 μg of pGL3-4κB-Luc and 0.2 μg of pCMV-β-gal was mixed with the LipofectAMINE Plus reagent and added to cells. After 18 hr, the cells were treated with CHE or LPS for 12 hr, and then lysed. Luciferase and β-galactosidase activities were determined as described previously.[19] Luciferase activity was normalized using β-galactosidase activity and was expressed relative to the activity of the control.

Electrophoretic mobility shift assay – Nuclear extracts

were prepared as previously described.[18] Two double-stranded deoxyoligonucleotides containing the NF-κB binding site (5'-GGGCRCTTTCC-3') were end-labeled with [γ-^{32}P]dATP. Nuclear extracts (5 μg) were incubated with 2 μg of poly (dI-dC) and the ^{32}P-labeled DNA probe in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml concentration each of aprotinin and leupeptin) for 10 min on ice. DNA binding was separated from the free probe using a 4.8% polyacrylamide gel in 0.5x TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA). Following electrophoresis, the gel was dried and subjected to autoradiography.

Statistical Analysis – All experiments were repeated at least three times. The Student's t-test was used to assess the statistical significance of differences. A confidence level of $p < 0.05$ was considered significant.

Results and Discussion

The inducible production of NO by macrophages appears to be important in the elimination of viruses and tumors.[9,20] Our results demonstrate that CHE stimulates NO production and is able to upregulate iNOS expression through NF-κB transactivation. After incubating macrophage with CHE for 24 hr CHE-induced NO production was assessed using the Griess reaction. The basal level of NO in untreated peritoneal macrophages was found to be less than 2 μg/mL (Fig. 1). However, upon CHE stimulation, NO release by peritoneal macrophages increased in a dose-dependent manner over the range of 1-20 μg/mL (Fig. 1). Moreover, this release had a cytotoxic action upon macrophages at concentrations exceeding 50 μg/mL. The potent macrophage activator LPS (0.5 μg/mL), when used as immunostimulator, also increased NO production 22 times compared to the control. Consistent with these findings, CHE also induced NO generation in a dose-dependent manner in RAW 264.7 cells. NO is involved in the killing and proliferative inhibition of virus and tumor by activated macrophages, and is a component of the nonspecific host defense.[21,22] The biological significance of the different effects of CHE on NO production in the resting and stimulated state needs to be determined. In 2012, Phil-Ok Koh demonstrated that a compound named ferulic acid isolated from CHE, mediates the expression levels of NOS isoforms in focal cerebral ischemia.[23] Also caffeic acid derivatives and cyclotartane triterpenes from rhizomes of CHE showed anti-tumor effects.[24,25] It is well known that roots of CHE exhibits analgesic, antibacterial, antiviral, febrifuge and

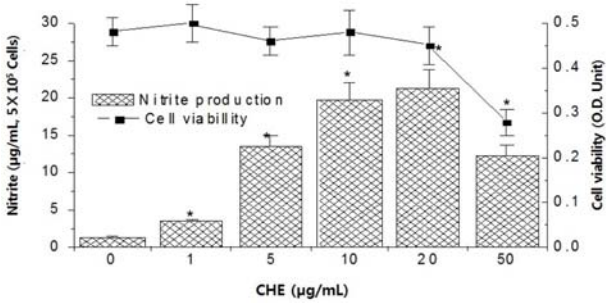

Fig. 1. Effects of CHE on NO production. Murine peritoneal macrophages (2×10^5 cells/mL) were cultured for 24 h in the presence of media alone, with the indicated concentrations of CHE. NO production was determined by measuring the accumulation of nitrite in the culture medium. Cell viability was assessed by MTT assay. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$, significantly different from the control.

Table 1. Effects of polymyxin B on NO secretion by CHE and LPS

[a]RAW 264.7 cells (5×10^5 cells/mL) cultured with CHE (10 μM) or LPS (0.5 μg/mL), in the presence or absence of polymyxin B (10 μg/mL). [b]Supernatants were harvested after being cultured for 24 h and assayed for NO. Values are the means ± SD of three individual experiments, performed in triplicate. *$P < 0.01$, significantly different from the LPS. **$P < 0.01$, significantly different from the CHE.

Treatment[a]	Nitrite (μg/mL)[b]
Control	1.98 ± 0.23*,**
CHE	19.62 ± 2.23*
CHE + polymyxin B	20.24 ± 2.27*
LPS	42.38 ± 6.31**
LPS + polymyxin B	8.43 ± 0.93*,**

sedative activites.[26] Macrophages can be induced to produce NO by LPS, endotoxins, or cytokines.[8] To confirm, that the observed ability of CHE to induce NO was not due to LPS contamination, the CHE was tested for the presence of contaminating LPS by using the *Limulus* amoebocyte lysate test. The level of LPS in CHE was found to be below the detection limit, which is typically below 12.5 pg/mL (data not shown). Polymyxin B sulfate has been used previously as a LPS inhibitor in macrophage cultures, and although CHE described no detectable activity in the *Limulus* amoebocyte lysate assay, we rechecked for possible LPS contamination in CHE, by adding polymyxin B (10 μg/mL) to cell cultures treated with CHE (10 μg/mL). As shown in Table 1, polymyxin B effectively inhibited the NO production induced by LPS, but had no effect on the CHE, which demonstrated that the production of NO by CHE was unlikely to have been resulted from LPS contamination of CHE.

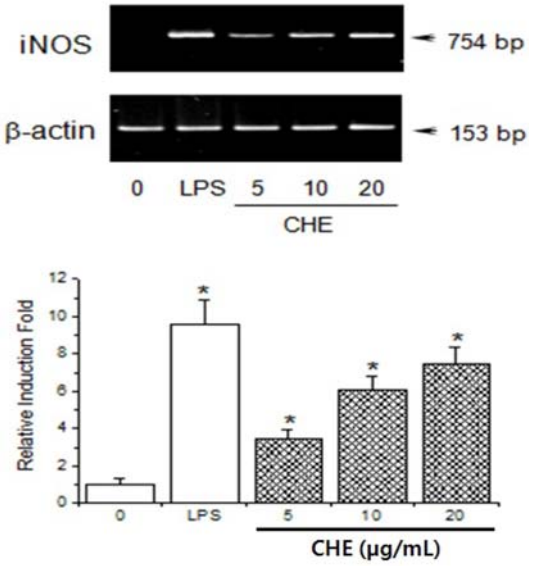

Fig. 2. Effects of CHE on iNOS mRNA expression. RAW 264.7 cells (1×10^6 cells/mL) were cultured for 6 hr in the presence of media alone, with the indicated concentrations of CHE, or with LPS (0.5 μg/mL). Cells were lysed and total RNA was prepared for the RT-PCR analysis of gene expression. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide. One of three representative experiments is shown. The ratio of the RT-PCR products of iNOS to β-actin was calculated. Induction-fold represents the mean ± SD of three separate experiments. $*P < 0.01$, significantly different from the control.

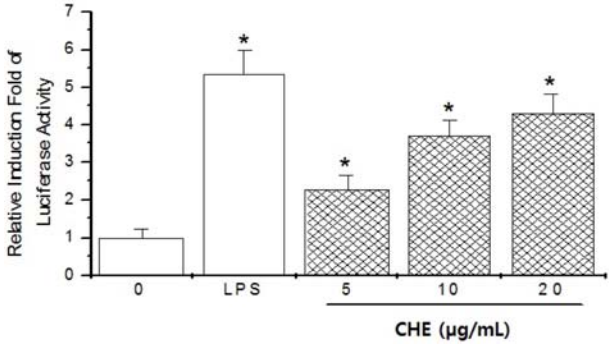

Fig. 3. Effects of CHE on NF-κB-dependent luciferase gene expression. RAW 264.7 cells (5×10^5 cells/mL) were transiently co-transfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 hr, cells were treated with the indicated concentrations of CHE or LPS (0.5 μg/mL) for 12 hr. Cells were then harvested, and their luciferase and β-galactosidase activities determined. Luciferase activities are expressed relative to the control. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. $*P < 0.01$, significantly different from the control.

As stated above, CHE induced No secretion from macrophage. In order to determine whether CHE regulates NO production at the mRNA level, a reverse transcription-polymerase chain reaction (RT-PCR) assay was undertaken. LPS was used as a positive control. Consistent with the results obtained from the NO assay, iNOS mRNA levels were markedly increased by CHE treatment (Fig. 2). This result indicates that CHE up-regulates NO accumulation in macrophages in a dose dependent manner. Therefore, we believe that increased NO production by CHE is regulated through transcriptional activation.

Activated macrophages have the capacity to produce relatively large quantities of NO and NO-derived species, such as $NO_2^{\bullet}$, NO_2^-, N_2O_3, N_2O_4, *S*-nitrosothiols, and peroxynitrite ($ONOO^-$). Moreover, DNA and proteins are targets of reactive nitrogen intermediates. In addition, nitrogen intermediates and reactive oxygen intermediates can synergistically interact through the formation of peroxynitrites.[2,6] Since cysteine proteases are critical components of the virulence or replicating ability of many viruses, bacteria, and parasites, and *S*-nitrosylation of pathogen cysteine proteases may be a general mechanism of the antimicrobial host defenses. NO has also been reported to interfere with specific stages in the life cycles of viruses. Since, NO inhibits a variety of viruses, it is possible that NO also inhibits the cellular processes necessary for viral replication.

NF-κB is a member of the Rel family, and is a common regulatory element in the promoter region of many cytokines. In activated macrophages, NF-κB, in synergy with other transcriptional activators, plays a central role in coordinating the expression of genes encoding iNOS, TNF-α, and IL-1.[8] To further investigate the role of CHE on iNOS gene expression, we assessed the effect of CHE on NF-κB-dependent gene expression by using the luciferase reporter gene assay. RAW 264.7 cells were transiently transfected with a plasmid containing 4 copies of the NF-κB binding sites, and the luciferase activities were measured. LPS, an immunostimulatory agent, was used as a positive control. When cells were stimulated with LPS a near 6-fold increase in luciferase activity was observed versus the unstimulated control cells. Consistent with NO production and iNOS mRNA measurement, CHE also significantly increased NF-κB-dependent luciferase activities in a dose dependent manner (Fig. 3). To determine the putative mechanism by which CHE activates iNOS, we monitored the effects of CHE on the activation of a family of transcription factors by electrophoretic mobility gel shift assay. NF-κB binding activity was determined because of its critical role in the regulation of iNOS and TNF-α. These results demonstrated that CHE induced a marked increase in NF-κB binding at its conserved site, which was visualized as a distinct band

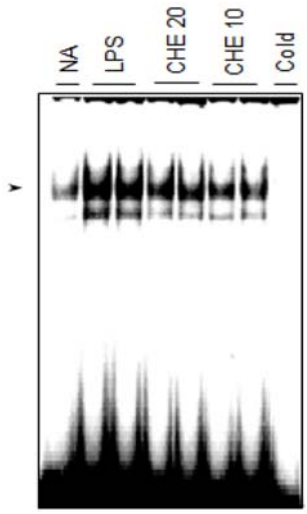

Fig. 4. Effects of CHE on NF-κB binding. RAW 264.7 cells were treated with LPS (0.5 μg/mL) or CHE (10, 20 μg/mL) for 1 hr. Nuclear extracts were isolated and used in an electrophoretic mobility shift assay with ^{32}P-labeled NF-κB oligonucleotide as a probe, as described in Experimental. The arrow indicates the NF-κB binding complex. Cold; 200-fold molar excess of non-labeled NF-κB probe. One of three representative experiments is shown.

(Fig. 4). Although we demonstrated the up-regulatory effect of CHE on iNOS gene expression through NF-κB transactivation in macrophages, the mechanism by which CHE stimulated this iNOS expression is not clear. Further studies on CHE are needed to prove its immunochemotherapeutic usefulness and its exact mechanism.

In summary, our results show for the first time that, CHE stimulates macrophage-derived NO production, and is able to up-regulate iNOS expression through NF-κB transactivation in murine macrophages. These actions may provide a mechanistic basis for the anti-viral and anti-tumor properties of CHE.

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