Garlic (Allium sativum) Extract Inhibits Lipopolysaccharide-Induced Toll-Like Receptor 4 Dimerization

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Received July 6, 2007; Accepted November 8, 2007; Online Publication, February 7, 2008
[doi:10.1271/bbb.70434]

Garlic has long been used as a folk medicine. Numerous studies have demonstrated that a garlic extract and its sulfur-containing compounds inhibited nuclear factor kappa B (NF-κB) activation induced by various receptor agonists including lipopolysaccharide (LPS). Toll-like receptors (TLRs) play a key role in sensing diverse microbial products and inducing innate immune responses. The dimerization of TLR4 is required for the activation of downstream signaling pathways, including NF-κB. Therefore, TLR4 dimerization may be one of the first lines of regulation in activating LPS-induced signaling pathways. We report here biochemical evidence that the ethyl acetate fraction of garlic inhibited the LPS-induced dimerization of TLR4, resulting in the inhibition of NF-κB activation and the expression of cyclooxygenase 2 and inducible nitric oxide synthase. Our results demonstrate for the first time that a garlic extract can directly inhibit the TLRs-mediated signaling pathway at the receptor level. These results shed a new insight into understanding how garlic modulates the immune responses that could modify the risk of many chronic diseases.

Key words: garlic; toll-like receptor; cyclooxygenase; nitric oxide synthase

Garlic (Allium sativum) has been considered as a medicinal plant for a long time.1) Garlic contains many organo-sulfur compounds that give the characteristic flavor and potent biological health benefits.2) Several epidemiological studies have demonstrated that a garlic extract and its sulfur-containing compounds had anti-cancer activity.3-5) The consumption of garlic and related sulfur compounds has been reported to reduce carcinogen-induced mammary, colon, lung, stomach, skin and liver cancers.4,9) The mechanisms of garlic for its biological activities have been ascribed to its potent antioxidative,10) antithrombotic1) and lipid-lowering11) activities, and to its stimulating ability for immunological responses.12)

Numerous studies have demonstrated that a garlic extract and its related compounds with anti-inflammatory effects inhibited the nuclear factor kappa B (NF-κB) activation induced by various receptor agonists, including tumor necrosis factor α (TNFα) and lipopolysaccharide (LPS),13,14) and the expression of inducible nitric oxide synthase (iNOS) in activated macrophages.15,16) NF-κB is a central transcription factor for pro-inflammatory gene expression. Increased activity of NF-κB is known to be associated with an enhanced risk of many chronic diseases such as arthritis, atherosclerosis, cardiovascular disease, Alzheimer’s disease and cancer.17) S-allylcysteine, the major water-soluble sulfur compound in a garlic extract, inhibits the NF-κB activation induced by hydrogen peroxide in human T cells.14) The lipid-soluble sulfur compounds such as diallyl disulfide and allicin also inhibit the NF-κB activation induced by LPS in macrophages.13,15) These effects suggest that the
anti-inflammatory and anti-cancer effects of garlic and its sulfur-containing compounds may be mediated through the modulation of NF-κB activation.

Toll-like receptors (TLRs) recognize conserved microbial structural elements and induce innate immune responses through NF-κB- and interferon (IFN)-regulatory factor (IRF)-dependent signaling pathways. There are at least 13 mammalian TLRs. The best characterized is TLR4 which recognizes Gram-negative bacterial product, LPS. TLR2, heterodimerizes with TLR1 or TLR6 to recognize diacyl- or triacyl-lipopeptide, respectively. TLR2 also recognizes various fungal and protozoal products. TLR5 recognizes bacterial flagellin. The anti-viral TLRs are TLR3 that recognizes double-stranded RNA, while TLR7 and TLR8 recognize single-stranded RNA. TLR9 recognizes the CpG motifs that exist in both bacteria and viruses.

Broadly speaking, TLRs can trigger the activation of two downstream signaling pathways: the myeloid differentiation factor 88 (MyD88)- and toll-receptor associated activator of interferon (TRIF)-dependent pathways. All TLRs, except for TLR3, activate the MyD88-dependent pathway, while TLR3 activates the TRIF-dependent pathway. However, TLR4 activates both the MyD88- and TRIF-dependent pathways. MyD88 is an immediate downstream adaptor molecule recruited by activated TLRs and recruits IL-1 receptor-associated kinase (IRAK)-4 and induces IRAK-4 phosphorylation. Phosphorylated IRAK-4 induces the phosphorylation of IRAK-1, leading to the degradation of IRAK-1. Phosphorylated IRAK-1 associates with TNF receptor associated factor 6 (TRAF6), leading to activation of the inhibitor κB (I-κB) kinase (IKK) complex (IKKα/β). IKKβ phosphorylates I-κBα, resulting in the subsequent degradation of I-κBα by 26S proteosome and leading to the nuclear translocation and DNA binding of NF-κB. NF-κB activation mediated through the MyD88-dependent signaling pathway leads to the induction of inflammatory gene products, including cytokines and cyclooxygenase-2 (COX-2).

TLR3 and TLR4 activate the TRIF-dependent signaling pathway, which leads to the expression of type I interferon and IFN-inducible genes. TRIF activates the downstream kinases, TNF receptor-associated factor family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) (also known as T2K) and IKKε (also known as IKKι), leading to the activation of IRF3. Activated IRF3 translocates into the nucleus to regulate the expression of target genes. The activation of the TRIF pathway also leads to the delayed activation of NF-κB. The C-terminal portion of TRIF has been shown to be associated with receptor interacting protein 1 (RIP1) for the delayed activation of NF-κB. Thus, TRIF is likely to use TBK1 and RIP1 for respective IRF3 and NF-κB activation. Since the TLR4 ligand-induced production of inflammatory cytokine was impaired in TRIF-deficient mice, signals from both the TRIF and MyD88 pathways may be required for the maximum expression of cytokines.

Although the medicinal properties of garlic have been intensively studied during the last 100 years, it still remains largely unknown how a garlic extract can mediate beneficial effects. The purpose of the present study is to find the direct molecular targets of a garlic extract having anti-cancer and anti-inflammatory effects. Human TLR4 is the first characterized mammalian Toll and is responsible for delivering the LPS signal. The LPS signal is triggered by dimerization of the cytoplasmic domain of TLR4 with subsequent activation of transcription factor NF-κB. We report here biochemical evidence for a garlic extract inhibiting LPS-induced dimerization of TLR4 resulting in the inhibition of NF-κB activation. A garlic extract can therefore inhibit the LPS-induced expression of such inflammatory gene products as COX-2 and iNOS. These results suggest the important possibility that TLR-mediated inflammatory responses and consequent risk of chronic inflammatory diseases can be modulated by certain dietary components.

**Materials and Methods**

**Reagents.** Garlic was extracted twice with methanol under reflux for 2h. The methanol extract was partitioned between ethyl acetate and water. The ethyl acetate-soluble fraction of garlic (EAG) was evaporated under reflux for 2h, dissolved in DMSO and lyophilized to completely remove the ethyl acetate. EAG was dissolved in DMSO for subsequent experiments. LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA), all other reagents also being purchased from Sigma unless otherwise described.

**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 the NF-κB luciferase reporter gene have been previously described. The cells were cultured in an RPMI 1640 medium containing recombinant murine IL-3 (70 U/ml), 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO-BRL, Grand Island, NY, USA). RAW 264.7 cells (a murine monocytic cell line; ATCC TIB-71, Rockville, MD, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM). The cells were maintained at 37 °C in a 5% CO₂/air environment.

**Transfection and reporter gene luciferase assay.** The NF-κB (2x)-luciferase reporter gene assay was performed as described previously. Cells were co-transfected with a luciferase plasmid and HSP70-β-galactosidase plasmid as an internal control by using the SuperFect transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Luciferase enzyme activities were determined by using the Luciferase Assay System (Promega, Madison, WI, USA).
Nitrite assay. RAW 264.7 cells were seeded at 6 x 10^5 cells/well in 48-well plates for 24 h and were activated by incubating in a medium containing LPS (1 μg/ml) and various concentrations of EAG dissolved in DMSO (0.1% final concentration in the medium) for 20 h with 1% FBS. NO released from the RAW 264.7 cells was assessed by determining the NO_2^- concentration in the culture supernatant. Samples (100 μl) of the culture medium were incubated with 150 μl of the Griess reagent (1% sulfanilamide and 0.1% naphthylethylene diamine in a 2.5% phosphoric acid solution) at room temperature for 10 min in a 96-well microplate. The absorbance at 540 nm was read by using an ELISA plate reader. The concentration of NO was determined by a standard calibration curve prepared with sodium nitrite as the standard.

PGE_2 assay. The level of PGE_2 was determined by using an enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI, USA) according to the manufacturer’s instruction, accumulated PGE_2 in the medium then being assessed. A standard curve was simultaneously prepared in the range of 0.06 to 6 ng/ml of PGE_2.

Immunoblotting. This was performed as previously described. The protein concentration in the lysate was determined by the Bradford method, and equal amounts of the cell extract were subjected to 10% SDS polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane. The membrane was blocked to prevent non-specific binding of antibodies with phosphate-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk. Immunoblotting was performed with the indicated antibodies for COX-2 (Cayman, Ann Arbor, MI, USA), iNOS (BD Bioscience, Franklin Lakes, NJ, USA), IRAK-1 (Santa Cruz, Santa Cruz, CA, USA) and actin (Sigma, St. Louis, MO, USA), and secondary antibodies conjugated to horseradish peroxidase (Cell Signaling, Danvers, MA, USA). The reactive bands were visualized following the recruitment of MyD88. MyD88 recruits IRAK-4 and induces the phosphorylation of IRAK-4. Phosphorylated IRAK-4 phosphorylates IRAK-1, leading to the degradation of IRAK-1 and activation of the IKK complex resulting in the activation of NF-κB. The activation of NF-κB induces the production of inflammatory gene products, including cytokines, COX-2 and iNOS.

To investigate the molecular target of the garlic extract on the TLR4 signaling pathway, we determined the effect of EAG on the LPS-induced activation of NF-κB and the expression of COX-2 and iNOS. Garlic and its sulfur-containing compounds are known to inhibit the NF-κB activation induced by various agonists (LPS, TNFα, and H_2O_2) in macrophages. EAG inhibited the LPS-induced activation of NF-κB and the expression of COX-2 and iNOS in RAW 264.7 cells (Fig. 1A and B). EAG inhibited the LPS-induced production of nitric oxide (Fig. 2A) and PGE_2 (Fig. 2B), which are respective products of iNOS and COX-2 in RAW 264.7 cells.

Inhibition of LPS-induced IRAK-1 degradation by the garlic extract

LPS induces the receptor dimerization of TLR4 following the recruitment of MyD88. MyD88 recruits IRAK-4 and induces the phosphorylation of IRAK-4. Phosphorylated IRAK-4 phosphorylates IRAK-1, leading to the degradation of IRAK-1 and activation of the IKK complex resulting in the activation of NF-κB. The activation of NF-κB induces the production of inflammatory gene products, including cytokines, COX-2 and iNOS.

To investigate the molecular target of the garlic extract on the TLR4 signaling pathway, we determined the degradation of IRAK-1 induced by LPS in macrophages. EAG inhibited the LPS-induced degradation of IRAK-1 (Fig. 3). This result suggests that the molecular target of the garlic extract might have been the upstream signaling components of IRAK-1, including TLR4 itself and/or MyD88.

Inhibition of LPS-induced TLR4 dimerization by the garlic extract

The LPS signal have been reported to be triggered by dimerization of the cytoplasmic domain in TLR4. We determined whether EAG would inhibit the LPS-induced dimerization of TLR4. We used for these studies IL-3-dependent Ba/F3 cells stably transfected with murine TLR4-Flag, and TLR4-GFP, MD2, CD14, and the NF-κB-luciferase reporter gene as previously described. We evaluated the dimerization of TLR4 by co-immunoprecipitation of TLR4-Flag and TLR4-GFP. EAG inhibited the LPS-induced dimerization of TLR4.
TLR4 in a dose-dependent manner (Fig. 4A). The garlic extract was used up to a concentration of 100 μg/ml for the most of our experiments, since higher concentrations exerted cytotoxicity after 20h of incubation with the cells. However, for the TLR4 dimerization experiment, we could increase the concentration of the garlic extract to 200 μg/ml, because the treatment time was only 90 min. The dimerization of TLR4 leads to the recruitment of MyD88. Interaction of MyD88 with the TIR domain of TLR results in the phosphorylation of IRAK-1, leading to its degradation. Phosphorylated IRAK-1 associates with TRAF6, leading to activation of the IKK

Fig. 1. Inhibition of LPS-Induced NF-κB Activation, COX-2 and iNOS Expression by the Garlic Extract. 
A, RAW 264.7 cells were transfected with the NF-κB luciferase reporter plasmid and pre-treated with EAG (20, 50, 75 and 100 μg/ml) for 1 h, before being treated with LPS (1 μg/ml) for an additional 6 h. Cell lysates were prepared, and the luciferase and β-galactosidase enzyme activities were measured as described in the Materials and Methods section. Relative luciferase activity (RLA) was normalized to the β-galactosidase activity. Each value is the mean ± SEM (n = 3). *Significantly different from LPS alone, p < 0.05. B, RAW 264.7 cells were pretreated with EAG (20, 50, 75 and 100 μg/ml) for 1 h and then further stimulated with LPS (1 μg/ml) for 20 h. Cell lysates were analyzed for COX-2, iNOS or actin protein by immunoblotting.

Fig. 2. Inhibition of LPS-Induced Nitric Oxide Production and PGE2 Formation by the Garlic Extract. 
A, RAW 264.7 cells were pretreated with EAG (20, 50, 75 and 100 μg/ml) for 1 h and then further stimulated with LPS (1 μg/ml) for 20 h. Nitric oxide formation was measured as described in the Materials and Methods section. Each value is the mean ± SEM (n = 3). *Significantly different from LPS alone, p < 0.01. B, RAW 264.7 cells were pretreated with EAG (20, 50, 75 and 100 μg/ml) for 1 h and then further stimulated with LPS (1 μg/ml) for 20 h. The prostaglandin E2 (PGE2) level (ng/well) was determined by an enzyme immunoassay, using the conditioned medium from the vehicle and treated cells. Each value is the mean ± SEM (n = 3). *Significantly different from LPS alone, p < 0.05.
complex and NF-κB transcription factor. Thus, the degradation of IRAK-1 and the activation of NF-κB were used to demonstrate the inhibitory effect of the garlic extract on the dimerization of TLR4. The garlic extract inhibited the degradation of IRAK-1 (Fig. 3) and the activation of NF-κB (Fig. 4B) induced by LPS in Ba/F3 cells.

**Discussion**

The major findings of the present study are that the garlic extract inhibited LPS-induced dimerization of TLR4. LPS induces TLR4 dimerization to trigger the activation of downstream signaling pathways. This receptor dimerization activates transcription factor NF-κB, leading to the induction of such inflammatory gene products as COX-2 and iNOS. Many studies have demonstrated that a garlic extract and its sulfur-containing compounds inhibited NF-κB activation induced by various receptor agonists, including TNFα and LPS.13,14) However, the direct molecular targets of garlic to exert an anti-inflammatory effect have not been fully identified. We have demonstrated here that the garlic extract inhibited the LPS-induced dimerization of TLR4 (Fig. 4A), resulting in suppression of the activation of downstream transcription factor, NF-κB (Fig. 4B). Our results therefore suggest a novel mechanism for the garlic extracts to suppress LPS activity and exert an anti-inflammatory effect (Fig. 5).

Garlic contains many organosulfur compounds such as S-allylcysteine, alliin, diallyl disulfide, diallyl trisulfide, ajoene and allicin. Numerous studies have shown that these sulfur-containing compounds had antioxidative, antibacterial, antiviral, antifungal and anticancer proper-
ties. The sulfur compounds showing this biological activity have specific sulfur chemotypes such as thiols, disulfides, thiosulfimates, sulfoxides, and sulfones. These sulfur chemotypes react with the thiol groups to reduce an oxidative stressor such as \( \text{H}_2\text{O}_2 \) to affect the function of redox-sensitive cysteine proteins and to disrupt the integrity of DNA. Allicin containing thiosulfinate chemotypes can react readily with cysteine to yield disulfide and sulfur dioxide. Among the sulfur-containing compounds of garlic, allicin has been extensively studied as an active ingredient of garlic, although there is no report regarding how garlic modulates TLR signaling pathways for its mechanism of biological activity.

We have demonstrated in the previous studies that polyphenols with the \( \alpha,\beta \)-unsaturated carbonyl group inhibited the dimerization of TLR4. It is well documented that molecules with the structural motif of an \( \alpha,\beta \)-unsaturated carbonyl group can react with the sulphydryl group of cysteine by Michael addition. We have also demonstrated that a gold salt, which had thiol binding affinity, inhibited the LPS-induced dimerization of TLR4. Therefore, cysteine residues have been implicated as potential targets for garlic, because the sulfur compounds in a garlic extract have high binding affinity for the thiol group. TLR4 has several cysteine residues in both cytoplasmic and extracellular domains which form disulfide bonds for dimerization of the receptors. It can therefore be speculated that a garlic extract with sulfur compounds may interact with the cysteine residue in TLR4, leading to the inhibition of TLR4 dimerization.

Toll-like receptors play a critical role in the induction of innate immune responses by recognizing invading microbial pathogens leading to the activation of adaptive immune responses. Deregulating the activation of TLRs causes severe systemic inflammation, including septic shock with high mortality. Moreover, chronic inflammation is known to induce various chronic diseases, including atherosclerosis, diabetes, and cancer. Recent studies have suggested the involvement of TLRs in these chronic diseases. TLR4 dimerization is induced by stimulation with LPS. This receptor dimerization may be one of the first lines of regulation in the activation of the TLR-mediated downstream signaling pathway and in the induction of subsequent immune and inflammatory responses. Therefore, disruption of the dimerization of TLR4 would result in the deregulation of TLR activation. Our results have demonstrated that a garlic extract suppressed LPS-induced TLR4 dimerization, suggesting this inhibition to be one of the mechanisms for the anti-inflammatory activity of garlic.

Our study demonstrates for the first time that garlic can modulate inflammatory responses through the suppression of TLR activation. The garlic extract inhibited the LPS-induced dimerization of TLR4, leading to the inhibition of NF-\( \kappa \)B activation and COX-2 and iNOS expression. These results provide new insight into understanding the mechanism by which a garlic extract exerts anti-cancer and anti-inflammatory effects.
Acknowledgments

This work was supported by grant from the Research Center for Women’s Diseases of the Korean Science and Engineering Foundation.

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