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Inhibitory effect of 2′-hydroxycinnamaldehyde on nitric oxide production through inhibition of NF-κB activation in RAW 264.7 cells

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Abstract

Cinnamomum cassia has been widely used for treating dyspepsia, gastritis, and inflammatory disease. In the present study, several of cinnamaldehyde derivatives were synthesized from various cinnamic acid based on the 2′-hydroxycinnamaldehyde isolated from the bark *C. cassia* Blume was investigated to compare their NO production and NF-κB activity from Raw 264.7 cell since nitric oxide (NO) and NF-κB have been shown to be implicated factors in the inflammatory disease. The results show that HCA, among the derivatives, most significantly inhibited lipopolysaccharide (LPS)-induced NO production and NF-κB transcriptional activity in a dose-dependent manner with an IC_{50} value of 8 and 22 μM, respectively. We next investigated putative possible mechanisms of inhibitory effect of HCA on NO production. The inhibition of NO by HCA was consistent with the inhibitory effect on LPS-induced inducible nitric oxide synthase (iNOS) expression. Moreover, HCA inhibited LPS-induced p50 and p65 translocation resulting in the inhibition of the DNA binding activity of the NF-κB, a central regulator of iNOS. The present results provided evidence that HCA, among cinnamaledhyde derivatives, has the most inhibitory effect on NO production through inhibition of NF-κB activation, and thus can be used as an anti-inflammatory agent.
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Keywords: 2′-Hydroxycinnamaldehyde; Inducible nitric oxide synthase; Nitric oxide; Nuclear transcription factor-κB; Cyclooxygenase 2; Lipopolysaccharide

1. Introduction

Cinnamomum cassia has been widely used for treating dyspepsia, gastritis, and inflammatory diseases. We previously isolated a cinnamaldehyde derivative, 2′-hydroxycinnamaldehyde (HCA), from the stem bark of *C. cassia*, and found it has an inhibitory effect on farnesyl transferase activity in vitro [1]. In addition, we also found that 2′-hydroxycinnamaldehyde has several other activities such as anti-angiogenic activity [2], immunomodulating activity [3], and also has to induce apoptotic cell death and growth inhibition of several human cancer cell lines including breast, leukemia, ovarian, lung, and colon tumor cells [4]. HCA exerts several biological effects as much as those of cinnamalehyde which in known to have anti-inflammatory effect. However, little is known about anti-inflammatory activities and possible mechanisms.

Exposure to outer bacterial toxins such as lipopolysaccharide (LPS) stimulates cellular inflammatory responses, and releases several inflammatory mediators including nitric oxide (NO), cytokines, tumor necrosis factor-α, prostaglandin E_2 and other eicosanoid mediators to promote inflammatory responses [5]. Nitric oxide synthase (NOS) enzymes are classified into two groups, one type (cNOS) is constitutively present in several cell types (e.g. neurons and endothelial cells) and is regulated predominantly at the posttranscriptional level by calmodulin in a Ca^{2+}-dependent manner [6]. In contrast, the inducible form (iNOS), expressed in various cell types, including vascular smooth muscle cells, macrophages, hepatocytes, and

Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; HCA, 2′-hydroxycinnamaldehyde; LPS, lipopolysacharide; NF-κB, nuclear transcription factor-κB; TNF-α, tumor necrosis factor-α

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astrocytes, is induced in response to pro-inflammatory cytokines and bacterial LPS. Activation of iNOS catalyzes the formation of large amount of NO, which plays a key role in the pathogenesis of a variety of inflammatory diseases [7–11]. Therefore, NO production induced by iNOS may reflect the degree of inflammation and provides an indicator to assess inflammatory process.

Activation of NF-κB by LPS is induced by a cascade of events leading to the activation of IKK, which phosphorylates IκB, leading to its degradation and translocation of NF-κB to the nucleus [12]. NF-κB regulates host inflammatory and immune responses, and cellular growth properties by increasing the expression of specific cellular genes [13]. These include the transcription of various inflammatory cytokines, such as IL-1, IL-2, IL-6, and IL-8 and TNF-α [14], as well as genes encoding cyclooxygenase-2 (COX-2) and iNOS [15]. As a result, inhibition of signal transduction proteins in the pathways leading to activation of NF-κB is now widely recognized as a valid strategy to combat inflammatory disease. In this study, to investigate anti-inflammatory effects of 2′-hydroxycinnamaldehyde, we determined inhibitory effect of HCA, and compared with other derivatives of cinnamaldehyde on the NO production and NF-κB activation in RAW 264.7 cells. Among the derivatives, HCA must effectively inhibited LPS-induced NO production, at the same time, inhibited LPS-induced expression of iNOS, COX-2 and TNF-α in RAW 264.7 cells. Using gel shift assay and NF-κB-luciferase assay, we show that HCA inhibited activation of the transcription factor NF-κB, a central regulator of iNOS and inflammatory response of body. We provide evidence that HCA has inhibitory effect on NO production through inhibition of NF-κB activation. These results suggest that HCA can be used for an anti-inflammatory agent.

2. Materials and methods

2.1. 2′-Hydroxycinnamaldehyde derivatives

Chemical reagents were purchased from Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise mentioned. A series of 2′-hydroxycinnamaldehyde derivatives was synthesized according to the report procedure [2].

2.2. Cell culture

RAW 264.7, a mouse macrophage-like cell line was obtained from the American Type Culture Collection (Cryosite, Lane Cove, NSW, Australia). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, and fetal bovine serum were purchased from Gibco Life Technologies (Rockville, MD, USA). RAW 264.7 cells were grown in DMEM with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2 humidified air.

2.3. Cell viability assay

RAW 264.7 cells were plated at a density of 10^4 cells/well in 96-well plates. To determine the appropriate dose that is not cytotoxic to the cells, the cytotoxic effect was evaluated in the cells cultured for 24, 48 and 72 h using the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide MTT assay. Briefly, MTT was dissolved in without phenol red at a concentration of 10 μg/ml; 10 μl of this solution was then added to cell cultured for designed time. After 4 h, cultures were removed from the incubator and the formazan crystals dissolved by adding 100 μl solubilization solution (0.04N HCl in isopropanol). Metabolic activity was quantified by measuring light absorbance at 570 nm.

2.4. Nitrite assay

RAW 264.7 cells were plated at 2×10^5 cells/well in 96-well plate and then incubated with or without LPS (1 μg/ml) in the absence or presence of various concentrations of HCA for 24 h. The nitrite accumulation in the supernatant was assessed by Griess reaction [16]. Each 50 μl of culture supernatant was mixed with an equal volume of Griess reagent [0.1% *N*-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phophoric acid] and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in an automated microplate reader, and a series of known concentrations of sodium nitrite was used as a standard.

2.5. Western blot analysis

About 1×10^6 cells were harvested and homogenized with lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM PMFS, 10 μl/ml aprotinin, 1% igapel 630 (Sigma Chemical Co., St. Louis, MO, USA), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate]. The extracts were centrifuged at 23,000 × *g* for 1 h. Equal amount of proteins (20 μg) were separated on a SDS/12% polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% Tween-20. The membrane was incubated for 5 h at room temperature with specific antibodies: rabbit polyclonal antibodies against COX-2 (1:500) and goat polyclonal for TNF-α (1:500), and mouse monoclonal iNOS antibody (1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the ECL Western blotting detection system. The relative density of the protein bands was scanned by densitometry

using MyImage (SLB, Seoul, South Korea), and quantified by Labworks 4.0 software (UVP Inc., Upland, CA, USA).

2.6. Gel electromobility shift assay

Gel shift assays were performed according to the manufacturer's recommendations (Promega, Madison, WI, USA). Briefly, 1×10^6 cells/ml was washed twice with 1× PBS, followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were spun down at 15,000 × *g* for 1 min, and the resulting supernatant was removed. Solution A (50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, and 0.5% Nonidet P-40) was added to the pellet in a 2:1 ratio (v/v), and allowed to incubate on ice for 10 min. Solution C (solution A + 10% glycerol and 400 mM KCl) was added to the pellet in a 2:1 ratio (v/v) and vortexed on ice for 20 min. The cells were centrifuged at 15,000 × *g* for 7 min, and the resulting nuclear extract supernatant was collected in a chilled Eppendorf tube. Consensus oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP for 10 min at 37 °C. Gel shift reactions were assembled and allowed to incubate at room temperature for 10 min followed by the addition of 1 μl (50,000–200,000 cpm) of ^{32}P-labeled oligonucleotide and another 20 min of incubation at room temperature. Subsequently, 1 μl of gel loading buffer was added to each reaction and loaded onto a 4% nondenaturing gel and electrophoresis until the dye was three-fourth of the way down the gel. The gel was dried at 80 °C for 1 h and exposed to film overnight at 70 °C. The relative density of the protein bands was scanned by densitometry using MyImage, and quantified by Labworks 4.0 software (UVP Inc.).

2.7. Transfection and assay of luciferase activity

RAW 264.7 cells were plated at a density of 1×10^5 cells per 24-well plate. After 24 h of growth to 90% confluence, the cell were transfected with pNF-κB-Luc plasmid (5× NF-κB; Stratagene, CA, USA) using a mixture of plasmid and lipofectAMINE PLUS in OPTI-MEN according to manufacture's specification (Invitrogen, Carlsbad, CA, USA). Luciferase activity was measured by using the luciferase assay kit (Promega) according to the manufacturer's instructions (WinGlow, Bad Wildbad, Germany).

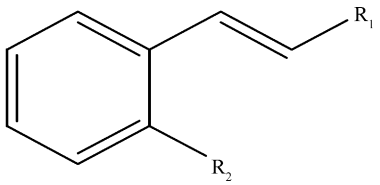
2.8. Data anaylsis

Statistical analysis. Data were analyzed using one-way analysis of variance followed by Tuckey test as a post hoc test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of HCA derivatives on LPS-induced NO production in RAW 264.7 cells

HCA is a derivative of cinnamaldehyde, however, HCA differs in structure that the H group in 2′ site of cinnamaldehyde displaces with hydroxy group. The chemical structures of 2′-hydroxycinnamaldehyde [3-(2′-hydroxy-



Compound	R_1group	R_2group	Name
1	COH	2'-OH	2'-hydroxycinnamaldehyde (HCA)
2	$COOCH_3$	2'-OH	3-(2'-methylcarboxyphenyl)-2-propenal
3	CH_2OH	2'-OH	3-(2'-hydroxyphenyl)-2-propenol
4	COH	2'-OCH_3	3-(2'-methoxyphenyl)-2-propenol
5	COH	2'-$OC(O)CH_3$	3-(2'-O-Acetylphenyl)-2-propenol
6	COH	2'-OCH_2Ph	3-(2'-O-Benzylphenyl)-2-propenol
7	COH	2'-OCH_2Ph-4-OCH_3	3-[2'-O-(4-Methoxybenzyl)-phenyl]-2-propenol
8	COH	2'-OCH_2Ph-4-CH_3	3-[2'-O-(4-Methylbenzyl)-phenyl]-2-propenol

Fig. 1. Chemical structures of HCA (2′-hydroxycinnamaldehyde) derivatives.

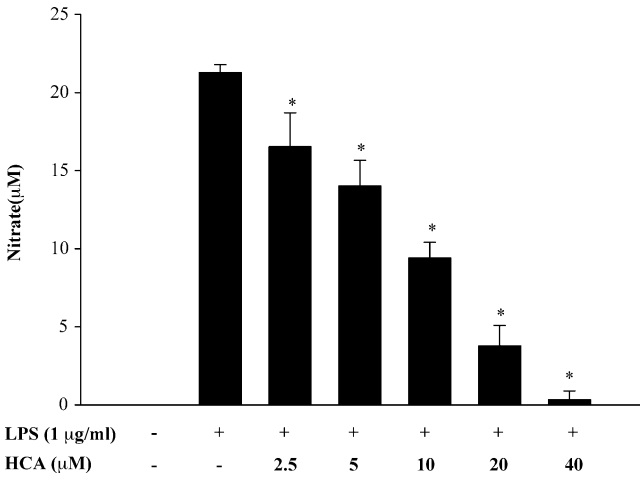


Fig. 2. Effect of HCA on LPS-induced nitrite production in RAW 264.7 macrophages. The cells were treated with 1 μg/ml of LPS only (LPS) or LPS plus different concentrations (5, 10, 20, 40 μM) of HCA at 37 °C for 24 h. At the end of incubation, 50 μl of the medium was removed to measure nitrite production. Control values were obtained in the absence of LPS. The effect of HCA on LPS-induced NO production in RAW 264.7 macrophages were investigated by measuring the accumulated nitrite, as estimated by the Griess reaction, in the culture medium. Unstimulated macrophages, after 24 h of incubation in culture medium, produced background levels of nitrite. After treatment with LPS (1 μg/ml) for 24 h, nitrite concentrations in the medium increased remarkably by about 20-fold (~25 μM). All values represent mean ± S.E.M. of three independent experiments performed in triplicate. $^{*}P < 0.05$ and indicate statistically significant differences from the LPS.

phenyl)-2-propenal](HCA) derivatives are shown in Fig. 1. The effect of 2′-hydroxycinnamaldehyde derivatives on LPS-induced NO production in RAW 264.7 cells was investigated by measuring the accumulated nitrite, as estimated by Griess reaction, in the culture medium. After co-treatment with LPS and 2′-hydroxycinnamaldehyde derivatives for 24 h, LPS-induced nitrite concentrations in the medium was decreased remarkably in a concentration-dependent manner. The IC_{50} value of HCA in inhibiting LPS-induced NO production was 8 μM (Fig. 2). Cytotoxic effect of HCA was evaluated in the absence or presence of LPS by MTT assay. HCA, even at the highest concentration (80 μM), did not affect the cell viability in RAW 264.7 cells (data not shown). Therefore, HCA inhibited LPS-induced NO production in RAW 264.7 cells without any toxic effect. To determine the key functional groups of HCA, a series of 2′-hydroxycinnamaldehyde derivatives was examined a structure-activity relationship in the inhibition of LPS-induced NO production. Cinnamic acids (compounds **2** and **3**), corresponding methoxy and alcohols showed no significant effect (Table 1). Compound **4** containing methoxy group inhibited the LPS-induced production of nitrate but compound **5** containing acyl group showed no significant effect. Compound **6** containing benzyl group improved the inhibitory activity, however, the only slightly improved activity of HCA was found. Changing the alkyl substituent R_2 from methyl to polar group such as methoxy was examined with the hope of improving activity in comparison to the parent compound **6**. As shown in Table 1, the polar group such as methoxy was not increased. The improving activity did not shown (compounds **7** and **8**). Therefore, these results suggested that the aldehyde group of the side chain seems to play a critical role in inhibitory activity of NO production of compounds and the free hydroxy or benzyl groups are also considered to improve the activity of the cinnamaldehydes.

3.2. Effect of HCA on NF-κB-luciferase activity and NF-κB DNA binding activity

NF-κB controls the expression of enzymes including iNOS and COX-2 whose products contribute to the pathogenesis of the inflammatory process [16]. To investigate whether 2′-hydroxycinnamaldehyde derivatives are able to attenuate LPS-induced NF-κB-mediated promoter activity, we used a luciferase reporter gene expressed under the control five κB *cis*-acting elements. RAW 264.7 cells were transiently transfected with the NF-κB-dependent luciferase reporter construct according to manufacture's specification (Invitrogen), and then cells were treated with LPS (1 μg/ml) or co-treated with LPS and HCA derivatives for 24 h. Luciferase activity was decreased by treatment with HCA derivatives, but cinnamic acid (compound **3**) had no significant effect (Table 1). Most of HCA derivatives containing aldehyde group decreased luciferase activity (Table 1), indicating that inhibition of pro-inflammatory cytokine expression correlates with decreased NF-κB-stimulated promoter activity. Among the HCA derivatives, HCA strongly inhibited LPS-induced NF-κB-mediated promoter activity. Treatment of cells with HCA resulted in dose-dependent suppression of luciferase activity

Table 1
Effect of HCA derivatives on LPS-induced NO production in RAW 264.7 cells

Compound	R_1 group	R_2 group	NO production (IC_{50}, μM)	NF-κB-luciferase (IC_{50}, μM)	Cytotoxicity (IC_{50}, μM)
1	COH	2′-OH	8	22	78
2	$COOCH_3$	2′-OH	>500	>500	>500
3	CH_2OH	2′-OH	>500	80	>500
4	COH	2′-OCH_3	11.7	74	77
5	COH	2′-$OC(O)CH_3$	>500	12.1	50
6	COH	2′-OCH_2Ph	6.7	43.2	49
7	COH	2′-OCH_2Ph-4-OCH_3	16.4	38	42
8	COH	2′-OCH_2Ph-4-CH_3	10.3	43.6	47

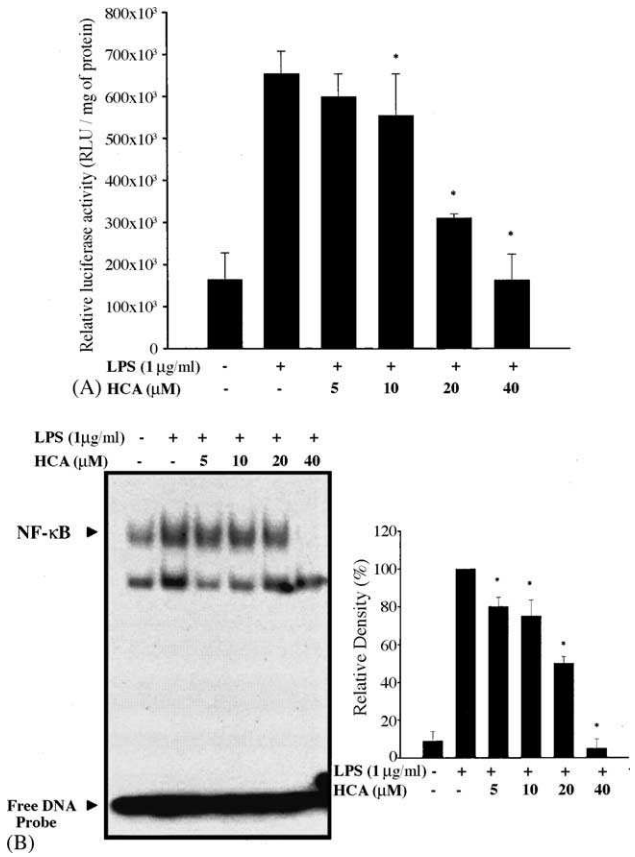


Fig. 3. Effect of HCA on LPS-induced NF-κB-dependent luciferase activity in RAW 264.7 cells (A). RAW 264.7 cells were transfected with pNF-κB-Luc plasmid (5× NF-κB) and then activated with LPS (1 μg/ml), in the LPS only (LPS) or LPS plus different concentrations (5, 10, 20 and 40 μM) of HCA at 37 °C, and then the luciferase activity was determined. Effect of HCA on LPS-induced NF-κB activation in RAW 264.7 cells (B). The activation of NF-κB was investigated using EMSA as described in Section 2. Nuclear extracts from RAW 264.7 cells with LPS alone (1 μg/ml) or with HCA (5, 10, 20 and 40 μM) were incubated in binding reactions of ^{32}P-end-labeled oligonucleotide containing the κB sequence. NF-κB DNA binding activity was determined by EMSA. All values represent mean ± S.E.M. of three independent experiments performed in triplicate. $^{*}P < 0.05$ and indicate statistically significant differences from the LPS.

induced by LPS (Fig. 3A). To investigate whether HCA can also inhibit NF-κB activation, cells were co-treated with LPS and HCA for 60 min which it is the time to activate NF-κB maximally LPS treatment (data not shown). Nuclear extracts from co-treated cells were prepared and assayed NF-κB DNA binding by EMSA. LPS-induced a strong NF-κB binding activity, which was attenuated by co-treatment with HCA in a dose-dependent manner (Fig. 3B).

3.3. Effects of HCA on LPS-induced iNOS, COX-2 and TNF-α expression

To investigate whether inhibitory effect of HCA on NO production via inhibition of corresponding gene expression, expressions of iNOS, COX-2 and TNF-α were determined by Western blot analysis since iNOS can be modulated by COX-2 and TNF-α through their products, PEG_2 and TNF-α. Time course study showed that iNOS expression was induced at early time (1 h) after LPS (1 μg/ml) treatment, but the elevated expression was continued until 48 h (data not shown). Upon LPS treatment for 24 h, iNOS expression was dramatically increased in RAW 264.7 cells, and co-treatment of cells with LPS and different concentration of HCA dose-dependent, inhibited iNOS in RAW 264.7 cells (Fig. 4A). This result is consistent with the profile of the inhibitory effects of HCA on NO production. A similar inhibitory effect of HCA on the LPS-induced COX-2 and TNF-α expression was found (Fig. 4B and C).

3.4. Effect of HCA on LPS-induced NF-κB activation and degradation of IκB

It has been demonstrated that LPS activates NF-κB transcription factor that leading to the induction of the expression of many immediate early genes [17]. To clarify the mechanism of action of HCA for the LPS-induced production of nitrate and TNF-α, inhibitory effect of HCA on LPS-induced activation of NF-κB was examined. Treatment with LPS (1 μg/ml) for 1 h increased nuclear translocation of NF-κB. In the presence of HCA at 5–40 μM, nuclear translocation of p50 and p65 was inhibited on dose-dependent manner (Fig. 5A and B). LPS-induced the transient degradation IκB-α in RAW 264.7 cells. HCA inhibited the LPS-induced degradation of IκB-α at 5–40 μM (Fig. 4C). These results indicate that HCA inhibit the LPS-induced activation of NF-κB, and this effect may result in the inhibition of the LPS-induced production of nitrate, COX-2 and TNF-α.

4. Discussion

The activation of iNOS catalyzes the formation of large amount of NO, which plays a key role in the pathogenesis of a variety of inflammatory diseases [7–11]. Therefore, drugs that inhibit iNOS expressing and/or enzyme activity resulting in decreased NO generation may have beneficial therapeutic effect in the treatment of disease due to overproduction of NO. Activation of NF-κB/Rel is critical in the induction of iNOS and is required to induce the expression of COX-2 in LPS-stimulated RAW 264.7 cells [18–19].

In this study, we showed that HCA derivatives have lowering effect on LPS-induced NO production, and HCA has the most effective inhibitory effect. This inhibitory effect was consistent with its inhibitory effect on the expressing of iNOS, COX-2 and TNF-α through inhibition of NF-κB activation in RAW 264.7 cells. Although, it has been reported that three commercially available cinnamaldehyde derivative compounds, cinnamyl alcohol, cinnamic acid, and eugenol have not or little inhibitory effects of NO

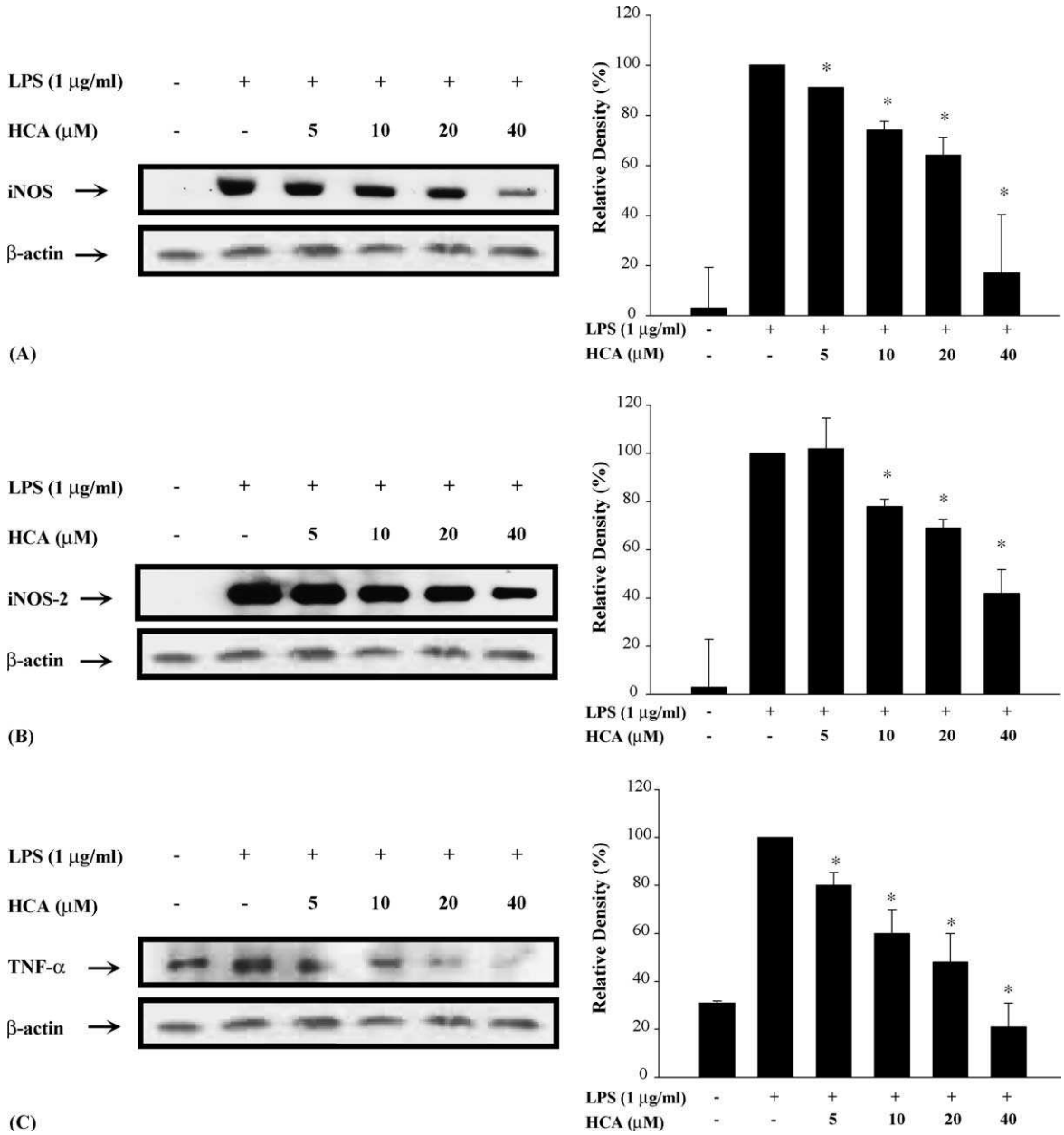


Fig. 4. Effect of HCA on LPS-induced expression of iNOS, COX-2 and TNF-α. The cells were treated with 1 μg/ml of LPS only (LPS) or LPS plus different concentrations (5, 10, 20 and 40 μM) of HCA at 37 °C for 24 h. Equal amounts of total proteins (50 μg/lane) were subjected to 10% SDS-PAGE, and expression of iNOS (A), COX-2 (B), and TNF-α (C) protein were detected by Western blotting using specific antibodies. β-Actin protein was used here as an internal control. Quantification of band intensities from three independent experimental results was determined by a densitometry (Imaging System). Data were described as mean ± S.E.M. from three experiments performed in triplicate for iNOS, COX-2, or TNF-α/β-actin. $^{*}P < 0.05$ indicate statistically significant differences from the LPS-treated group.

production in RAW 264.7 cells [20], the key functional group of the cinnamaldehyde-related compounds in the inhibitory effects of NO production and it's molecular mechanisms are not well known.

We previously isolated a cinnamaldehyde derivative, 2′-hydroxy-cinnamaldehyde (HCA) [1] and, cinnamaldehyde and related compounds were synthesized from various cinnamic acids based on the 2′-hydroxycinnamaldehyde. We examined effect of HCA derivatives on the inhibition of LPS-induced NO production and LPS-induced NF-κB-mediated promoter activity in RAW 264.7 cells. HCA was more potent in roughly three-fold of IC_{50} value than cinnamaldehyde (about 22 μM). HCA derivatives including unsaturated aldehydes inhibited the LPS-induced production of NO production and NF-κB-mediated promoter activity (Table 1). The aldehyde group of the side chain seems to play a critical role in the antitumor activity or biological activity of the compounds. Free hydroxy acyl-substituted groups are also considered to improve the activity of the cinnamaldehydes [2,21]. These results suggested a possibility that the propenal group and 2′-substituted groups of cinnamaldehyde may improve the

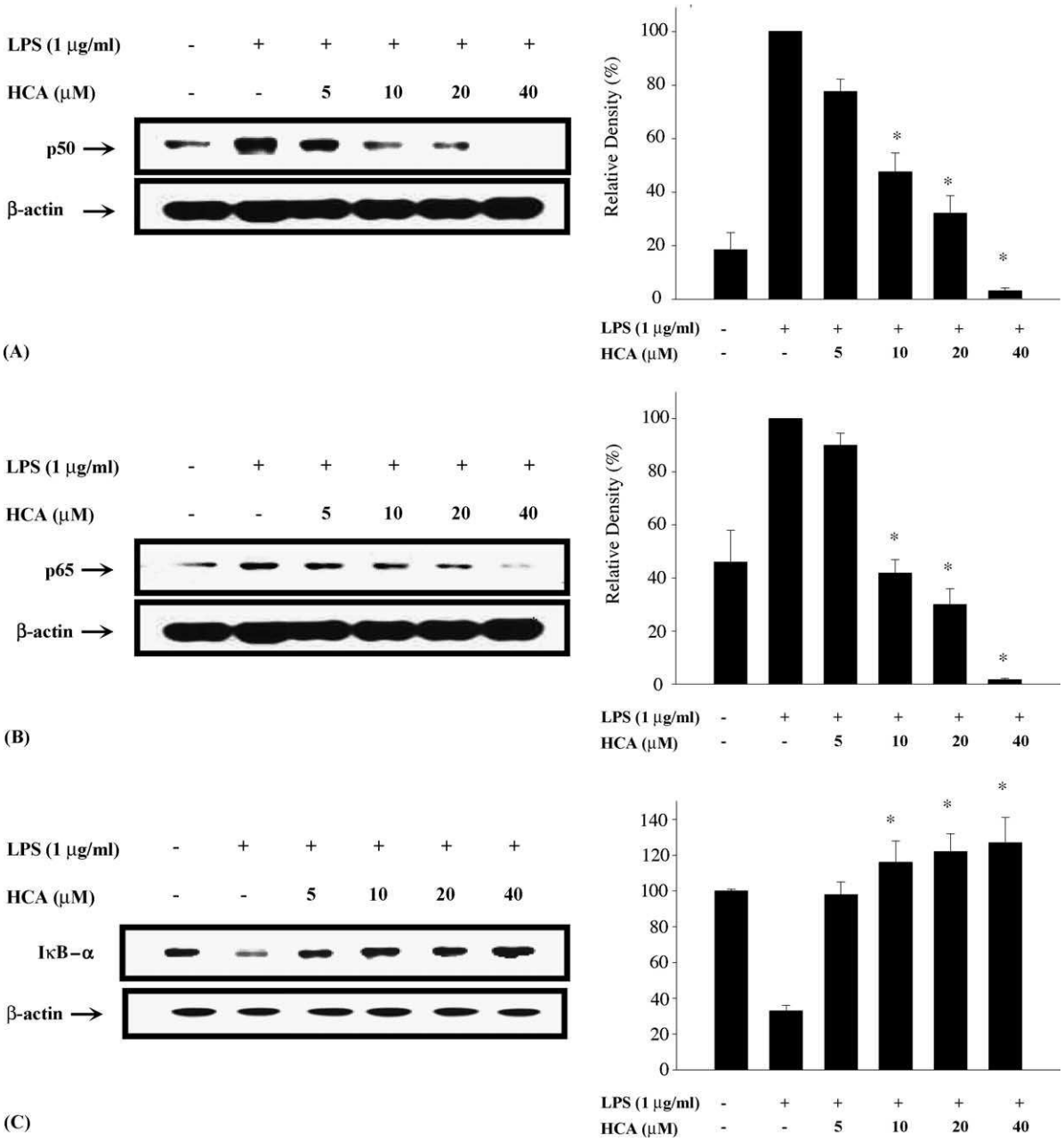


Fig. 5. Effect of HCA on LPS-induced NF-κB activity and degradation of IκB. The cells were treated with 1 μg/ml of LPS only or LPS plus different concentrations (5, 10, 20 and 40 μM) of HCA at 37 °C for 1 h. Equal amounts of total proteins (50 μg/lane) were subjected to 10% SDS-PAGE. Nuclear translocation of p50 (A) and p65 (B), and degradation of IκB (C) were detected by Western blotting using specific antibodies. β-Actin protein was used here as an internal control. Quantification of band intensities from three independent experimental results was determined by a densitometry (Imaging System). Data was described as mean ± S.E.M. from three experiments performed in triplicate for p50, p65, or IκB/β-actin. $^{*}P < 0.05$ indicate statistically significant differences from the LPS-treated group.

inhibitory activity of compound against NO production and NF-κB-mediated promoter activity.

This inhibitory effect of NO production could be related with gene expression of inducible nitric oxide synthase since HCA inhibited iNOS protein in RAW 264.7 cells. HCA also inhibited LPS-induced COX-2 as well as TNF-α expression. These results showed HCA could interfere LPS-induced signaling involving the production of pro-inflammatory molecules. The activation of LPS-induced signaling has been known to increase NO production through NF-κB dependent enhancement of iNOS and COX-2 expression. It is well known that macropharges play a crucial role in both non-specific and acquired immune responses, and marcrophage activation by LPS leads to a functionally diverse series of responses including the production of pro-inflammatory cytokines such as TNF-α [22]. TNF-α exerts its inflammatory effects by activating a diverse spectrum of signaling cascades in the cells [23] that lead to the induction of inducible iNOS and COX-2 [24]. Therefore, the inhibition of COX-2 and

TNF-α expression may be also related to inhibitory effect of HCA on the LPS-induced NO production.

Activation of NF-κB is thought to play a key role in the LPS-induced expression of iNOS, COX-2, and TNF-α. Consistent with inhibitory activity on NO production, HCA decreased NF-κB transcriptional activity. HCA also inhibited NF-κB-specific DNA binding on dose-dependent manner. At the gene level, the expression of murine macrophage: NOS is largely regulated by transcriptional activation. The promoter of the iNOS gene contains two major discrete regions synergistically functioning for binding of transcription factors: one for NF-κB, which is mainly activated by LPS, and the other one for interferon-related transcription factors, such as interferon regulatory factor-1 [17,25–27]. Therefore, inhibition of NF-κB activation could contribute the inhibitory effect of HCA on iNOS, COX-2 and TNF-α.

The way that HCA can interfere NF-κB activation is not clear. However, it is noteworthy that HCA is a α,β-unsaturated carbonyl compounds, which is known to react with nucleophiles, especially with cysteine sulfhydryl groups, in Michael-type addition. Data presented herein show that HCA treatment significantly inhibits NF-κB activation by reducing the degradation of IκB. These results suggest a possibility that HCA inhibited the upstream proteins of IκB such as IKK or 26s proteasome. Interestingly, three other IKKβ inhibitors have been recently shown to target the cysteine residue of IKKβ [28–29]. The α,β-unsaturated carbonyl group of prostaglandin A_1 was shown to be essential for the covalent modification of IKKβ, putatively via interaction with cysteine 179 [29]. Similarly, modification of cysteine 179 of IKKβ has been proposed to mediate the pathological effects of arsenite and parthenolide [30]. The α,β-unsaturated carbonyl group of HCA may be essential for the covalent modification of IKKβ via interaction with cysteine as above compounds.

Peptide aldehydes are well-characterized inhibitors of two other classes of proteolytic enzymes, serine and cysteine proteases. They inhibit these enzymes reacting with the catalytic hydroxyl or thiol groups in the active sites of those enzymes to form a reversible hemi(thio)acetal, which resembles a transition state analogue of the enzymatic reaction [31]. Because HCA have an aldehyde group and seems to play a critical role in several biological activities such as antitumor and anti-angiogenesis, the aldehyde of HCA have a possibility that react with the catalytic hydroxyl or thiol groups in the active sites of those enzymes to form a reversible hemi(thio)acetal as peptide aldehyde.

Several studies also have shown that an α,β-unsaturated carbonyl compounds react directly with cysteine sulfhydryl groups of NF-κB subunit or alters expression of the NF-κB target gene product [33–34]. In NF-κB/p65, cysteine L1 (L1-Cys, position 38) participates in DNA binding by forming a hydrogen bond with the sugar/phosphate backbone of the κB-DNA motif. 4-Hydroxy-2-nonenal (HNE), an α,β-unsaturated carbonyl compound which acts as a specific inhibitor of NF-κB, blocked both the activation of the NF-κB-binding proteins and the production of NO in vascular smooth muscle cells (VSMCs) stimulated by lipopolysaccharide (LPS) [32]. HCA have a possibility that resulted in decrease of DNA binding activation of NF-κB through react directly with cysteine sulfhydryl groups of NF-κB subunit molecule similar to the above mention way.

On the basis of the current results and those of other reports, we propose that HCA prevents binding of the essential transcription factor NF-κB to the iNOS promoter, which in turn leads to decreased transcription of the iNOS gene. Our results show that HCA inhibit the expression of iNOS, COX-2 and TNF-α, and NO production through inhibition of NF-κB DNA binding activity as well as transcriptional activation, and suggest that HCA may be useful as an anti-inflammatory agent.

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References

[1] Kwon BM, Cho YK, Lee SH, Nam JY, Bok SH, Chun SK, et al. 2′-Hydroxycinnamaldehyde from stem bark of *Cinnamomum cassia*. Planta Med 1996;62:183–4.
[2] Kwon BM, Lee SH, Cho YK, Bok SH, So SH, Youn MR, et al. Synthesis and biological activity of cinnamaldehydes as angiogenesis inhibitors. Bioorg Med Chem Lett 1997;7:2473–6.
[3] Koh WS, Yoon SY, Kwon BM, Jeong TC, Nam KS, Han MY. Cinnamaldehyde inhibits lymphocyte proliferation and modulates T cell differentiation. Int J Immunopharmacol 1998;20:643–60.
[4] Lee CW, Hong DH, Han SB, Park SH, Kim HK, Kwon BM, et al. Inhibition of human tumor growth by 2′-hydroxy- and 2′-benzoyloxycinnamaldehydes. Planta Med 1999;65:263–6.
[5] Chen YC, Shen SC, Chen LG, Lee TJF, Yang LL. Wogonin, baicalin and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide. Biochem Pharmacol 2001;61:1417–27.
[6] Liu RH, Hotchkiss JH. Potential genotoxicity of chronically elevated nitric oxide: a review. Mutat Res 1995;339:73–89.
[7] Rockey DC, Chung JJ, McKee CM, Noble PW. Stimulation of inducible nitric oxide synthase in rat liver by hyaluronan fragments. Hepatology 1998;27:86–92.
[8] Nunokawa Y, Ishida N, Tanaka S. Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. Biochem Biophys Res Commun 1993;191:89–94.
[9] Lyons CR, Orloff GJ, Cunningham JM. Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. J Biol Chem 1992;267:6370–4.
[10] Geller DA, Lowenstein CJ, Shapiro RA, Nussler AK, Kisilvio M, Wang SC, et al. Molecular cloning and expression of inducible nitric

oxide synthase from human hepatocytes. Proc Natl Acad Sci USA 1993;90:3491–5.
[11] Galea E, Feinstein DL, Reis DJ. Induction of calciumindependent nitric oxide synthase activity in primary rat glial cultures. Proc Natl Acad Sci USA 1992;89:10945–9.
[12] Griscavage JM, Wilk S, Ignarro LJ. Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-κB. Proc Natl Acad Sci USA 1996;93:3308–12.
[13] Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J Clin Invest 2001;107:135–42.
[14] Haefner B. NF-κB: arresting a major culprit in cancer. Drug Discov Today 2002;7:653–63.
[15] Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. Oncogene 1999;18:6853–66.
[16] Nath J, Powledge A. Modulation of human neutrophil inflammatory responses by nitric oxide: studies in unprimed and LPS-primed cells. J Leukoc Biol 1997;62:805–16.
[17] Baeuerle P. The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochim Biophys Acta 1991;1072:63–80.
[18] Qiao-wen X, Yuki K, Carl N. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J Biol Chem 1994;269:4705–8.
[19] Daniel H, Byeong CJ, Gang Y, Mary B. Expression of mitogeni-inducible cyclooxygenase induced by lipopolysaccharide. Biochem Pharm 1997;54:87–96.
[20] Lee HS, Kim BS, Kim MK. Suppression effect of *Cinnamomum cassia* derived component on nitric oxide synthase. J Agric Food Chem 2002;50:7700–3.
[21] Kwon BM, Lee SH, Choi UC, Park SH, Lee CO, Cho YK, et al. Synthesis and in vitro cytotoxicity of cinnamaldehydes to human solid tumor cells. Arch Pharm Res 1998;21:147–52.
[22] Chen CC, Chiu KT, Sun YT, Chen WC. Role of the cyclic amp-protein kinase a pathway in lipopolysaccharide-induced nitric oxide synthase expression in raw 264.7 macrophages involvement of cyclooxygenase-2. J Bio Chem 1999;274:31559–64.
[23] Nam KW, Je KH, Lee JH, Han HJ, Lee HJ, Kang SK. Inhibition of COX-2 activity and proinflammatory cytokines (TNF-alpha andIL-1beta) production by water-soluble sub-fractionated parts from bee (*Apis mellifera*) venom. Arch Pharm Res 2003;26: 383–8.
[24] Lee AK, Sung SH, Kim YC, Kim SG. Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF-alpha and COX-2 expression by sauchinone effects on IκB α-phosphorylation, C/EBP and AP-1 activation. Br J Pharmacol 2003;139:11–20.
[25] Muller JM, Loms Ziegler-Heitbrock HW, Baeuerle PA. Nuclear factor kappa B, a mediator of lipopolysaccharide effects. Immunobiology 1993;187:233–56.
[26] Baldwin Jr AS. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649–81.
[27] Thanos D, Maniatis T. NF-κB: a lesson in family values. Cell 1995; 80:529–32.
[28] Kapahi P, Takahashi T, Natoli G, Adams SR, Chen Y, Tsien RY, et al. Inhibition of NF-κB activation by arsenite through reaction with a critical cysteine in the activation loop of IκB kinase. J Biol Chem 2000;275:36062–6.
[29] Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, et al. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. Nature 2000;403:103–8.
[30] Kwok BHB, Koh B, Ndubuisi MI, Elofsson M, Crews CM. The anti-inflammatory natural product patenolide from the medicinal herb Feverfew directly binds to and inhibits IκB kinase. Chem Biol 2001;8:759–66.
[31] Kisselev AF, Goldberg AL. Proteasome inhibitors: from research tools to drug candidates. Chem Biol 2001;8:739–58.
[32] Yoshiyuki H, Sachiko H, Kikuo K. 4-Hydroxynonenal prevents NO production in vascular smooth muscle cells by inhibiting nuclear factor-κB-dependent transcriptional activation. Arterioscler Thromb Vasc Biol 2001;21:1179–83.
[33] Alfonso J, Garcia P, Victor C, Gerardo M, Thomas JS, Elisabeth S, et al. Cystein 38 in p65/NF-κB plays a crucial role in DNA binding inhibition by sesquiterpene lactones. J Biol Chem 2001;276:39713–20.
[34] Chuan JI, Kevin RK, Lawrence JM. IκB kinase, a molecular target for inhibition by 4-hydroxy-2-nonenal. J Biol Chem 2001;276: 18223–8.