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Cinnamaldehyde suppresses toll-like receptor 4 activation mediated through the inhibition of receptor oligomerization

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ABSTRACT

Toll-like receptors (TLRs) play a critical role in induction of innate immune and inflammatory responses by recognizing invading pathogens or non-microbial endogenous molecules. TLRs have two major downstream signaling pathways, MyD88- and TRIF-dependent pathways leading to the activation of NFκB and IRF3 and the expression of inflammatory mediators. Deregulation of TLR activation is known to be closely linked to the increased risk of many chronic diseases. Cinnamaldehyde (3-phenyl-2-propenal) has been reported to inhibit NFκB activation induced by pro-inflammatory stimuli and to exert anti-inflammatory and anti-bacterial effects. However, the underlying mechanism has not been clearly identified. Our results showed that cinnamaldehyde suppressed the activation of NFκB and IRF3 induced by LPS, a TLR4 agonist, leading to the decreased expression of target genes such as COX-2 and IFNβ in macrophages (RAW264.7). Cinnamaldehyde did not inhibit the activation of NFκB or IRF3 induced by MyD88-dependent (MyD88, IKKβ) or TRIF-dependent (TRIF, TBK1) downstream signaling components. However, oligomerization of TLR4 induced by LPS was suppressed by cinnamaldehyde resulting in the downregulation of NFκB activation. Further, cinnamaldehyde inhibited ligand-independent NFκB activation induced by constitutively active TLR4 or wild-type TLR4. Our results demonstrated that the molecular target of cinnamaldehyde in TLR4 signaling is oligomerization process of receptor, but not downstream signaling molecules suggesting a novel mechanism for anti-inflammatory activity of cinnamaldehyde.

1. Introduction

Cinnamaldehyde (3-phenyl-2-propenal) is the major constituent of the essential oil of cinnamon bark isolated from *Cinnamomum* trees. It is a spice compound in cinnamon and has been widely used as a component in perfumes, a fungicide, and a flavoring agent in foodstuffs such as chewing gum, ice cream, candy and beverages. Cinnamaldehyde is an

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α,β-unsaturated carbonyl derivative with a mono-substituted benzene ring. Cinnamaldehyde has been known to have various biological activities including anti-inflammatory and anti-bacterial properties. It was observed that cinnamaldehyde suppressed lipopolysaccharide (LPS)-induced NFκB activation [1]. However, the molecular mechanism as to how cinnamaldehyde exerts anti-inflammatory activity has not been clearly elucidated.

The exposure to LPS, a cell wall component of gram-negative bacteria, causes septic shock through intensive systemic inflammatory responses. Toll-like receptor 4 (TLR4) has been identified as the receptor for LPS. TLR4 is a type I transmembrane receptor composed of an extracellular domain containing leucine-rich repeat (LRR) motifs, a transmembrane domain, and a cytoplasmic Toll/interleukin-1 receptor (TIR) homology domain. One of the initial steps of TLR4 activation after LPS engagement to TLR4 is the oligomerization of the receptor [2]. Subsequently, adaptor molecules are recruited to the cytoplasmic TIR domain of oligomerized TLR4. Activation of NFκB mediated through TLR4 was completely abolished in MyD88/TRIF double-knockout cells demonstrating that MyD88 and TRIF are the major adaptor molecules required for TLR4 signaling pathways [3]. The interaction of MyD88 with TIR domain of TLR results in the phosphorylation of IRAK-1 leading to degradation of IRAK-1. The phosphorylated IRAK-1 associates with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) which forms complex with transforming growth factor-β-activated kinase-1 (TAK-1) [4] leading to the activation of IKKα/β/γ complex and NFκB transcription factor. TRIF is another adaptor molecule responsible for the activation of MyD88-independent signaling pathways. TRIF induces NFκB activation with a delayed kinetics while MyD88-dependent NFκB activation occurs in the earlier time [5]. RIP1 is known to be involved in NFκB activation in TRIF signaling pathway [6,7]. TRIF also activates TBK1 and IKKε leading to the activation of IRF3 and the expression of IFNβ and IFN-inducible genes [8,9].

The activation of TLR4 signaling by bacterial ligands leads to the expression of pro-inflammatory gene products such as cytokines, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). TLR4 can be also activated by non-microbial components such as fibronectin, saturated fatty acid, and modified low-density lipoprotein resulting in the induction of sterile inflammatory responses [10–12]. Indeed, the accumulating evidences show that the activation or the suppression of TLR4 is implicated in the development and progression of various inflammatory diseases [13]. These suggest that TLR4 and the signaling components can be excellent therapeutic targets for chronic diseases of which etiology is closely linked to inflammation. Therefore, we investigated the modulation of TLR4 activation by cinnamaldehyde and the underlying mechanism to elucidate the anti-inflammatory effects of cinnamaldehyde.

2. Materials and methods

2.1. Reagents

Cinnamaldehyde and 2-phenylpropionaldehyde were purchased from Sigma–Aldrich (St. Louis, MO), and diluted in DMSO. Purified LPS was obtained from List Biological Lab. Inc. and dissolved in endotoxin-free water. Antibody for IRAK-1 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other reagents were purchased from Sigma unless otherwise described.

2.2. Cell culture

RAW 264.7 cells (a murine monocytic cell line, ATCC TIB-71) and 293 T (human embryonic kidney cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 units/ml Penicillin, and 100 μg/ml Streptomycin (Invitrogen). Ba/F3 cells, an IL-3-dependent murine pro-B cell line, expressing TLR4 (Flag or GFP-tagged), CD14, MD2 (Flag-tagged), and NFκB luciferase reporter gene were cultured as previously described [2]. Cells were maintained at 37 °C in a 5% CO_2/air environment.

2.3. Plasmids

A NFκB(2x)-luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). An IFNβ PRDIII-I-luciferase reporter plasmid and a wild-type TBK1 expression plasmid were kind gifts from Kate Fitzgerald (University of Massachusetts Medical School). Heat shock protein 70 (HSP70)-β-galactosidase reporter plasmid was from Robert Modlin (University of California, Los Angeles, CA). A constitutively active chimeric CD4-TLR4 was obtained from C.A. Janeway, Jr. (Yale University, New Haven, CT). A wild-type TLR4 expression plasmid was obtained from Adeline Hajjar (University of Washington, Seattle, WA). A wild-type of MyD88 was provided by Jurg Tschopp (University of Lausanne, Switzerland). A TRIF expression plasmid was provided by Shizo Akira (Osaka University, Japan). A wild-type IKKβ was obtained from Michael Karin (University of California, San Diego, CA). All DNA constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen, Chatsworth, CA) for transfection.

2.4. Transfection and luciferase assay

These were performed as described in our previous studies [14,15]. Briefly, RAW264.7 or 293T cells were transfected with a luciferase plasmid and various expression plasmids of signaling components using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. HSP70-β-galactosidase plasmid was co-transfected as an internal control. The total amount of transfected plasmids was equalized by supplementing with the corresponding empty vector. Luciferase and β-galactosidase enzyme activities were determined using the Luciferase Assay System and β-galactosidase Enzyme System (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity.

2.5. Immunoblotting

These were performed essentially the same as previously described [16,17]. Equal amounts of cell extracts were resolved

on SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane. The membranes were blotted with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL). The reactive bands were visualized with the enhanced chemiluminescence system (Amersham Biosciences).

2.6. *Immunoprecipitation*

These were performed essentially the same as previously described [2,18]. Protein extracts from Ba/F3 cells expressing TLR4 (Flag or GFP-tagged), CD14, MD2 (Flag-tagged), and NFκB luciferase reporter gene were prepared. Supernatants were incubated with monoclonal anti-Flag antibody (Sigma–Aldrich) for 4 h, and further incubated with 70 μl of 50% (v/v) protein A-agarose (Amersham, Arlington Heights, IL) for overnight at 4 °C with rocking. Immune complexes were solubilized with Laemmli sample buffer after five times of washing with lysis buffer. The solubilized immune complex was resolved on 8% SDS-PAGE. The membranes were immunoblotted with anti-Flag antibody and rabbit anti-GFP antibody (Molecular Probes Inc., Eugene, OR).

2.7. *Quantitative real-time reverse transcription (RT)-PCR analysis of interferon-beta (IFNβ) expression*

These were performed essentially the same as previously described [14,19]. Briefly, total RNAs were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Five micrograms of total RNAs were reverse-transcribed and amplified through PCR. The specificity of the amplified PCR products was assessed by a melting curve analysis. IFNβ expression was expressed as fold inductions after normalization with beta-actin expression.

2.8. *The determination of protein thiols*

These were performed essentially the same as previously described [20,21]. Cinnamaldehyde was incubated with bovine serum albumin (1 mg/ml) in phosphate-buffered saline (pH 7.4) for the indicated period. DTNB (250 μM final concentration) was then added and, after 20 min, the absorbance was measured at 412 nm. Protein thiol levels were calculated on the basis of a glutathione calibration curve.

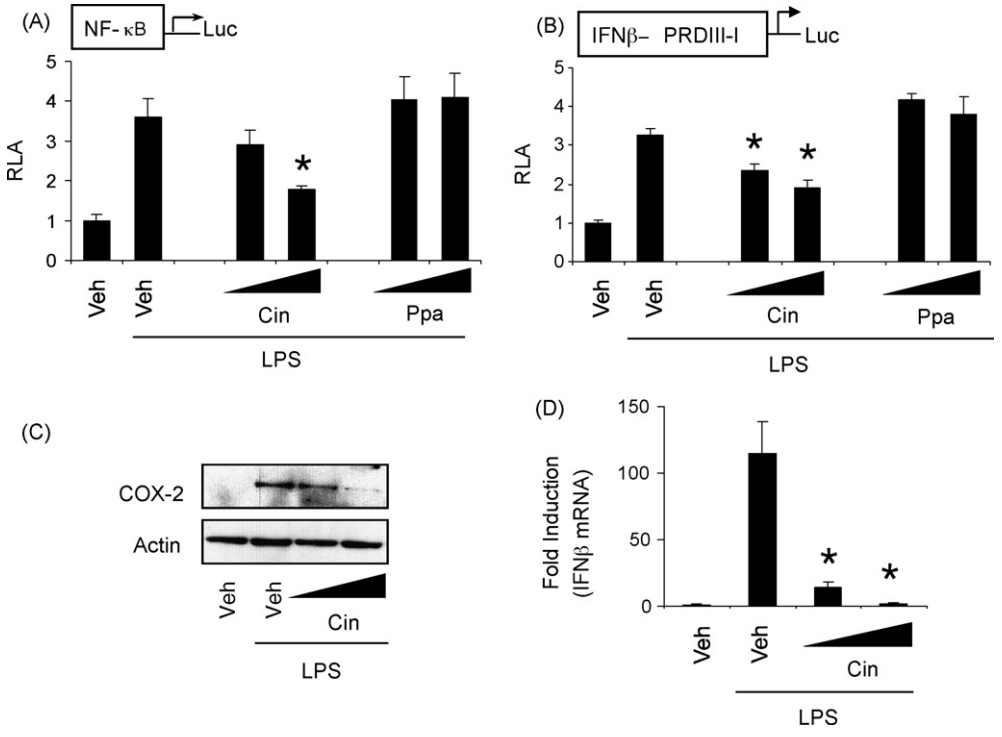

Fig. 1 – Cinnamaldehyde inhibits LPS-induced activation of TLR4 signaling and expression of target genes. (A and B) RAW264.7 cells were transfected with a luciferase reporter plasmid containing NFκB binding site(2x) or IFNβ promoter domain of IRF3 binding site (IFNβ PRDIII-I). Cells were pre-treated with cinnamaldehyde (Cin; 20, 50 μM) or 2-phenylpropionaldehyde (Ppa; 20, 50 μM) for 1 h, and then stimulated with LPS (5 ng/ml) for additional 6 h. Cell lysates were analyzed for luciferase and β-galactosidase enzyme activities as described in Section 2. Relative luciferase activity (RLA) was calculated after normalization with β-galactosidase activity. Values are mean ± S.E.M. ($n = 3$). (C) RAW264.7 cells were pre-treated with Cin (20, 50 μM) and further stimulated with LPS (5 ng/ml) for 6 h. Cell lysates were analyzed for COX-2 and actin protein by immunoblotting. (D) RAW264.7 cells were pre-treated with Cin (20, 50 μM) and further stimulated with LPS (5 ng/ml) for 4 h. Total RNAs were extracted and the levels of IFNβ expression were determined by the Quantitative real-time RT-PCR analysis. IFNβ expression was normalized with β-actin (internal control) expression. The results were presented as fold inductions compared with the vehicle control. The panels are representative data from more than three independent experiments. *Significantly different from LPS alone, $p < 0.05$. Veh, vehicle.

3. Results

3.1. Cinnamaldehyde suppresses LPS-induced activation of TLR4

The major transcription factors known to be activated in TLR4 signaling include NFκB and IRF3. To investigate whether cinnamaldehyde modulates the activation of TLR4, we determined the effect of cinnamaldehyde on TLR4 agonist-induced activation of these transcription factors. Activation of NFκB and IRF3 induced by LPS (a TLR4 agonist) was suppressed by cinnamaldehyde in macrophages (RAW264.7) (Fig. 1A and B). The inhibition of transcription factor activation by cinnamaldehyde resulted in the decrease of LPS-induced expression of TLR4 target genes, COX-2 and IFNβ (Fig. 1C and D).

On the contrary, a structural analog, 2-phenylpropionaldehyde did not inhibit LPS-induced activation of NFκB and IRF3 (Fig. 1A and B). Cinnamaldehyde possesses α,β-unsaturated carbonyl moiety while 2-phenylpropionaldehyde does not. These results suggest that α,β-unsaturated carbonyl moiety present in cinnamaldehyde may play a role in the inhibitory effect on TLR4 activation.

3.2. Cinnamaldehyde does not suppress the activation of NFκB or IRF3 induced by downstream signaling components of TLR4

MyD88- and TRIF-dependent signaling pathways are two main downstream signaling pathways to activate NFκB and IRF3 and to induce the expression of COX-2 and IFNβ in TLR4 signaling. Therefore, we investigated whether the inhibitory effects of cinnamaldehyde on activation of NFκB and IRF3 are mediated through the inhibition of downstream signaling components.

Cinnamaldehyde did not inhibit NFκB activation induced by MyD88 itself or IKKβ, a major kinase downstream of MyD88 in 293T cells (Fig. 2A and B).

IRAK-1 is recruited to MyD88 and phosphorylated upon stimulation of TLR4 by ligand. Phosphorylated IRAK-1 undergoes degradation process. Therefore, IRAK-1 degradation was used as a surrogate marker for ligand-induced activation of MyD88-dependent pathway of TLR4. Our results showed that LPS stimulation induced the degradation of IRAK-1 in RAW264.7 cells and this was reversed by cinnamaldehyde in a dose-dependent manner (Fig. 2C).

These results demonstrate that cinnamaldehyde inhibits TLR4 signaling activated by LPS, but not by MyD88 and its downstream signaling component.

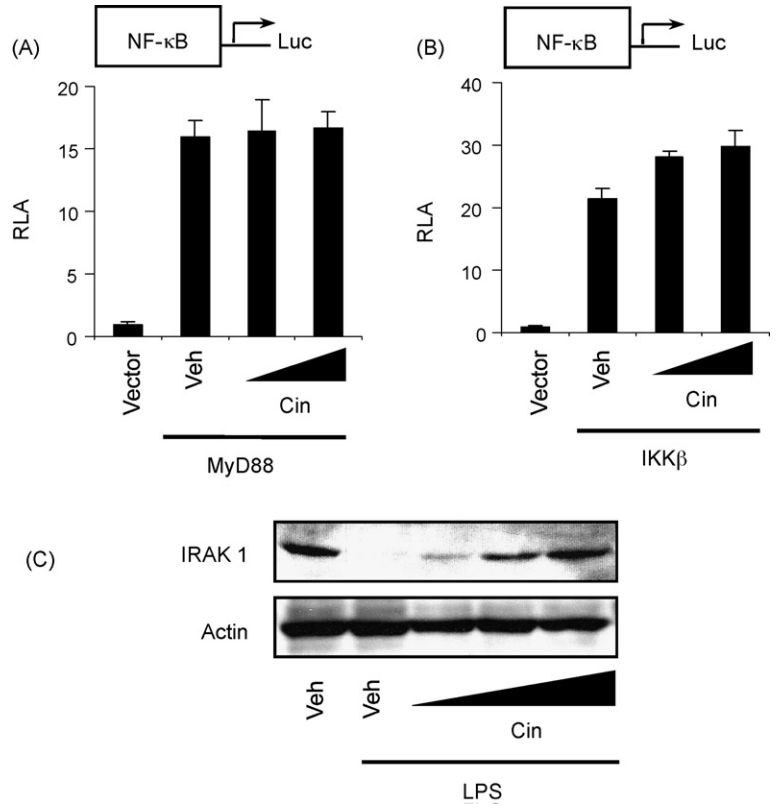

Fig. 2 – Cinnamaldehyde does not inhibit NFκB activation induced by MyD88-dependent signaling components. (A and B) 293T cells were transfected with a luciferase reporter plasmid containing NFκB binding site(2x) and the expression plasmid of MyD88 or IKKβ. Cells were further treated with cinnamaldehyde (Cin; 20, 50 μM) for 8 h. Relative luciferase activity (RLA) was determined as described in the legend of Fig. 1. Values are mean ± S.E.M. (n = 3). (C) RAW264.7 cells were pretreated with Cin (20, 50, 100 μM) for 1 h and then further stimulated with LPS (5 ng/ml) for 30 min. Cell lysates were analyzed for IRAK-1 and actin protein by immunoblotting. The panels are representative data from more than three independent experiments. Veh, vehicle.

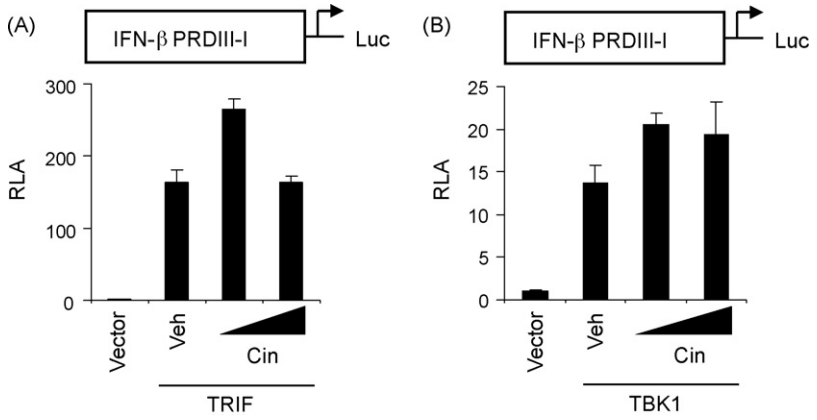

Fig. 3 – Cinnamaldehyde does not inhibit IRF3 activation induced by TRIF-dependent signaling components. 293T cells were transfected with a luciferase reporter plasmid containing IFNβ promoter domain of IRF3 binding site (IFNβ PRDIII-I) and the expression plasmid of (A) TRIF or (B) TBK1. Cells were further treated with cinnamaldehyde (Cin; 20, 50 μM) for 8 h. Relative luciferase activity (RLA) was determined as described in the legend of Fig. 1. Values are mean ± S.E.M. (n = 3). The panels are representative data from more than three independent experiments. Veh, vehicle.

The activation of IRF3 in TLR4 signaling is mainly mediated through TRIF-dependent signaling pathways. Therefore, we determined if the inhibition of IRF3 activation by cinnamaldehyde is mediated through the modulation of TRIF-dependent signaling components. Cinnamaldehyde did not inhibit IRF3 activation induced by TRIF itself or TBK1, a major kinase activating IRF3 in TRIF-signaling pathways (Fig. 3A and B).

These results suggest that the target of cinnamaldehyde is not MyD88- nor TRIF-dependent downstream signaling components including adaptor molecules themselves. The target may be components upstream of adaptor molecules including TLR4 itself or events leading to TLR4 activation by agonist.

3.3. *Cinnamaldehyde suppresses LPS-induced oligomerization of TLR4*

The oligomerization of TLR4 is one of the critical steps to initiate the activation of TLR4 signaling after engagement of agonist to receptor. Therefore, we investigated if cinnamaldehyde affects the oligomerization of TLR4 as determined by co-immunoprecipitation experiment using differently tagged TLRs. The oligomerization of TLR4 induced by LPS was suppressed by cinnamaldehyde in Ba/F3 cells expressing Flag- and GFP-tagged TLR4 (Fig. 4A). The suppression was well correlated with the downregulation of NFκB activation by cinnamaldehyde in Ba/F3 cells (Fig. 4B). These results demonstrate that the inhibitory target of cinnamaldehyde is the receptor oligomerization process.

Cinnamaldehyde suppressed LPS-independent activation of NFκB induced by constitutively active TLR4 (CD4-TLR4) or wild-type TLR4 (Fig. 5A and B). These results suggest that the interaction of ligand with TLR4 may not be the critical target for cinnamaldehyde-inhibitory effect. These further support that cinnamaldehyde disrupts TLR4 oligomerization process resulting in the decreased activation of downstream transcription factors and the attenuated expression of target genes.

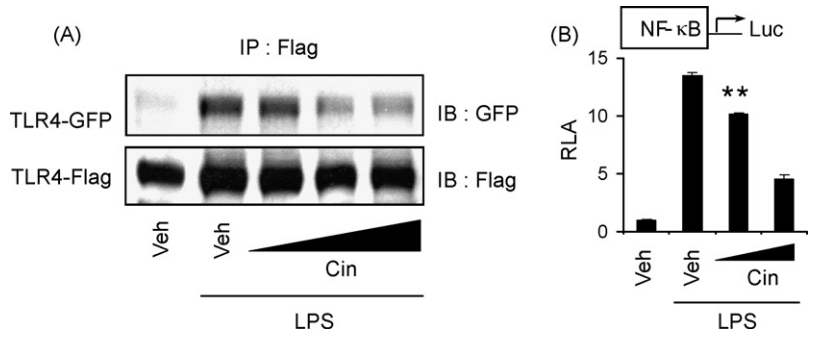

Fig. 4 – Cinnamaldehyde inhibits LPS-induced oligomerization of TLR4. (A) Ba/F3 cells expressing TLR4-Flag, TLR4-GFP, MD2-Flag, CD14 and NFκB luciferase were pre-treated with cinnamaldehyde (Cin; 20, 50, 100 μM) for 1 h and then stimulated with LPS (50 ng/ml) for 20 min. Cells were then subjected to immunoprecipitation with anti-Flag antibody and immunoblotted with anti-GFP (upper) and anti-Flag (lower) antibody. (B) The same Ba/F3 cells in (A) were pretreated with Cin (20, 50 μM) for 1 h and then stimulated with LPS (5 ng/ml) for additional 8 h. Cell lysates were prepared and luciferase enzyme activities were measured as described in the legend of Fig. 1. Values are mean ± S.E.M. (n = 3). **Significantly different from LPS alone, $p < 0.01$. The panels are representative data from more than three independent experiments. Veh, vehicle.

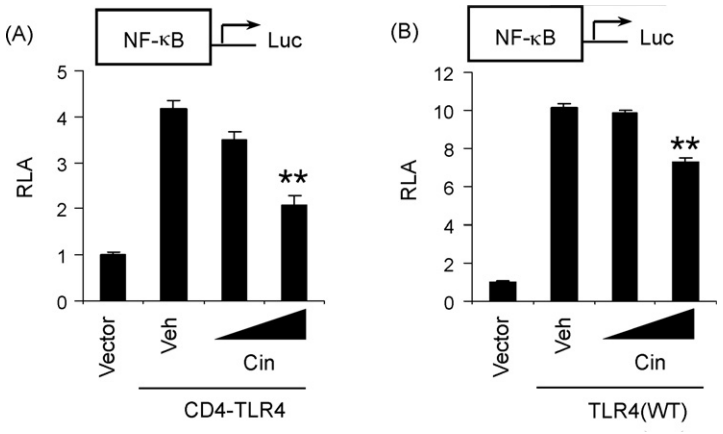

Fig. 5 – NFκB activation induced by constitutively active TLR4 or wild-type TLR4 is inhibited by cinnamaldehyde. 293T cells were transfected with a luciferase reporter plasmid containing NFκB binding site(2x) and the expression plasmid of CD4-TLR4 or wild-type (WT) TLR4. After 24 h, cells were further treated with cinnamaldehyde (Cin; 20, 50 μM) for 8 h. Relative luciferase activity (RLA) was determined as described in the legend of Fig. 1. Values are mean ± S.E.M. ($n = 3$). **Significantly different from CD4-TLR4 alone (A) or TLR4 alone (B), $p < 0.01$. The panels are representative data from more than three independent experiments. Veh, vehicle.

Cinnamaldehyde is known to react with sulfhydryl group and decrease intracellular thiol levels possibly due to the α,β-unsaturated carbonyl moiety [22,23]. Our results showed that protein thiol level in bovine serum albumin was decreased by cinnamaldehyde (Fig. 6A). To determine whether sulfhydryl-modifying activity of cinnamaldehyde is related to the suppressive effect on TLR4 signaling and oligomerization, we performed competition experiments using thiol-donors,

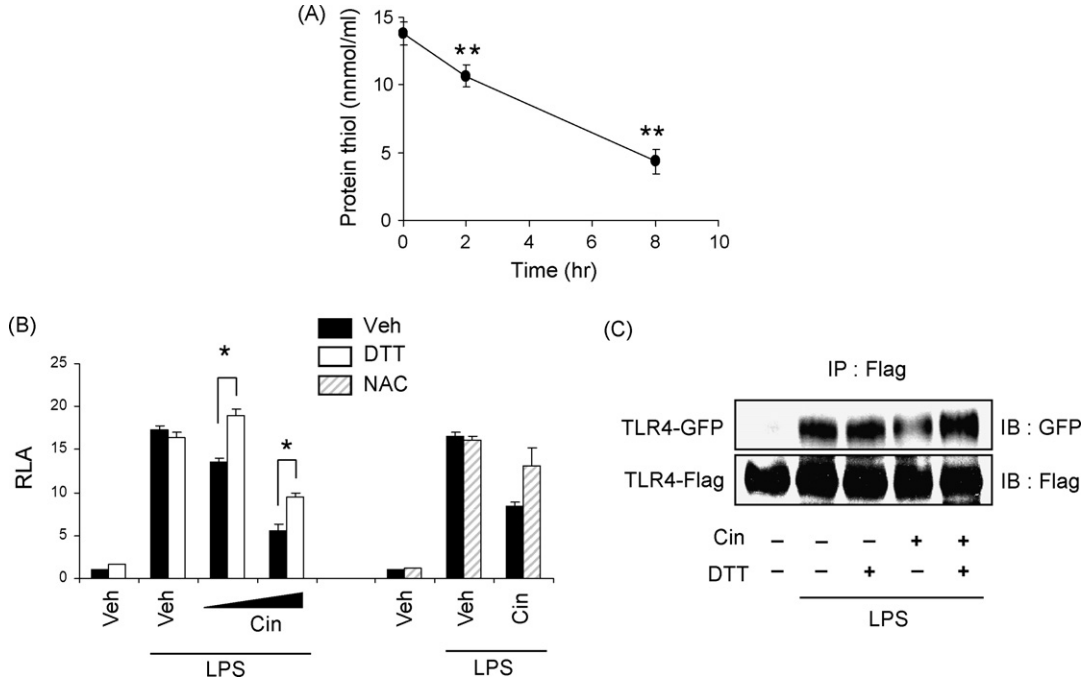

Fig. 6 – The inhibitory effects of cinnamaldehyde on NFκB activation and TLR4 oligomerization are reversed by thiol-donors. (A) Cinnamaldehyde (Cin; 50 μM) was incubated with bovine serum albumin (1 mg/ml) in phosphate-buffered saline (pH 7.4) for the indicated period. Total protein thiol levels were determined. Values are mean ± S.E.M. ($n = 3$). **Significantly different from 0 h, $p < 0.01$. (B) Ba/F3 cells expressing TLR4-Flag, TLR4-GFP, MD2-Flag, CD14 and NFκB luciferase were pre-treated with either Cin (20, 50 μM) with dithiothreitol (DTT; 300 μM) or Cin 50 μM with *N*-acetyl-L-cysteine (NAC; 2 mM) for 1 h. Cells were further stimulated with LPS (1 ng/ml) for additional 8 h. Cell lysates were prepared and luciferase enzyme activities were measured. Values are mean ± S.E.M. ($n = 3$). *$p < 0.05$. Veh, vehicle. (C) The same Ba/F3 cells in Fig. 6B were pretreated with Cin (50 μM) in the presence of DTT (400 μM) for 1 h and then stimulated with LPS (50 ng/ml) for 20 min. Cells were then subjected to immunoprecipitation with anti-Flag antibody and immunoblotted with anti-GFP (upper) and anti-Flag (lower) antibody.

dithiothreitol (DTT) and *N*-acetyl-L-cysteine (NAC). The inhibition of LPS-induced NFκB activation by cinnamaldehyde was reversed by DTT or NAC in Ba/F3 cells expressing TLR4 (Fig. 6B). Furthermore, DTT restored LPS-induced TLR4 oligomerization which was disrupted in the presence of cinnamaldehyde (Fig. 6C). These results suggest that sulfhydryl-modifying activity of cinnamaldehyde is correlated with the suppressive effects on TLR4 signlaing and oligomerization.

4. Discussion

Oligomerization of toll-like receptor (TLR) is an important step for the recognition of ligands and the activation of signaling. TLR4 oligomerization was induced by the stimulation with LPS and well correlated with IRAK-1 degradation which reflects the activation of TLR signaling [2]. The deletion of cytosolic domain of TLR4 did not abolish LPS-induced receptor oligomerization suggesting that extracellular domain participates ligand-induced oligomerization [2]. The replacement of extracellular domain of TLR4 with CD4 resulted in CD4-mediated oligomerization of receptors and conferred the constitutive activity to activate NFκB [24]. The recognition of double-stranded RNA and subsequent activation of signaling are dependent on TLR3 oligomerization [25]. The deletion of certain leucine-rich repeat (LRR) domain of TLR3 affects TLR3 signaling suggesting that the modification of extracelluar domain results in the change of receptor-receptor association [25]. Heterodimerization of TLR2 with TLR1 or TLR6 discriminates different structural moiety of TLR2 agonists. Diacylated mycoplasmal lipopeptides are recognized by TLR2 and TLR6 dimer while TLR1 and TLR2 dimer responds to triacylated bacterial lipopeptides [26,27]. Co-expression of CD4-TLR2 with CD4-TLR6 or CD4-TLR2 with CD4-TLR1 resulted in the production of cytokine in macrophages possibly due to CD4-induced heterodimerization [28]. Our results demonstrated that cinnamaldehyde did not inhibit activation of NFκB and IRF3 induced by adaptor molecules and the downstream signaling components of TLR suggesting that the target of cinnamaldehyde is upstream of these signaling molecules. Our results further showed that cinnamaldehyde suppressed LPS-induced oligomerization of TLR4 which is one of the initial steps for receptor activation. This suppression resulted in the downregulation of LPS-induced activation of transcription factor, NFκB. Certain receptor signaling can be modulated by cellular redox status or anti/pro-oxidants through the changes of its affinity to ligand or the transducing function of receptor. Hayakawa et al. demonstrated that antioxidants such as *N*-acetyl-L-cysteine inhibit TNF-induced signaling by reducing the affinity of receptor to TNF [29]. TNF receptor is susceptible to redox status modulated by pro-oxidants due to changes in thiols in extracellular domains [30]. Our results showed that ligand-independent activation of NFκB induced by constitutively active TLR4 or wild-type TLR4 was suppressed by cinnamaldehyde. These suggest that ligand affinity of TLR4 may not be the critical target for cinnamaldehyde and that the inhibitory target of cinnamaldehyde is the interaction of receptors or the proximal events leading to receptor activation. Therefore, our results demonstrate that the inhibition of TLR4 oligomerization is the novel mechanism for anti-inflammatory activity of cinnamaldehyde. These further suggest that ligand-induced oligomerization can be a beneficial therapeutic target for anti-inflammatory agents.

The results from our previous reports have shown that curcumin and gold compounds which are also known to have anti-inflammatory activity disrupted oligomerization of TLR4 [18,31]. In contrast, other anti-inflammatory compounds, resveratrol and EGCG, did not inhibit TLR4 oligomerization. Curcumin has α,β-unsaturated carbonyl moiety which is highly reactive with sulfhydryl group in protein [32]. Gold compounds are also known to react with sulfhydryl group [33] while resveratrol and EGCG do not exert high reactivity to sulfhydryl moiety [18,19]. Cinnamaldehyde which has α,β-unsaturated carbonyl moiety exerted suppressive effect on TLR4 oligomerization while 2-phenylpropionaldehyde which does not possess α,β-unsaturated carbonyl moiety did not inhibit TLR4-mediated signaling (Fig. 1). Cinnamaldehyde is known to react with sulfhydryl group and to decrease intracellular thiol levels [22,23]. Moon and Pack have reported that cinnamaldehyde reduced the content of sulfhydryl group in L1210 cell sonicates and reacted with sulfhydryl group of cysteine [22]. Ka et al. showed that cinnamaldehyde decreased the level of intracellular GSH and protein thiols in human leukemia HL-60 cells [23]. Our results also showed that protein thiol level of albumin was decreased by cinnamaldehyde (Fig. 6). In addition, the inhibitory effects of cinnamaldehyde on NFκB activation and TLR4 oligomerization were reversed by thiol donors, DTT or NAC (Fig. 6). These results suggest that sulfhydryl-modifying activity of different chemicals is positively correlated with the suppressive effect on oligomerization of TLR4. This further suggests that sulfhydryl modification may be an important contributing factor for the regulation of TLR4 activation. Sulfhydryl group of cysteine residue is critical for biological activities for many proteins [34]. TRPA1, a member of the Transient Receptor Potential (TRP) family of ion channels, can be activated by covalent modification of reactive cysteines by cysteine-modifying agents including cinnamaldehyde [35]. TLRs contain several cysteine residues in extracellular and cytoplasmic domains which may be involved in disulfide bond formation for receptor oligomerization [36,37]. However, biochemical mechanism as to how cinnamaldehyde modifies receptor oligomerization remains to be further determined.

Although curcumin, gold compound, and cinnamaldehyde have common to disrupt TLR4 oligomerization, they show different pattern for inhibition of downstream signaling pathways of TLR4. Curcumin suppresses MyD88-dependent signaling pathways due to the inhibition of IKKβ [18] while gold compounds are able to inhibit multiple steps in both MyD88-dependent and TRIF-dependent signaling pathways in addition to oligomerization [31]. In contrast, cinnamaldehyde does not suppress downstream signaling components of MyD88 or TRIF, but inhibits oligomerization of TLR4. The reason for these differences is not clearly understood. However, it may be related to differences in chemical potency to react with sulfhydryl group, metabolism or distribution in cells, and sensitivity of signaling components to sulfhydryl modification.

Cinnamaldehyde affects the action of other inflammatory cytokines in addition to LPS. Cinnamaldehyde inhibits the

enzymatic activity of COX-2 increased by IL-1β, but not the protein expression of COX-2 in rat cerebral microvascular endothelial cells [38]. IL-1β receptor has MyD88 as an adaptor molecule which is also involved in TLR4 signaling. Our results that cinnamaldehyde does not inhibit MyD88-induced NFκB activation are consistent with no inhibitory effect of cinnamaldehyde on IL-1β-induced gene expression. Our results that NFκB activation induced by MyD88 and IKKβ is not inhibited by cinnamaldehyde suggest that the molecular target of cinnamaldehyde is not the downstream signaling molecules.

Cinnamaldehyde derivatives, 2′-hydroxycinnamaldehyde and 2′-benzoxycinnamaldehyde, suppressed lymphoproliferation and antibody production induced by LPS stimulation in mouse splenocytes [39]. It is well established that the activation of TLR4 is critical for inducing both innate and adaptive immune responses. Therefore, our results suggest that the suppression of TLR4 signaling by cinnamaldehyde may be the mechanism for the decreased adaptive immune activity.

Lipid rafts are detergent insoluble membrane microdomains enriched with cholesterol and sphingolipids. Lipid rafts play a role as a signaling platform to recruit and concentrate molecules to facilitate cellular signaling. Recently, lipid rafts are implicated in LPS-mediated cellular stimulation. Olsson and Sundler showed that LPS induced the translocation of co-receptor CD14 and MAP kinases to lipid rafts in macrophages [40]. Triantafilou et al. showed that TLR4 and its downstream signaling molecules such as MyD88 and JNK/SAPK were present in lipid rafts after LPS stimulation and that lipid rafts-disrupting agents, nystatin and methyl-beta-cyclodextrin, decreased LPS-induced TNF-alpha production [41]. Lipid rafts-disrupters, filipin and methyl-beta-cyclodextrin, suppressed LPS-induced NFκB activation in intestinal epithelial cells [42]. Therefore, the functional maintenance of lipid rafts would be important for TLR4 activation and the following cellular responses. Both TLR4 oligomerization and translocation of the receptor to lipid rafts are two important initial events to transmit ligand engagement to activation of intracellular signaling pathways. However, it has not been clearly elucidated how these two events are correlated or regulated by each other. The understanding of the relationship between these two events would help to reveal the detail mechanism of LPS-induced TLR4 activation and to understand the action mechanism for certain anti-inflammatory agents.

TLR4 is critical for regulating immune and inflammatory responses of which dysregulation would be one of the key etiological factors for the development of chronic diseases. Therefore, it will be important to find the beneficial way to modulate TLR4 activity in order to prevent or treat chronic diseases. TLR4 activation can be modulated by endogenous molecules and dietary factors including saturated fatty acid and *n*-3 polyunsaturated fatty acids [17]. Our results show that TLR activity can be regulated by certain phytochemicals such as cinnamaldehyde mediated through the modulation of receptor oligomerization leading to decreased inflammatory gene expression. These results provide a new paradigm in identifying specific molecular targets of anti-inflammatory agents. Furthermore, these results suggest that TLRs and their downstream signaling components are important molecular targets for preventive and therapeutic strategy against many chronic diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.08.033.

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