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Inhibition of homodimerization of Toll-like receptor 4 by curcumin

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Abbreviations:
LPS, lipopolysaccharide
COX, cyclooxygenase
NF-κB, nuclear factor κB
MyD88, myeloid differential factor 88
TRIF, TIR domain-containing adapter inducing IFN-β
IFN, interferon
RIP1, receptor interacting protein 1
IKK, IκB kinase
TBK1, TANK-binding kinase 1
IRF3, IFN-regulatory factor 3
IRAK, IL-1 receptor-associate kinase
GFP, green fluorescent protein

ABSTRACT

Toll-like receptors play a key role in sensing microbial components and inducing innate immune responses. Ligand-induced dimerization of TLR4 is required for the activation of downstream signaling pathways. Thus, the receptor dimerization may be one of the first lines of regulation in activating TLR-mediated signaling pathways and induction of subsequent immune responses. LPS induces the activation of NF-κB and IRF3 through MyD88- or TRIF-dependent pathways. Curcumin, a polyphenol found in the plant *Curcuma longa*, has been shown to suppress the activation of NF-κB induced by various pro-inflammatory stimuli by inhibiting IKKβ kinase activity in MyD88-dependent pathway. Curcumin also inhibited LPS-induced IRF3 activation. These results imply that curcumin inhibits both MyD88- and TRIF-dependent pathways in LPS-induced TLR4 signaling. However, in TRIF-dependent pathway, curcumin did not inhibit IRF3 activation induced by overexpression of TRIF in 293T cells. These results suggest that TLR4 receptor complex is the molecular target of curcumin in addition to IKKβ. Here, we report biochemical evidence that phytochemicals (curcumin and sesquiterpene lactone) inhibit both ligand-induced and ligand-independent dimerization of TLR4. Furthermore, these results demonstrate that small molecules with non-microbial origin can directly inhibit TLRs-mediated signaling pathways at the receptor level. These results imply that the activation of TLRs and subsequent immune/inflammatory responses induced by endogenous molecules or chronic infection can be modulated by certain dietary phytochemicals we consume daily.

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1. Introduction

Toll-like receptors (TLRs) recognize conserved microbial structural molecules and induce innate immune responses that are essential for host defense against invading microbial pathogens [1–4]. It was demonstrated that ligand-induced homotypic oligomerization is proceeded for LPS-induced activation of TLR4 signaling pathways [5]. TLR2 is known to heterodimerize with TLR1 or TLR6 recognizing diacyl- or triacyl-lipopeptide, respectively [6–8]. These results suggest that ligand-induced receptor dimerization for TLR4 and TLR2 is required for the activation of downstream signaling pathways. In addition, ligand-independent dimerization of TLR4 leads to the activation of the downstream signaling pathways [9–11]. Therefore, the receptor dimerization may be one of the first lines of regulation in activating TLR-mediated signaling pathways and induction of subsequent innate and adaptive immune responses.

Broadly, TLRs can activate two branches of downstream signaling pathways: MyD88-dependent and MyD88-independent pathways [1]. MyD88 is a common downstream adaptor molecule for all mammalian TLRs [1]. MyD88 is an immediate downstream adaptor molecule recruited by activated TLRs through their TIR domain. MyD88 in turn recruits IRAK-4 and induces IRAK-4-induced phosphorylation and degradation of IRAK-1. IRAK-1 associates with TRAF6 leading to the activation of MAP kinases and IKK complex resulting in the activation of AP-1 and NF-κB transcription factor, respectively. The activation of MyD88-dependent signaling pathway leads to the induction of inflammatory gene products including cytokines and cyclooxygenase-2 (COX-2) [10].

TLR3 and TLR4 activate MyD88-independent signaling pathway mediated through TIR domain-containing adaptor inducing IFNβ (TRIF) leading to the expression of type I interferon and IFN-inducible genes. The activation of TRIF pathway also leads to the delayed activation of NF-κB. TRIF interacts with TBK1 and RIP1 [12–14]. The C-terminal portion of TRIF was shown to be associated with RIP1, and embryonic fibroblast from RIP1-deficient mice showed impaired NF-κB activation and the expression of ICAM-1 in response to TLR3 agonist [12]. Thus, TRIF is likely to use TBK1 and RIP1 for IRF3- and NF-κB activation, respectively [15]. Since TLR4 ligand-induced inflammatory cytokine production was impaired in TRIF-deficient mice [1], signals from both TRIF and MyD88 pathways may be required for the maximum expression of cytokines.

Numerous studies demonstrated that certain phytochemicals including polyphenols and sesquiterpene lactones possessing anti-inflammatory effects inhibit NF-κB activation induced by various receptor agonists including TNFα and LPS [16]. However, the direct molecular targets for such anti-inflammatory phytochemicals are largely unknown. It was demonstrated that some polyphenols inhibit NF-κB activation and target gene expression induced by different receptor agonists mediated through the inhibition of IKKβ activity or DNA binding of p65 [17,18]. One of these compounds is curcumin which is a naturally occurring yellow pigment found in the plant *Curcuma longa*. Curcumin was shown to inhibit NF-κB activation induced by LPS, PMA, TNF-α and hydrogen peroxide mediated through the inhibition of IKKβ that phosphorylates IκBα leading to ubiquitinylation and degradation [17,19]. Curcumin inhibited induction of nitric oxide synthase induced by LPS/IFN-γ in RAW264.7 cells [20]. Curcumin also suppressed LPS-induced COX-2 gene expression by inhibiting NF-κB and AP-1 DNA bindings in BV2 microglial cells [21].

Forced dimerization of TLR4 by replacing the entire extracellular domain with CD4 [9], integrin [11], or deletion of leucine-rich repeat (LRR) domain [10] confers ligand-independent activation of the receptor. These results suggest that the receptor dimerization is required to activate downstream signaling pathways. Indeed, the dimerization of TLR4 was shown to be a prerequisite for the ligand-induced receptor activation [22]. LPS activates both MyD88- and TRIF-dependent pathways. It was demonstrated that curcumin inhibits LPS-induced NF-κB activation by inhibiting IKKβ that lies downstream of the MyD88-dependent pathway of TLR4. However, it is not known whether curcumin inhibits TRIF-dependent pathway or not. Here, we report biochemical evidence that curcumin inhibits the dimerization of TLR4. Therefore, curcumin inhibits LPS-induced activation of both MyD88- and TRIF-dependent pathways of TLR4 resulting in the inhibition of both NF-κB and IRF3. The results from these studies open important possibility that TLR-mediated inflammatory responses and consequent risk for chronic inflammatory diseases can also be modulated by dietary phytochemicals.

2. Materials and methods

2.1. Reagents

Curcumin and helenalin were purchased from Biomol (Plymouth Meeting, PA). Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) was purchased from Sigma–Aldrich (St. Louis, MO). Purified LPS was obtained from List Biological Lab. Inc. GFP and IRAK-1 antibodies were purchased from Molecular Probes Inc. (Eugene, OR) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively. All other reagents were purchased from Sigma unless otherwise described.

2.2. Cell culture

Ba/F3 cells, an IL-3-dependent murine pro-B cell line, expressing TLR4 (Flag or GFP-tagged), CD14, MD2 (Flag-tagged), and NF-κB luciferase reporter gene were described previously [5]. Cells were cultured in RPMI1640 medium containing recombinant murine IL-3 (70 U/ml), 10% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 units/ml Penicillin, and 100 μg/ml Streptomycin (GIBCO-BRL). RAW264.7 cells (a murine monocytic cell line, ATCC TIB-71) and 293T cells (human embryonic kidney) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FBS, 100 units/ml Penicillin, and 100 μg/ml Streptomycin. Cells were maintained at 37 °C in a 5% CO_2/air environment.

2.3. Transfection and reporter gene luciferase assay

NF-κB(2x)- and COX-2 luciferase reporter gene assay were performed as described previously [23,24]. Cells were co-

transfected with a luciferase plasmid and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Various expression plasmids or equal amounts of empty vector for signaling components were co-transfected. Luciferase enzyme activities were determined using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity.

2.4. *Immunoblotting*

These were performed the same as previously described [23]. Equal amounts of cell extracts were subjected to 10% SDS polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membrane for COX-2, IRAK-1, pJNK1, β-actin, JNK and GAPDH immunoblot analysis. The membrane was blocked to prevent nonspecific binding of antibodies in phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk. Immunoblotting was performed with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (Amersham Biosciences, Arlington Heights, IL). The reactive bands were visualized with ECL Western blot detection reagents (Amersham Biosciences, Arlington Heights, IL).

2.5. *Immunoprecipitation*

Immunoprecipitation was performed the same as previously described [5,23]. Protein extracts from Ba/F3 cells expressing TLR4 (Flag or GFP-tagged), CD14, MD2 (Flag-tagged), and NF-κB luciferase reporter gene for immunoprecipitation were prepared as described [5]. The samples were immunoprecipitated with mouse-GFP antibody (Molecular Probes Inc., Eugene, OR) for overnight. The solubilized immune complex was resolved on SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane. The membrane was blocked with phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk and was blotted with the Flag antibodies (Sigma–Aldrich, St. Louis, MO) for overnight. Thereafter, the blot was exposed

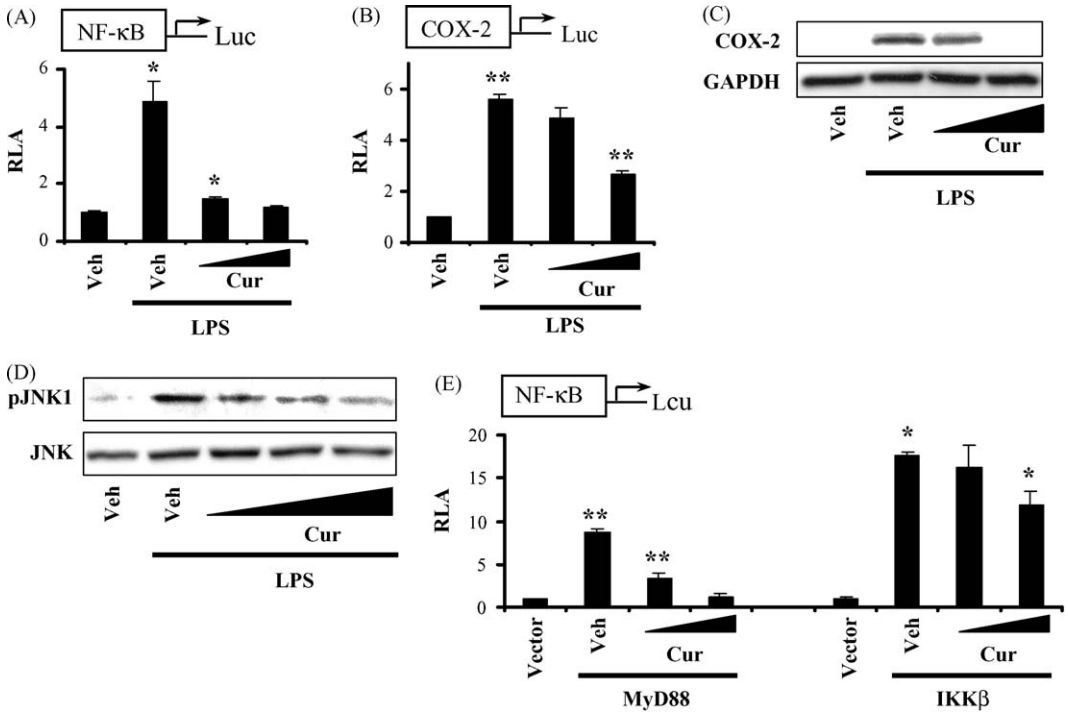

Fig. 1 – Curcumin inhibits the activation of NF-κB and the expression of COX-2 induced by LPS or the transfection of MyD88 or IKKβ expression plasmid. (A, B) RAW264.7 cells were transfected with (A) NF-κB or (B) COX-2 luciferase reporter plasmid and pre-treated with curcumin (10, 20 μM) for 1 h, and then treated with LPS (5 ng/ml) for an additional 6 h. Cell lysates were prepared and luciferase and β-galactosidase enzyme activities were measured as described in Section 2. Relative luciferase activity (RLA) was normalized with β-galactosidase activity. Values are mean ± S.E.M. ($n = 3$). *Significantly different from LPS alone (A), $p < 0.05$. **Significantly different from LPS alone (B), $p < 0.01$. (C) RAW264.7 cells were pretreated with curcumin (10, 20 μM) for 1 h and then further stimulated with LPS (5 ng/ml) for 6 h. Cell lysates were analyzed for COX-2 and GAPDH protein by immunoblots. (D) RAW264.7 cells were pretreated with curcumin (10, 20, 50 μM) for 1 h and then further stimulated with LPS (50 ng/ml) for 30 min. Cell lysates were analyzed for phospho-JNK1 and JNK protein by immunoblots. (E) 293T cells were co-transfected with NF-κB-luciferase reporter plasmid and an expression plasmid for MyD88 or IKKβ. pcDNA was used as a vector control for MyD88 and IKKβ. After 24 h, cells were treated with curcumin (10, 20 μM) for 6 h. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. Values are mean ± S.E.M. ($n = 3$). **Significantly different from MyD88 plus vehicle, $p < 0.01$. *Significantly different from IKKβ plus vehicle, $p < 0.05$. The panels are representative data from more than three independent experiments. Veh, vehicle; Cur, curcumin.

to horseradish peroxidase-conjugated secondary antibodies for 1 h, and detected with ECL Western blot detection reagents. The blot was reprobed with rabbit GFP antibodies (Molecular Probes Inc., Eugene, OR).

3. Results

3.1. *TLR4 ligand-induced activations of NF-κB, IRF3, and target gene (COX-2) expression were inhibited by curcumin. However, curcumin did not inhibit TRIF-induced IRF3 activation suggesting that the target of inhibition is the TLR4 receptor complex*

IKKβ is the key kinase in the canonical pathway for NF-κB activation induced by various agonists including ligands for TLRs. The activation of IKKβ by LPS (TLR4 ligand) is mediated through MyD88-dependent pathway. Curcumin is known to inhibit NF-κB activation induced by various agonists (LPS, PMA, TNFα, and H_2O_2) mediated through the inhibition of IKKβ [17,19]. However, it is not known whether curcumin inhibits TRIF-dependent LPS signaling pathways as well. The results showed that curcumin inhibited LPS-induced NF-κB activation and COX-2 expression in RAW264.7 cells (Fig. 1A–C). Curcumin also inhibited LPS-induced JNK posphorylation (Fig. 1D). NF-κB activation induced by the overexpression of MyD88 or IKKβ in 293T cells was inhibited by curcumin (Fig. 1E). The results suggest that curcumin inhibits NF-κB activation induced through MyD88-dependent pathway of TLR4. The TRIF-dependent pathway of TLR4 leads to the activation of IRF3 transcription factor mediated through TBK1 [14]. Curcumin also inhibited the LPS-induced activation of IRF3 (the readout for TRIF-dependent pathway) as determined by IRF3 specific IFNβ promoter reporter gene assay (Fig. 2A) [14]. However, curcumin did not inhibit TRIF or TBK1 (downstream components of MyD88-independent pathway)-induced IRF3 activation (Fig. 2B–D), but resveratrol, which was shown to inhibit TRIF-dependent pathway, inhibited TRIF or TBK1-induced IRF3 activation (Fig. 2B and D) [25]. Together, these results suggest that the target of inhibition by curcumin is TLR4 receptor complex in addition to IKKβ.

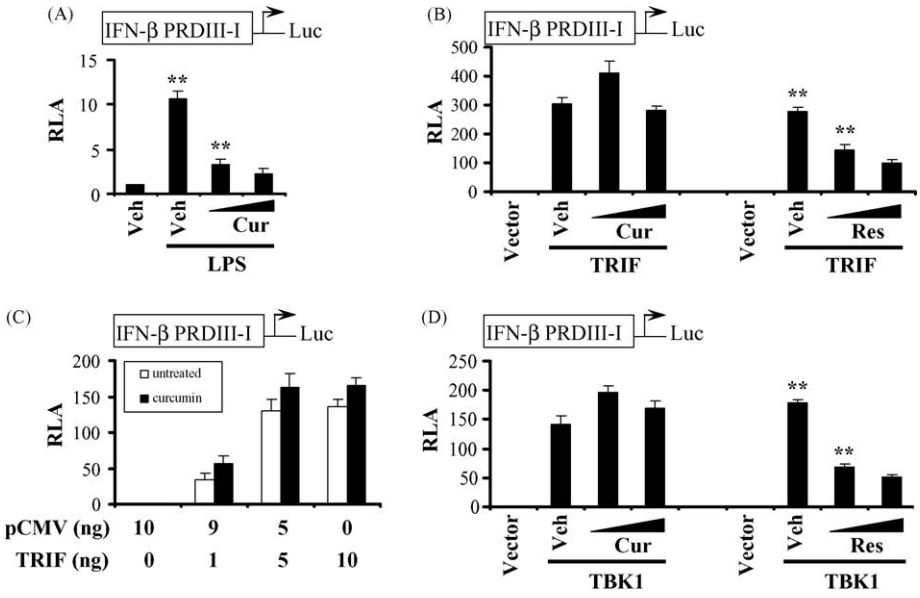

Fig. 2 – Curcumin inhibits TLR4 ligand (LPS)-induced activation of IRF3, but it does not inhibit the activation of IRF3 induced by the downstream components (TRIF or TBK1) of TLR4. (A) RAW264.7 cells were transfected with IFNβ promoter with specific IRF3 binding site (IFNβ PRDIII-I) luciferase reporter plasmid [14] and pre-treated with curcumin (10, 20 μM) for 1 h and then treated with LPS (5 ng/ml) for an additional 6 h. Cell lysates were prepared and luciferase and β-galactosidase enzyme activities were measured as described in the legend of Fig. 1. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. Values are mean ± S.E.M. ($n = 3$). **Significantly different from LPS alone, $p < 0.01$. (B) 293T cells were co-transfected with IFNβ promoter (IFNβ PRDIII-I)-luciferase reporter plasmid and an expression plasmid for TRIF. pCMV for TRIF was used as a vector control. After 24 h, cells were treated with curcumin (10, 20 μM) or resveratrol (10, 20 μM) for 6 h. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. Values are mean ± S.E.M. ($n = 3$). **Significantly different from TRIF plus vehicle, $p < 0.01$. (C) 293T cells were co-transfected with IFNβ promoter (IFNβ PRDIII-I)-luciferase reporter plasmid and an indicated concentration of expression plasmid for TRIF. After 24 h, cells were treated with curcumin (20 μM) for 6 h. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. Values are mean ± S.E.M. ($n = 3$). (D) 293T cells were co-transfected with IFNβ promoter (IFNβ PRDIII-I)-luciferase reporter plasmid and an expression plasmid for TBK1. pcDNA for TBK1 was used as a vector control. After 24 h, cells were treated with curcumin (10, 20 μM) or resveratrol (10, 20 μM) for 6 h. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. Values are mean ± S.E.M. ($n = 3$). **Significantly different from TBK1 plus vehicle, $p < 0.01$. The panels are representative data from more than three independent experiments. Veh, vehicle; Cur, curcumin; Res, resveratrol.

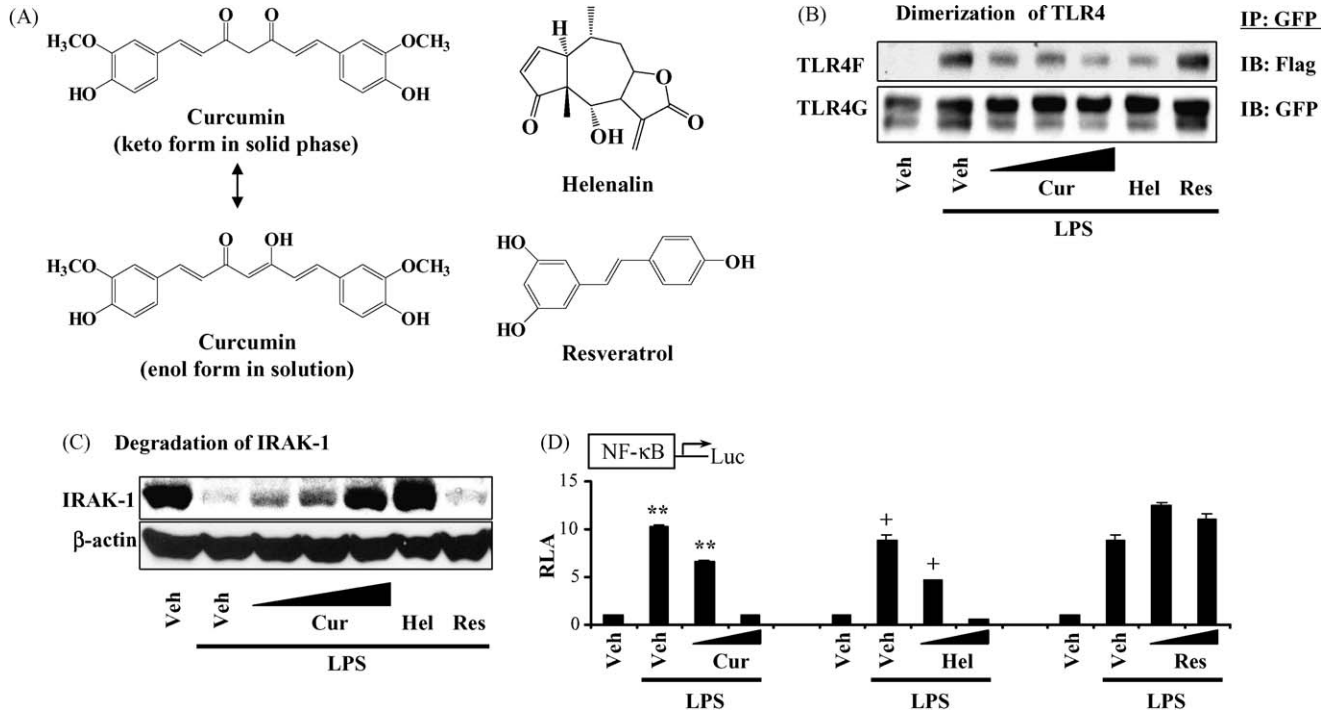

Fig. 3 – Curcumin and helenalin inhibit LPS-induced TLR4 dimerization, but resveratrol does not inhibit the dimerization. (A) The chemical structures of curcumin, helenalin, and resveratrol. (B) Ba/F3 cells expressing TLR4-Flag (TLR4F), TLR4-GFP (TLR4G), MD2-Flag (MD2F), CD14, and NF-κB luciferase were pre-treated with curcumin (10, 20, 50 μM), helenalin (5 μM), or resveratrol (50 μM) for 1 h and then treated with LPS (50 ng/ml) for 20 min. Cells were then subjected to immunoprecipitation with anti-mouse GFP antibody and immunoblotted with anti-Flag (upper) or anti-rabbit GFP (lower) antibody. (C) The same Ba/F3 cells in (B) were pre-treated with curcumin (10, 20, 50 μM), helenalin (5 μM), or resveratrol (50 μM) for 1 h and then treated with LPS (50 ng/ml) for 30 min. Cell lysate was subjected to SDS-PAGE and probed with anti-IRAK-1 (upper) or β-actin (lower) antibody. (D) The same Ba/F3 cells in (B) were pre-treated with curcumin (10, 20 μM), helenalin (1, 2 μM) or resveratrol (30, 50 μM) for 1 h and then treated with LPS (5 ng/ml) for an additional 6 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, $p < 0.01$. $^{+}$Significantly different from LPS alone, $p < 0.01$. The panels are representative data from more than three independent experiments. Veh, vehicle; Cur, curcumin; Hel, helenalin; Res, resveratrol.

3.2. *The dimerization of TLR4 and the degradation of IRAK-1 were inhibited by curcumin in Ba/F3 cells stably transfected with TLR4 (Flag or GFP-tagged), CD14, MD2, and NF-κB-luciferase reporter gene*

Therefore, we next determined whether curcumin inhibits ligand-induced dimerization of TLR4. For these studies, we used IL-3-dependent Ba/F3 cells stably transfected with murine TLR4-Flag and TLR4-GFP, MD2, CD14, and NF-κB-luciferase reporter gene as described previously [5]. The induction of the dimerization of TLR4 by LPS was demonstrated in this cell line [5]. We evaluated the dimerization of TLR4 by three criteria: co-immunoprecipitation of TLR4-Flag and TLR4-GFP (Fig. 3B), the degradation of IRAK-1 (Fig. 3C), and the activation of NF-κB (Fig. 3D). The receptor dimerization of TLRs leads to the recruitment of MyD88, and MyD88 in turn recruits IRAK-4. IRAK-4 phosphorylates IRAK-1 leading to its degradation. Thus, the degradation of IRAK-1 and NF-κB activation were used as additional readouts for the dimerization of TLR4. Curcumin inhibited the dimerization of TLR4 (Fig. 3B), the degradation of IRAK-1 (Fig. 3C), and NF-κB activation (Fig. 3D) induced by LPS in a dose-dependent manner. For most of our luciferase reporter assays, a curcumin concentration of up to 20 μM was used since the higher concentrations, like 50 μM, would exert cytotoxicity over 7 h time course. However, for the western blottings of TLR4 dimerization and IRAK-1 degradation (Fig. 3B and C), we could increase curcumin concentration up to 50 μM because the treatment time was only 90 min. Interestingly, another phytochemical sesquiterpene lactone, helenalin (Fig. 3A) also inhibited the dimerization of TLR4, IRAK-1 degradation and NF-κB activation induced by LPS (Fig. 3B–D). However, resveratrol (Fig. 3A) did not inhibit LPS-induced dimerization of TLR4, IRAK-1 degradation, and NF-κB activation in Ba/F3 cells (Fig. 3B–D). Resveratrol can inhibit LPS-induced NF-κB activation by inhibiting TRIF complex in macrophages [25].

3.3. *Ligand-independent dimerization of TLR4 was also inhibited by curcumin*

It is possible that curcumin may interfere with the binding of LPS to TLR4-MD2 complex, and thereby leads to the inhibition

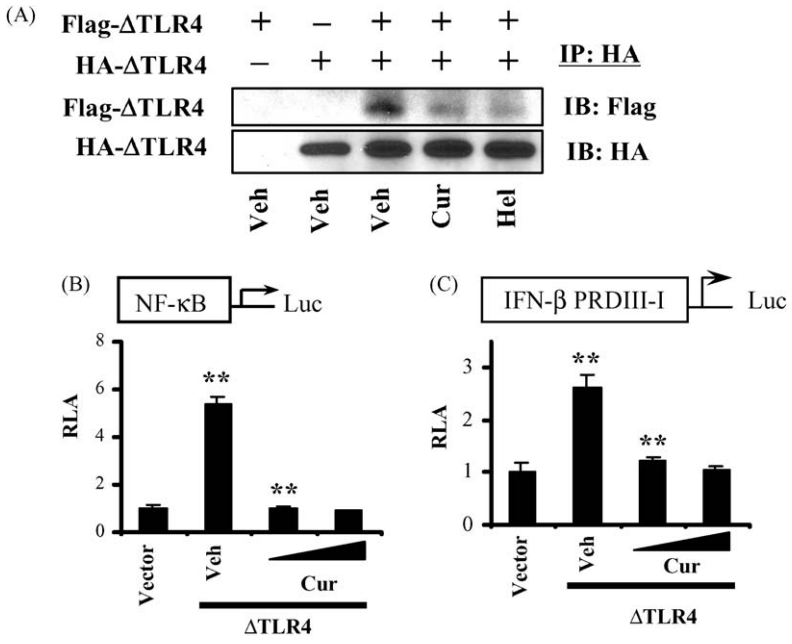

Fig. 4 – Curcumin inhibits ligand-independent TLR4 dimerization. (A) 293T cells were transfected with HA- and Flag-ΔTLR4. After 24 h, cells were treated with curcumin (50 μM) or helenalin (5 μM) for 3 h. Cells were then subjected to immunoprecipitation with anti-HA antibody and immunoblotted with anti-Flag (upper) or anti-HA (lower) antibody. (B, C) RAW264.7 cells were co-transfected with (B) NF-κB- or (C) IFNβ promoter with specific IRF3 binding site (IFNβ PRDIII-I)-luciferase reporter plasmid and an expression plasmid for constitutively active TLR4 (ΔTLR4). pcDNA was used as a vector control for ΔTLR4. After 24 h, cells were treated with curcumin (10, 20 μM) for 6 h. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity. Values are mean ± S.E.M. ($n = 3$). **Significantly different from vehicle control, $p < 0.01$. The panels are representative data from more than three independent experiments. Veh, vehicle; ΔTLR4, constitutively active TLR4; Cur, curcumin; Hel, helenalin.

of dimerization instead of directly inhibiting the receptor dimerization. Thus, we determined whether curcumin inhibits ligand-independent dimerization of TLR4. We previously prepared truncated murine TLR4 (ΔTLR4) lacking the leucine-rich region (LRR) of extracellular domain but with N-terminal 53 amino acids (aa 21–73) that contain two cysteine residues [10]. The gain of function by the truncated TOLL protein lacking LRR domain was also demonstrated in *Drosophila* [26]. This truncated murine ΔTLR4 is constitutively active [10] and can be dimerized without ligand similar to the chimeric constructs in which entire extracellular domain of TLR4 was replaced with either CD4 or integrin subunit [9,11]. Therefore, we determined whether curcumin inhibits the ligand-independent dimerization of TLR4 using this ΔTLR4 construct.

The dimerization of ΔTLR4-Flag and ΔTLR4-HA transfected into 293T cells was inhibited by curcumin and helenalin (Fig. 4A). Curcumin inhibited ΔTLR4-induced NF-κB and IRF3 activation (Fig. 4B and C). The inhibition of ΔTLR4-induced NF-κB activation by curcumin (Fig. 4B) may be due to the inhibition of IKKβ by curcumin; thus, this inhibition may not be a specific readout for the inhibition of ligand-independent dimerization of ΔTLR4. However, since curcumin does not inhibit TRIF or TBK1-induced IRF3 activation (Fig. 2B–D), the inhibition of ΔTLR4-induced IRF3 activation by curcumin (Fig. 4C) suggests that the target of curcumin is located above TRIF: that is ΔTLR4. Together, these results also demonstrate that curcumin inhibits ligand-independent dimerization of ΔTLR4.

4. Discussion

Curcumin has been shown to suppress the activation of NF-κB induced by various pro-inflammatory stimuli by inhibiting IKKβ kinase activity [18]. If IKKβ is the only target of curcumin, curcumin should not inhibit LPS (TLR4 agonist)-induced activation of TRIF pathway of TLR4. LPS induces the activation of NF-κB through both MyD88- and TRIF-dependent pathways, and the activation of IRF3 through TRIF-dependent pathway. Curcumin inhibited LPS-induced NF-κB (Fig. 1A) and IRF3 (Fig. 2A) activation in RAW264.7 cells. This result suggests that curcumin inhibits both MyD88- and TRIF-dependent pathways in LPS-induced TLR4 signaling. In MyD88-dependent pathway, the activation of NF-κB induced by the overexpression of MyD88 or IKKβ in 293T cells was inhibited by curcumin (Fig. 1D). However, in TRIF-dependent pathway, curcumin did not inhibit IRF3 activation induced by overexpression of TRIF or TBK1 in 293T cells (Fig. 2B–D) although curcumin inhibits LPS-induced IRF3 activation (Fig. 2A). Chen et al. also reported that small doses of curcumin inhibited JNK signaling pathway but it did not directly inhibit JNK activity [27]. They suggest that curcumin may inhibit the JNK pathway by affecting the upstream of the MAPKKK level. Together, these results suggest

that the molecular targets of curcumin include TLR4 receptor complex in addition to IKKβ.

LPS induces the dimerization of TLR4, and forced dimerization of TLR4 confers ligand-independent activation of the receptor. These results suggest that the receptor dimerization is required to activate downstream signaling pathways. Therefore, we determined whether curcumin inhibits LPS-induced and ligand-independent dimerization of TLR4. Curcumin inhibited the dimerization of TLR4 induced by LPS (Fig. 3B). Curcumin also inhibited the dimerization of truncated ΔTLR4 that can be dimerized without the presence of the ligand (Fig. 4A). Interestingly, another phytochemical, helenalin also inhibited LPS-induced and ligand-independent dimerization of TLR4 (Figs. 3B and 4A). However, resveratrol did not inhibit LPS-induced dimerization of TLR4 (Fig. 3B). The results showing that curcumin and helenalin inhibit LPS-induced dimerization of full length TLR4 in Ba/F3 cells (Fig. 3B) and the dimerization of truncated ΔTLR4 (Fig. 4A) suggest that these compounds inhibit both ligand-induced and ligand-independent dimerization of TLR4.

Curcumin, helenalin, and resveratrol have been shown to suppress the activation of NF-κB induced by LPS [28–30]. However, resveratrol did not suppress the activation of NF-κB induced by LPS in Ba/F3 cells (Fig. 3C). Previously, we reported that resveratrol inhibited NF-κB activation mediated through only TRIF-dependent pathway of TLR3 or TLR4 [25]. Therefore, this result suggests that Ba/F3 cells may not have the component of the TRIF pathway. Curcumin and helenalin inhibit the dimerization of TLR4 but resveratrol does not. Therefore, the next question is how curcumin and helenalin inhibit the dimerization of TLR4. Fang et al. [31] showed that curcumin binds to catalytically active cysteine residue of thioredoxin reductase by a Michael addition to inhibit enzyme activity. It has been well documented that molecules with the structural motif of α,β-unsaturated carbonyl group can react with biological nucleophiles such as sulfhydryl group by a Michael addition. Both curcumin and helenalin have the structural motif (α,β-unsaturated carbonyl group) conferring the Michael addition. However, resveratrol does not have this structural motif. Helenalin possess two α,β-unsaturated carbonyl structures such as α-methylene–γ-lactone and α,β-unsubstituted cyclopentenones. Thus, bifunctional sesquiterpene lactones (with two α,β-unsaturated carbonyl group) inhibit NF-κB activation 10 times more than monofunctional sesquiterpene lactones (with one α,β-unsaturated carbonyl group) [32]. This may be the reason why helenalin is more potent than curcumin in inhibiting the dimerization, the degradation of IRAK-1, and the activation of NF-κB (Fig. 3B–D).

Many plant polyphenols with inhibitory effects on NF-κB activation contain the structural motif that can react with biological nucleophiles such as sulfhydryl group by a Michael addition [33,34]. The activation loop of IKKβ also contains cysteine residue that is known to be modified by the Michael addition [35]. Curcumin with α,β-unsaturated carbonyl group can react with –SH group in cysteine in the activation loop of IKKβ, and thereby can inhibit the kinase activity of IKKβ. Sesquiterpene lactones also react with two cysteine residues (Cys^{38} and Cys^{120}) in the p65/NF-κB, thereby preventing DNA binding of the transcription factor NF-κB [32,36]. Also, numerous studies demonstrated that certain phytochemicals with α,β-unsaturated carbonyl group interact with sulfhydryl group of cysteine by Michael addition [32–34].

The dimerization of TLR4 was shown to be a prerequisite for the ligand-induced receptor activation [22]. TLRs are Type I transmembrane receptors, consisting of leucine-rich repeats (LRRs) and cysteine-rich region in the extracellular domain and TIR region (Toll/IL-1R homologous region) in the cytoplasmic domain. TLRs have several cysteine residues in both cytoplasmic and extracellular domains, which may form disulfide bonds for the dimerization of the receptors [37]. These results further suggest conceptual possibility that phytochemicals with the structural motif conferring Michael addition may interact with cysteine residue in TLR4, leading to inhibition of TLR4 dimerization.

It is now recognized that enhanced inflammation is one of key etiological conditions in the development of many chronic diseases including cancer, atherosclerosis and insulin resistance. Our results suggest that anti-inflammatory, chemopreventive and other beneficial effects of certain dietary phytochemicals may be at least in part mediated through the modulation of inflammatory responses resulting from TLR activation induced by endogenous molecules or chronic infection.

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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.2006.03.022.

REFERENCES

[1] Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005;17(1):1–14.
[2] Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol 2001;1(2):135–45.
[3] O'Neill LA. TLRs: Professor Mechnikov, sit on your hat. Trends Immunol 2004;25(12):687–93.
[4] Vogel SN, Fitzgerald KA, Fenton MJ. TLRs: differential adapter utilization by toll-like receptors mediates TLR-specific patterns of gene expression. Mol Interv 2003;3(8):466–77.
[5] Saitoh S, Akashi S, Yamada T, Tanimura N, Kobayashi M, Konno K, et al. Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. Int Immunol 2004;16(7):961–9.
[6] Hajjar AM, O'Mahony DS, Ozinsky A, Underhill DM, Aderem A, Klebanoff SJ, et al. Cutting edge: functional interactions

between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. J Immunol 2001;166(1):15–9.
[7] Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol 2001;13(7):933–40.
[8] Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 2002;169(1):10–4.
[9] Medzhitov R, Preston-Hurlburt P, Janeway Jr CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997;388(6640):394–7.
[10] Rhee SH, Hwang D. Murine TOLL-like receptor 4 confers lipopolysaccharide responsiveness as determined by activation of NF kappa B and expression of the inducible cyclooxygenase. J Biol Chem 2000;275(44):34035–40.
[11] Zhang H, Tay PN, Cao W, Li W, Lu J. Integrin-nucleated Toll-like receptor (TLR) dimerization reveals subcellular targeting of TLRs and distinct mechanisms of TLR4 activation and signaling. FEBS Lett 2002;532(1–2):171–6.
[12] Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, et al. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol 2004;5(5):503–7.
[13] Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, et al. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol 2003;171(8):4304–10.
[14] Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;4(5):491–6.
[15] Kawai T, Akira S. Pathogen recognition with Toll-like receptors. Curr Opin Immunol 2005;17(4):338–44.
[16] Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3(10):768–80.
[17] Pan MH, Lin-Shiau SY, Lin JK. Comparative studies on the suppression of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-regulation of IkappaB kinase and NFkappaB activation in macrophages. Biochem Pharmacol 2000;60(11):1665–76.
[18] Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, et al. Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity. J Immunol 1999;163(6):3474–83.
[19] Singh S, Aggarwal BB. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. J Biol Chem 1995;270(42):24995–5000.
[20] Brouet I, Ohshima H. Curcumin, an anti-tumour promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthase in activated macrophages. Biochem Biophys Res Commun 1995;206(2):533–40.
[21] Kang G, Kong PJ, Yuh YJ, Lim SY, Yim SV, Chun W, et al. Curcumin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression by inhibiting activator protein 1 and nuclear factor kappaB bindings in BV2 microglial cells. J Pharmacol Sci 2004;94(3):325–8.
[22] Miyake K. Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. Trends Microbiol 2004;12(4):186–92.
[23] Lee JY, Zhao L, Youn HS, Weatherill AR, Tapping R, Feng L, et al. Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. J Biol Chem 2004;279(17):16971–9.
[24] Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, et al. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. J Biol Chem 2003;278(39):37041–5.
[25] Youn HS, Lee JY, Fitzgerald KA, Young HA, Akira S, Hwang DH. Specific Inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by Resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. J Immunol 2005;175(5):3339–46.
[26] Winans KA, Hashimoto C. Ventralization of the Drosophila embryo by deletion of extracellular leucine-rich repeats in the Toll protein. Mol Biol Cell 1995;6(5):587–96.
[27] Chen YR, Tan TH. Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. Oncogene 1998;17(2):173–8.
[28] Chan MM. Inhibition of tumor necrosis factor by curcumin, a phytochemical. Biochem Pharmacol 1995;49(11):1551–6.
[29] Gertsch J, Sticher O, Schmidt T, Heilmann J. Influence of helenanolide-type sesquiterpene lactones on gene transcription profiles in Jurkat T cells and human peripheral blood cells: anti-inflammatory and cytotoxic effects. Biochem Pharmacol 2003;66(11):2141–53.
[30] Tsai SH, Lin-Shiau SY, Lin JK. Suppression of nitric oxide synthase and the down-regulation of the activation of NFkappaB in macrophages by resveratrol. Br J Pharmacol 1999;126(3):673–80.
[31] Fang J, Lu J, Holmgren A. Thioredoxin reductase is irreversibly modified by curcumin: A novel molecular mechanism for its anticancer activity. J Biol Chem 2005.
[32] Rungeler P, Castro V, Mora G, Goren N, Vichnewski W, Pahl HL, et al. Inhibition of transcription factor NF-kappaB by sesquiterpene lactones: a proposed molecular mechanism of action. Bioorg Med Chem 1999;7(11):2343–52.
[33] Dinkova-Kostova AT, Massiah MA, Bozak RE, Hicks RJ, Talalay P. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. Proc Natl Acad Sci USA 2001;98(6):3404–9.
[34] Siedle B, Garcia-Pineres AJ, Murillo R, Schulte-Monting J, Castro V, Rungeler P, et al. Quantitative structure–activity relationship of sesquiterpene lactones as inhibitors of the transcription factor NF-kappaB. J Med Chem 2004;47(24):6042–54.
[35] Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, et al. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. Nature 2000;403(6765):103–8.
[36] Garcia-Pineres AJ, Castro V, Mora G, Schmidt TJ, Strunck E, Pahl HL, et al. Cysteine 38 in p65/NF-kappaB plays a crucial role in DNA binding inhibition by sesquiterpene lactones. J Biol Chem 2001;276(43):39713–20.
[37] Tao X, Xu Y, Zheng Y, Beg AA, Tong L. An extensively associated dimer in the structure of the C713S mutant of the TIR domain of human TLR2. Biochem Biophys Res Commun 2002;299(2):216–21.