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Lipopolysaccharide Receptor, Toll -Like Receptor 4, Mediated Signaling Pathways Leading to Cyclooxygenase-2 Expression in Murine Macrophages.

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LIPOPOLYSACCHARIDE RECEPTOR, TOLL-LIKE RECEPTOR 4, MEDIATED SIGNALING PATHWAYS LEADING TO CYCLOOXYGENASE-2 EXPRESSION IN MURINE MACROPHAGES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Biological Sciences

by

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ABSTRACT

Genetic evidence indicating that Toll-like receptor 4 (TLR4) is the lipopolysaccharide (LPS) receptor in mice was reported. However, biochemical evidence that murine TLR4 confers LPS responsiveness has not been convincingly demonstrated. Inducible cyclooxygenase (COX-2) is selectively expressed in LPS-stimulated macrophages in part mediated through activation of NFκB. Thus, we determined whether murine TLR4 confers LPS responsiveness as evaluated by activation of NFκB and COX-2 expression. Expression of the constitutively active form (ΔTlr4) of TLR4 in murine macrophage cell line (RAW 264.7) is sufficient to activate NFκB and COX-2 expression and to stimulate prostaglandin E₂ synthesis. The truncated form [ΔTlr4 (P712H)] of the missense mutant Tlr4 (P712H) found in LPS-hyporesponsive mouse strain (C3H/HeJ) inhibits LPS-induced NFκB activation and COX-2 expression. Inability of ΔTlr4 (P712H) to activate NFκB and to induce COX-2 expression is rescued by a constitutively active adaptor protein (MyD88) which interacts directly with the cytoplasmic domain of TLR proteins. Furthermore, MyD88 is co-immunoprecipitated with the wild type ΔTlr4, but not with ΔTlr4 (P712H) mutant. Together, these results indicate that TLR4 confers LPS responsiveness in RAW 264.7 cells, and suggest that hyporesponsiveness of C3H/HeJ mice to LPS is due to disruption of TLR4-mediated signaling pathways resulting from inability of the mutant TLR4 (P712H) to interact with MyD88. In addition, the involvement of Lyn, Src-protein tyrosine kinase family, in LPS-induced signaling pathways leading to COX-2
expression was studied. All together, the results from this study may help pave the way
to understanding the signal transduction pathways for COX-2 gene expression.
PART 1

MURINE TOLL-LIKE RECEPTOR 4 CONFERS LIPOPOLYSACCHARIDE RESPONSIVENESS AS DETERMINED BY ACTIVATION OF NFκB AND EXPRESSION OF THE INDUCIBLE CYCLOOXYGENASE

1. REVIEW OF LITERATURE

1.1. Lipopolysaccharide

In the United States alone, 20,000 people die each year as a result of septic shock caused by Gram-negative infection (1,2,3,4). The symptom is characterized by refractory hypotension leading to inadequate organ perfusion, multi-organ failure, and death (5,6). The pathogenesis of gram-negative septic shock is presumed to be due to excess stimulation of host cells by bacterial lipopolysaccharide (LPS) endotoxin (7,8,9). LPS is found on the outer monolayer of the outer membrane of most Gram-negative bacteria so as to work like a tight shield (Fig. 1.1). It consists of the unique molecules, such as lipid A, core oligosaccharide, and O-specific oligosaccharide chain (Fig. 1.2) (10).

Lipid A is a β, 1-6 linked disaccharide of glucosamine which is acylated with R-3-hydroxymyristate at positions 2,3,2’, and 3’, and phosphorylated at positions 1 and 4’. The two R-3-hydroxy-acyl groups of nonreducing glucosamine are further esterified with laurate and myristate. However, various molecular species of lipid A are found in
pathogenic gram negatives. This region gives rise to biological responses that LPS induces (Fig. 1.3) (10,11).

![Diagram showing major components of the gram-negative bacterial envelope.](image)

**Fig. 1.1. Major components of the gram-negative bacterial envelope.** The structure of the two bacterial membranes. An endotoxin-rich outer membrane is an identifying feature of gram-negative bacteria. Lipopolysaccharides or LPS, are cell membrane components of gram-negative bacteria (e.g., *E. coli*). The lipid portion of the outer layer of the outer membrane is exclusively composed of LPS molecules. A single *E. coli* cell contains about 2 million LPS molecules.
Fig. 1.2. Chemical structure of the lipopolysaccharide. It consists of a polysaccharide, or long chain of sugar, and lipid A. The polysaccharide, which varies from one bacterial species to another, is composed of the O-specific chain (O-antigen) and the two-part core (Outer core and Inner core). Lipid A virtually includes two glucosamine sugars modified by phosphate and a variable number of fatty acids. It also has negatively charged phosphate groups.
Fig. 1.3. Predominant molecular species of lipid A found in pathogenic gram-negative bacteria.

<table>
<thead>
<tr>
<th>Bacterium</th>
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<td><em>Salmonella typhimurium</em></td>
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<tr>
<td><em>Hemophilus influenzae</em></td>
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<td><em>Neisseria gonorrhoeae</em></td>
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<td><em>Pseudomonas aeruginosa</em></td>
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Core region of LPS is divided into the inner core and the outer core. The former part is linked to the lipid and the latter is connected to the O-specific chain. The 6’ position of lipid A is glycosylated with eight-carbon sugar, 3-deoxy-D-manno-octulosonic acid (KDO). KDO is found in most endotoxins. Thus, KDO may be a potential target of therapy. Other core sugars include L-glycero-D-manno-heptose, glucose, galactose, and N-acetylglucosamine (11,12). The O-specific chain typically consists of 20-40 repeating saccharides, but the composition is usually different from each bacterial species. This variable region elicits production of different antibodies. While the structure of LPS is highly complex due to these three segments, lipid A is sufficient to evoke the signaling events of LPS. However, the entire lipid component of LPS molecule is needed for optimal activity. The individual elements may combine into a three-dimensional shape to facilitate interaction with the host cells (11,12).

1.2. LPS-induced Signaling Mechanism

Comparatively less is known about intracellular signaling events after LPS stimulation. There is evidence to suggest that G protein (13), phospholipase C (14), protein kinase A, and protein kinase C (15) are involved in LPS responsiveness. However, strong evidence indicates that MAP kinases (MAPKs) signaling cascades play critical roles in LPS signaling. Weinstein et al. first showed that within minutes of LPS stimulation, numerous proteins become tyrosine phosphorylated, in particular MAP kinases p42 (ERK2), p44 (ERK1), and p38 as targets (16,17,18). The c-Jun kinase (JNK) also becomes enzymatically activated within minutes after LPS treatment of macrophages (19). MAPKs require dual phosphorylation: threonine and tyrosine...
residues in TXY consensus sequence must be phosphorylated to be enzymatically activated. Dual specificity protein kinases known as a MAP Kinase Kinases (MAPKKs) or MEKs, which are activated by upstream serine-threonine kinases, MAPKKKs or MEKKs, catalyzes these phosphorylations (20).

Rel family members which is transcription factor, such as NFκB, have been implicated in activation of LPS-inducible gene expression (21). So far, it is believed that MAPK pathway and NFκB pathway are two major signaling events by LPS stimulation. Such signaling events lead to expression and release of a plethora of pro-inflammatory marker-gene products.

1.3. Complex Formation of LPS-LBP and CD14

LSP itself is not intrinsically harmful. Instead, it acts by inducing myeloid and/or nonmyeloid cells to produce a number of proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukins, inducible nitric oxide synthase (iNOS), and COX-2, which lead to fever, inadequate organ perfusion, multi-organ failure, and death observed in septic shock (5,6,15,22,23,24). During the last few decades, two major discoveries have been reported that describe the molecular mechanisms of LPS-induced cell activation. The first was the discovery of LPS binding protein (LBP). LBP is 60 KDa glycoprotein that binds to lipid A of LPS, resulting in LPS-LBP complex (25). The second was the identification of CD14 that acts as a membrane receptor for LPS-LBP complex. CD14 is a 55 KDa protein of myeloid cells. There are two types of CD14, a soluble form (sCD14) and a membrane bound form (mCD14). mCD14 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein, but sCD14 lacks the
GPI-anchor (26). While it was defined that mCD14 is involved in LPS activation of myeloid cells, such as macrophage cells, sCD14 participates in activation of nonmyeloid cell types such as endothelial or epithelial cells that normally do not express mCD14 (27,28). The unique feature of CD14 is the presence of a leucine-rich repeat (LRR) domain in its extracellular region (29). LPS-LBP complex binds to mCD14 on the host cell membrane, and the current understanding is that LPS-LBP bound mCD14 leads to activation of LPS signaling. mCD14 however does not have transmembrane domain to transduce LPS signaling from extracellular environment to cytosol. Thus, it has been a puzzling question as to how mCD14 lacking a transmembrane domain transmits the LPS signaling to a downstream cytoplasmic pathway.

1.4. Genetic Regulation of LPS Response

Decreased sensitivity to the proinflammatory and lethal effects of LPS were subsequently observed in the C3H/HeJ mouse strain (30). C3H/HeJ mice were naturally resistant to LPS preparations from *Salmonella typhosa* 0-901 and *Escherichia coli* 0127:B8, tolerating 20 and 38 times the medial lethal dose, respectively for other C3H sublines that are normally responsive to LPS. In addition, LPS, or Lipid A is not cytotoxic in vitro for macrophages from C3H/HeJ, but directly cytotoxic for those from LPS-responsive mice (31). It was also suggested that B lymphocytes from C3H/HeJ mice was specifically hyporesponsive to LPS, but responded normally to other B-cell stimulators (32). LPS responses were also defective in T lymphocytes and fibroblasts from C3H/HeJ mice (33-35). These results indicated that C3H/HeJ mice were
hyporesponsive to LPS (36). Genetically, it was demonstrated that these LPS hyporesponsive mouse strains have a defective \textit{Lps} allele (\textit{Lps}^d) in chromosomal assignment of the \textit{Lps} locus on chromosome 4, while \textit{Lps}^n is characteristic of most inbred mouse strains that are LPS responsive (37,38)

Two other LPS hyporesponsive mouse strains have been identified, C57BL/10ScCr and its progenitor C57BL/10ScN (also known as C57BL/10ScNCr). Coutinho et al showed that the LPS hyporesponsive phenotype of C57BL/10ScCr was also due to a mutation at the \textit{Lps} locus, and crosses between C57BL/10ScCr (\textit{Lps}^d) and C57BL/10Sn (\textit{Lps}^n) have yielded only the high-LPS responder phenotype, indicating that this \textit{Lps} mutation is recessive (39). In contrast, F1 progeny of crosses between C3H/HeJ and most LPS-responder strains have intermediate responsiveness, suggesting that this may be due to a dominant negative effect of the C3H/HeJ \textit{Lps}^d allele in vivo (40).

Over the last three decades, the nature of the \textit{Lps} mutation has been a central question in molecular basis of LPS hyporesponsiveness. Using several techniques including cDNA selection, genomic sequencing or randomly-subcloned DNA, and comparative mapping, a candidate for \textit{Lps} gene product was identified and assigned as the Toll-like receptor 4 (Tlr4) (41,42). Based on Northern blot and RT-PCR analyses, the LPS hyporesponsive C57BL/10ScCr and C57BL/10ScNCr mouse strains do not transcribe Tlr4. Southern blots of genomic DNA probed with Tlr4 identified a chromosomal deletion involving this gene in the C57BL/10ScCr strain (an LPS hyporesponsive subline) (42). The mouse Tlr4 gene contains one open reading frame of
2505 nucleotides, predicted to encode a protein of 835 amino acids. This protein consists of an extracellular domain formed by a tandem arrangement of 22 leucine-rich repeat motifs connected by a single transmembrane domain to an intracellular signaling domain that shares homology with the Interleukin-1 receptor (IL-1R). Furthermore, nucleotide sequencing of the entire coding region of the Tlr4 gene in endotoxin-tolerant C3H/HeJ mice revealed a single missense mutation consisting of a C to A transversion at nucleotide 2135 predicting a substitution of proline for histidine at codon 712 within the signaling domain [Tlr4 (P712H)]. This missense mutation within the cytoplasmic domain of Tlr4 may be a clue to explain how C3H/HeJ mice are hyporesponsive to LPS (41). Identification of distinct independent mutations of the same gene in these strains of endotoxin-tolerant mice provided compelling evidence that Tlr4 is the gene encoded by Lps (41,42).

1.5. Toll Superfamily (IL-1R/TLR Superfamily)

The experimental approaches to understand Toll were started by the studies on the development of Drosophila embryo. The Toll gene was originally identified in the Drosophila embryo as it plays a crucial role in defining the dorsal-ventral pattern formation (43,44). Cloning and sequencing of the Drosophila Toll gene revealed a family of gene with homology to Toll in mammals, plants, and insects, defined as Toll-like receptors (Tlr) as described in Fig.1.4 (45,46).

Most of Toll gene products are type 1 transmembrane protein containing unique structural properties, such as N-terminal leucine rich-repeat (LRR) extracellular
Fig. 1.4. Schematic comparison of IL-1R/TIR superfamily protein. All members contain a conserved cytosolic region termed the TIR domain (Toll/IL-1R domain). IL-1R subfamily contains extracellular Ig domain and TLR subfamily has the unique extracellular domain, termed Leucine-rich repeat (LRR) domain (47).
domain, a single-pass transmembrane domain, and a C-terminal cytoplasmic signaling domain. The LRR is a widely dispersed motif found in over 70 proteins of diverse function and origin. It has been shown that LRR domain mediates direct protein-protein interactions, cell adhesion and signal transduction (48). But the precise function of LRR in the Toll gene product is unknown. The cytoplasmic signaling domain is highly homologous to that of the interleukin-1 receptor (IL-1R), termed the Toll/IL-1R homology domain (TIR domain) (49). The TIR domain is responsible for cytoplasmic signal transduction of Toll gene products. Due to the conserved TIR domain between Toll gene product and IL-1R, Toll-like receptors were also named IL-1R/TLRs.

Expressions and functions of IL-1/TLR superfamily proteins have been studied in insects, plants, and mammals such as human and mouse. As shown in Fig. 1.4, in Drosophila, the Toll-family proteins have been identified: Toll, 18-wheeler (18W), Mst, and STSDm2245 (44,50,51). When Toll was identified first, it was thought to participate only in the establishment of dorso-ventral polarity in the developing Drosophila embryo (44). However another Drosophila Toll family protein, 18-wheeler, was identified to participate in antibacterial responses in specifically adult fly by activating the REL protein Dorsal-like immunity factor (Dif) (52).

In plants, IL-1R/TLR family proteins such as N (DRgN) (53,54), L6, M (55), and Rpp5 (56) were identified. While these plant IL-1R/TLR superfamily proteins are not transmembrane receptors, but cytosolic proteins, they have TIR domain and LRR domain (53). The cellular functions of Toll gene products in plants are also involved in innate immunity against invading pathogen. N protein from tobacco activates an
enhanced response in the plant during infection, which leads to necrosis of infected plant tissue, thereby halting pathogen growth and spread (53,54). L6 and M in flax are required for resistance to flax rust (55). Several other plant proteins with similarity to TLRs are involved in disease resistance, including Rpp5 in *Arabidopsis* (56). In mammals, so far, ten Tlr genes were identified from human and mouse, respectively (57,58). The mammalian Tlrs are transmembrane protein, composed of LRR ectodomain, transmembrane domain, and cytosolic TIR domain.

While the cytoplasmic domains and TIR domains are highly conserved between Tlrs and IL-1R, the ectodomains are totally different. Tlrs contain LRR ectodomain, however all of IL-1R subfamily proteins have immunoglobulin (Ig) domains in their extracellular regions (59). Recently, it has been one of the most intensive research areas to elucidate what is the role of the mammalian Tlr proteins. While their cellular function was not well established, based on the genetic evidence resulting from the LPS-hyporesponsive mice as mentioned above, it is believed that Tlr4 might be involved in transducing the LPS-stimulated signaling event. Meanwhile, Yang et al. (60) and Kirschning et al. (61) showed that human Toll-like receptor 2 (Tlr2) mediates LPS-induced cellular signaling by in vitro transfection system with human embryonic kidney 293 cells, as measured by activation of NFκB or induction of a NFκB-responsive reporter gene. Kirschning et al also showed that human TLR4 can constitutively activate NFκB, but fails to confer LPS-responsiveness (61). Thus, it could be assumed that TLR2 would be a receptor for LPS. However, it was still controversial whether TLR2 is LPS receptor or not, because the genetic evidences
describing TLR4 is more relevant to explain the phenotype of LPS-hyporesponsive mice. In addition, the activation of NFκB and expression of certain NFκB-induced gene products were impaired in LPS-stimulated macrophages derived from C3H/HeJ mouse strain (62,63). Furthermore, Chow et al. identified that human Tlr4 transfection into 293 cells induced LPS response in CD14 dependent manner as measured by the expression of NFκB-luciferase reporter gene that was activated by LPS. Furthermore, they showed that LPS antagonist, E5531, blocked Tlr4-mediated LPS response implying Tlr4 is the LPS receptor (64). Faure et al. showed that overexpression of the mutant Tlr4 derived from C3H/HeJ mice in human dermal endothelial cells results in inhibition of LPS-induced NFκB activation (65). Thus, conclusive biochemical evidence is still needed to show that murine Tlr4 indeed confers LPS responsiveness.

1.6. Signal Transduction of IL-1R/TLR proteins

1.6.1. Mammalian Toll-like Signaling Pathways

1.6.1.1. IL-1R Signaling Cascade

IL-1R/TLR proteins are ligand-stimulated receptor molecules that elicit their pleiotropic effects through activation of the transcription factors NFκB and AP-1. The cytoplasmic domain of Tlr proteins (i.e. the TIR domain) is homologous to that of IL-1R, although the extracellular Ig domain in IL-1R is quite different from the LRR domain in Tlr. Thus, the investigation for the cellular mechanism from Tlr activation was approached on the basis of understanding IL-1R signaling pathway. IL-1R requires another IL-1 receptor family, IL-1 receptor accessory protein, (IL-1AcP) to be activated. IL-1R engagement by ligand induces dimerization of IL-1R and IL-
AcP (66,67). IL-1AcP has a critical role in IL-1R activation, recruiting the downstream signaling molecules: Toll-interacting protein (Tollip) and IL-1 receptor associated kinase (IRAK). IL-1AcP, not IL-1R, directly interacts with IRAK (68,69). Recently, Volpe et al. showed that Tollip is present in a complex with IRAK, and the Tollip-IRAK complex is recruited to the activated receptor complex through the association of Tollip with IL-1AcP. Meanwhile, Burns et al. showed that an adapter protein, myeloid differentiation factor 88 (MyD88), was co-immunoprecipitated with the activated IL-1R in an IL-1-dependent manner. MyD88 has N-terminal death domain (DD) that associates with another DD in IRAK and with the C-terminal TIR domain to interact with the cytoplasmic TIR domain of IL-1R/Toll superfamily protein (70). Such co-recruitment of MyD88 and Tollip-IRAK complex allows for MyD88-IRAK interaction through their death domain and then triggers IRAK autophosphorylation. In turn, the phosphorylated IRAK is dissociated from Tollip, leading to the association with another signaling molecule for transmission of the receptor mediated signal (71). Indeed, it has been demonstrated that using co-immunoprecipitations in IL-1 treated cells, IRAK directly interacts with TRAF6 which is a member of the TNF receptor-associated factor (TRAF) family. The family of proteins is characterized by a ring and zinc finger motif in their N terminal and a conserved C-terminal TRAF domain (72). Six TRAF family members, including TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, and TRAF6, have been identified and the C-terminal TRAF domain is involved in receptor association and homo- and hetero-oligomerization of TRAFs and serves as a docking site for other signaling proteins.
The N-terminal zinc binding domains are involved in mediating downstream signaling events (75,76). Green et al. showed that a dominant negative mutant form of TRAF6 inhibited NFκB activation by IRAK and IL-1. Thus, TRAF6 is a downstream signaling molecule of IRAK (77). Song et al. showed the association of TRAF family members with NFκB inducing kinase (NIK) through the conserved “WKI” motif within the C-terminal TRAF domain (74). NIK is a novel member of the mitogen activated protein kinase kinase kinase family (78). It was originally identified as a TRAF2-interaction protein and subsequently found to interact with all TRAF molecules, except TRAF4 (73). The overexpression of NIK potentially activates NFκB (73,78) and the dominant negative mutant form of NIK (NIK-KM) inhibited NFκB activation by TNF, interleukin-1, CD27, human T-cell leukemia virus type 1 TAX, and Epstein-Barr virus-transforming protein latent infection membrane protein 1 (73,78-82). These results indicate that NIK participates in activating NFκB-inducing signaling cascade.

Consequently, the activated NIK activates inhibitor κB (IκB) kinase (IKKα and IKKβ) complex and activated IKKs phosphorylate IκB, resulting in the ubiquitin mediated degradation of IκB (83.84). IκB makes a complex with NFκB to keep NFκB inactivated. Thus, IκB degradation leads to release the free NFκB molecule. These events ultimately result in the translocation of NFκB to the nucleus and the initiation of gene transcription. It was suggested that NIK, IKK, and IκB are part of a large complex of proteins, with a molecular weight in the region of 900 KDa, which has been termed the signalsome (85,86). Together, IL-1 induced signaling pathway is following the
cascade: IL-1 induced IL-1R-IL-1RcP complex/ MyD88/ IRAK/ TRAF6/ NIK/IKK/ IκB/NFκB (Fig.1.5).

Recently, a new molecule designated Evolutionarily-Conserved Signaling Intermediate in Toll pathways (ECSIT) was identified. ECSIT is specific for the IL-1R/TLR signaling pathways including IL-1R signaling and appears to bridge TRAF6 to MEKK1 by directly interacting with TRAF6 and MEKK1 (87). MEKK1 is a 195KDa protein that is believed to be activated upon proteolytic cleavage by a caspase into an active 80 KDa fragment (88). The activation of MEKK1 mediated by ECSIT leads to the activation of JNK. Together, the activation of IL-1R/TLR signaling pathway provides the activation of both AP-1 and NFκB.

1.6.1.2. Toll-like Receptor 4 Signaling Cascade

The cytoplasmic domain TLR is highly similar to that of IL-1R, and these regions are referred to as Toll/IL-1R (TIR) domain. This TIR homology suggests that TLR proteins may activate similar signaling pathway (57). Indeed, it has been demonstrated that TLR signaling pathway takes advantage of the same signaling components as IL-1R signaling pathway (46). However, the involvement of Tollip was not verified for TLR-mediated signaling cascade, although it was shown for IL-1R signaling pathway. While post-receptor signaling pathways share the same signaling components, the receptor activation procedures are different between Tlr and IL-1R. LPS interacts with LBP to give rise to LPS-LBP complex which binds to CD14 (89).
Fig. 1.5. The parallel and conserved signaling pathways in mammalian TNFR, IL-1R, TLR, and Drosophila Toll derived NFκB activation. The activation of NFκB signaling pathway by mammalian TNFR, TLR, and IL-1R shares the same signaling molecules and it is conserved between mammalian NFκB activating pathway and Drosophila Toll derived pathway. LPS, lipopolysaccharide; LBP, LPS binding protein; TNF; tumor necrosis factor; TNFR, tumor necrosis factor receptor; TRADD, TNF receptor associated death domain; RIP, receptor interacting protein; IL-1R, interleukin-1 receptor; MyD88, myeloid differentiation factor 88; IRAK, IL-1 receptor associated kinase; TRAF, TNF receptor associated factor; NIK, NFκB inducing kinase; IKK, IκB kinase; IκB, inhibitor κB; NFκB, nuclear factor κB; DLAK, Drosophila LPS-activated kinase; PM, plasma membrane.
However, CD14 can not transduce LPS signaling through the cell membrane, because CD14 does not have a transmembrane domain. Thus, it is believed that CD14 functions as the co-receptor for Tlr4. Indeed, Chow et al. showed that the presence of CD14 and human Tlr4 induced dramatically LPS-stimulated NFκB reporter gene expression, while human Tlr4 alone induced the reporter gene expression slightly (64). Meanwhile, in the case of Tlr2, Yang et al. demonstrated that CD14 co-immunoprecipitated with Tlr2 in the presence of LPS. They also showed that Tlr2 forms a homocomplex through its extracellular LRR domain of TLR2 (90). However, it is not clear if Tlr4 also forms a homocomplex (or heterocomplex) by LPS stimulation as Tlr2 does. Furthermore, it remains uncertain if CD14 even interacts with Tlr4, although it is assumed that LPS/LBP/CD14 complex interacts with Tlr4. Meanwhile, Kimoto et al. showed that LPS response mediated through Tlr4 requires the presence of MD2, a cell surface protein that co-immunoprecipitated with Tlr4 (91). Chow et al. also demonstrated that when MD2, a protein associated with the extracellular domain of Tlr4, was expressed, there was a marked increase in Elk-1 activity as well as ERK, JNK, and p38 MAP kinase phosphorylation in response to LPS. They also showed that Tlr4 mediated NFκB reporter activity and IL-8 production was enhanced by the expression of MD2 (92). In addition, Miyake et al. showed that MD2 expression in Tlr4-expressing cells enhanced LPS-induced NFκB activation (93). Together, these results imply that MD2 interacts with Tlr4 and with LPS stimulation. All these components form the complex of LPS/LBP/CD14/MD2/Tlr4, as described in Fig.1.6.
1.6.2. *Drosophila* Toll Signaling Pathway

On *Drosophila* Toll, recent studies revealed the striking similarities between *Drosophila* Toll signaling pathway and mammalian Tlr signaling pathway (Fig. 1.5). The current model of Toll signaling pathway proposes that this receptor is activated by ligand-dependent receptor dimerization, induced by an endogenous peptide ligand, Spatzle, which is proteolytically activated by the protease, Easter (94,95). The activated Toll recruits the adapter protein, Tube, and the serine-threonine kinase, Pelle. Tube and Pelle are homologous to MyD88 and IRAK, respectively, in mammalian Tlr signaling cascade (Fig.1.5) (96). Pelle interacts with an additional downstream molecule, the *Drosophila* TRAF6 (dTRAF6). The activated dTRAF6 results in the activation of the *Drosophila* IKK, *Drosophila* LPS-Activated Kinase (DLAK), which phosphorylates *Drosophila* IκB, called Cactus. Like the role of IκB in mammals, Cactus interacts with *Drosophila* NFκB like proteins, Dorsal and dorsal-like immunity factor (Dif) to keep them inactivated. Consequently, the activation of Cactus by DLAK releases Dorsal or Dif. The free Dorsal or Dif translocates to the nucleus where they induce gene transcription (97). Recently, Kopp et al. identified *Drosophila* ECSIT (dECSIT) which interacts with dTRAF6 as in mammal (87). Thus, it is clear that *Drosophila Toll* signaling pathway is similar to the mammalian Tlr signaling cascade both in terms of structure and function.
Fig. 1.6. TLR4 activated signaling pathways mediated through NFκB and JNK activation. PM, plasma membrane; JNK, C-Jun N-terminal kinase; TIR, Toll/IL-1R domain; Tollip, Toll-interacting protein; ECSIT, evolutionarily-conserved signaling intermediate in Toll pathways; DD, death domain.
1.7. Innate Immunity: Biological Significance of IL-1/TLR Superfamily

The significance of Toll gene products has been underscored because they are involved in innate immunity. In the *Drosophila* embryo, Toll participates in dorsal-ventral development, but in adult flies, Toll induces the antifungal peptide, drosomycin, which is mediated through the activation of Dorsal (98). In comparison with Toll, 18-wheeler causes the expression of the anti-bacterial peptide, attacin, through the activation of Dif. While Dorsal and Dif are *Drosophila* NFkB proteins, Dorsal is selectively activated by Toll and Dif is specifically activated by 18-wheeler (98,99).

Several cytoplasmic plant disease-resistance genes with sequence homology to members of Tlr family have also been cloned and shown to mediate host defense against specific viral and fungal plant pathogens as previously mentioned (54,100). So far, 400 peptides have been reported to participate in innate immunity, not only of insects, but also of all multicellular organisms that were investigated, including human and plants. Among these peptide, Defensins are representative. Defensins have wide spectra of activity directed against various bacteria, fungi, and enveloped viruses (101,102). Although the molecular mechanisms are not fully understood, they may involve the transient appearance of channel-like structures. Defensins and most other antimicrobial peptides act by permeabilizing the cell membranes of microorganisms, resulting in the efflux of solutes (103). As in *Drosophila*, antimicrobial peptides also counter infection in mammals (102,103). α-Defensins are major constituents of the microbicidal granules of blood granulocytes and are also abundantly expressed in
intestinal epithelial cells specialized for host defense functions. A constitutively expressed human epithelial β-defensin is abundant in the kidney and the urogenital tract, and an infection- or cytokine-inducible β-defensin is abundant in the skin. In addition to defensins, mammals produce cathelicidins which is a group of myeloid antimicrobial peptides (102,104). In addition, the complement cascade is one of the major roles in mammalian innate immunity. It is activated either directly or indirectly by microorganisms and leads to the phagocytosis through opsonization or the assembly of a pore-forming membrane attack complex on the surface of microorganism (105).

The innate immune system that is an earlier evolutionary form of host defense serves the second function, such as stimulating and orienting the primary adaptive immune response by controlling the expression of co-stimulatory molecules. Indeed, mammalian Tlr activation leads to expression and release of a plethora of cytokines, such as interleukins. These cytokines play direct or indirect role in adaptive immune system. Based on recent data on Tlr protein, it is suggested that Tlr4 is involved in LPS-induced signaling pathway. In contrast to Tlr4, it was suggested that Tlr2 is the receptor for bacterial lipoprotein (106). Recently, Hemmi et al. demonstrated that TLR9 is activated by bacterial CpG dinucleotide (58). Such cellular function of Tlr proteins implies that Tlr protein works as the tollgates for pathogen selection.

It is clear that disruptions in innate immunity predispose human to infection as described in the following examples. In the severely burned patient, the disruption of skin as not merely a barrier, but an organ adorned with antimicrobial peptides and first-line effector cells like macrophages, poses great risks to infection. In patients with
cystic fibrosis, the alterations in salinity of the bronchial airway fluid appear to disable the function of antimicrobial peptides that are found in the respiratory epithelium, thereby leading to colonization and infection with organisms like Staphylococci and Pseudomonas (107,108). As Toll-LBP-CD14 pathway was elucidated, new targets that modify these pathways may prove effective in designing drugs for the treatment of septic shock. Finally, the ability to produce large amounts of both insect and mammalian antimicrobial peptides may provide new classes of antibiotics.

1.8. Eicosanoid

‘Eidosanoid’ is a term to describe compounds derived from C₂₀ fatty acids and the term is now generally used to identify the paracrine hormones derived from free arachidonic acid (109). The eicosanoids are divided into two major groups. One is the leukotrienes that are formed by the initial conversion of free arachidonate to leukotriene (LTA₄) by the enzyme 5’-lipoxygenase. Subsequent metabolism of LTA₄ to a variety of other bioactive compounds (e.g. LTB₄, LTC₄) is determined by the presence or absence of enzymes that further metabolize LTA₄ and its products (Fig.1.7). The other is the prostnoids that are formed by the initial conversion of arachidonate to a common intermediate, PGH₂, by the enzyme prostaglandin H synthase (PGHS). Prostaglandin synthase is able to carry out two distinct, sequential biochemical reactions. The cyclooxygenase activity of PGHS converts free arachidonic acid to prostaglandin G₂ (PGG₂) that is then converted to PGH₂ by the hydroperoxidase activity of PGHS.
Fig.1.7. The pathways of prostanoid and leukotriene biosynthesis. The activation of cPLA$_2$ by any extracellular stimuli releases arachidonic acids from membrane phospholipids. The released AA is converted to LTA$_4$ by 5'-lipoxygenase and PGH$_2$ mediated through PGG$_2$ by cyclooxygenase (Prostaglandin H synthase, PGHS). The presence or absence of the appropriate enzymes determines the production of the subsequent leukotrienes and prostanoids. PG, prostaglandin; LT, leukotriene; cPLA$_2$, cytosolic phospholipase A$_2$. 

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PGH₂ is the common precursor for all the prostanoids. The subsequent synthesis of the prostanoids, such as the prostaglandins (PGs), thromboxanes, and prostacyclins, in various tissues and cells is determined by the presence of the biosynthetic enzymes that convert PGH₂ to the various prostanoids. PGs play a role for normal physiology including motility, vascular tone, angiogenesis, mucosal protection, and immune responsiveness (110). PGs are also important mediators of inflammation. Thromboxanes mediate a platelet aggregation, while prostacyclin (PGI₂) produced in endothelial cells inhibits a platelet aggregation (111, 265). PGs are synthesized and secreted by a wide variety of cells only when stimulated by a multitude of cell perturbations, ranging from mechanical to chemical stimuli. Once released, PGs act as autocrine or paracrine factors to regulate the functions of various differentiated cells. The ubiquitous presence of PG-metabolizing enzymes makes these mediators unstable and their actions short-lived. Due to the short half-lives of PGs in general, the levels and activities of PG synthetic enzymes determine the bioactivities of PGs within tissues (112, 113).

In arthritis, PGs contribute to synovial inflammation by increasing local blood flow and potentiating the effects of mediators such as bradykinin and interleukin (IL)-1 that induce increases in vasopermeability (114). PGs also may induce osteoclastic bone resorption, suggesting a mechanism through which they may contribute to joint erosion (115). In patients with inflammatory joint disease, there is an increase in synovial and cartilage PGHS activity (116).
1.9. Cyclooxygenase (COX)

Prostaglandin H synthase (PGHS) or Cyclooxygenase (COX) is the key enzyme that catalyzes the conversion of arachidonic acid and other eicosanoids. COX has two distinct enzyme activities: a cyclooxygenase activity to catalyze the conversion of arachidonic acid to PGG₂, and a peroxidase activity to catalyzes the conversion of PGG₂ to PGH₂. Consequently, PGH₂ is converted to PGD₂, PGE₂, PGF₂α, PGI₂, or thromboxane A₂ (TXA₂) by distinct synthases. In addition, several results showed that the cyclooxygenase and peroxidase sites are physically and functionally separate within the enzyme (117-119). Mizuno et al demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) compete directly with arachidonate for binding to the cyclooxygenase site and inhibit cyclooxygenase activity, but have no effect on peroxidase activity (120).

Two isoforms of the enzyme have been characterized: a constitutive COX-1 and a mitogen-inducible COX-2 (121-123). At the deduced amino acid level, the COX-1 and COX-2 polypeptides are 61% identical (119, 124). Two N-terminal N-linked glycosylation sites, the axial and distal heme-ligating histidines, active-site tyrosine, and aspirin-modified serine are conserved. In addition, both polypeptides end with the sequence STEL, as sequence that is reminiscent of the endoplasmic reticulum retention sequence KDEL. The 3'-untranslated region (UTR) of the COX-2 transcripts is estimated to be 2.5 kb in size. The human COX-2 mRNA contains at least 12 copies of the AUUUA, “Shaw-Kamen” motif that is implicated in the rapid degradation of many cytokine and oncogene mRNAs (125). The COX-1 protein contains a 17 amino acid sequence near its amino terminus that is not present in the inducible COX-2 protein.
In contrast, COX-2 contains an 18 amino acid sequence near its carboxyl terminus that is not present in the COX-1 molecule and a putative N-linked glycosylation site is found at the C-terminal unique 18 amino acids (Fig. 1.7). The presence of this 18 amino acid epitope has been used to prepare antibodies that can distinguish the COX-2 protein from the COX-1 protein in immunoblot assay (127).

COX-1 is the constitutive form of the enzyme and ubiquitously expressed in most cells. It provides certain homeostatic functions, such as maintaining normal gastric mucosa and aiding in blood clotting by abetting platelet aggregation (128). COX-2 is the inducible form and expressed in response to inflammatory and other physiologic stimuli, growth factors, tumor promoters, hormones, bacterial endotoxins, and cytokines (129-132). Although both COX-1 and COX-2 convert arachidonic acid to prostaglandins, there are many differences between the COX isoforms. One major distinction is that COX-2 is the principal isoform that participates in inflammation (133). Of the two isoforms, the inducible COX-2 enzyme is thought to make the more important contribution to synthesis of PGs at sites of inflammation as suggested by studies of cultured inflammatory cell (22) or of synoviocytes from rheumatoid arthritis and osteoarthritis patients (134). COX-2 expression is inhibited by glucocorticoids such as dexamethasone at the level of transcription and translation, while these agents have little effect on the expression of COX-1 (135, 136, 289). COX-1 is also not upregulated by inflammatory or other stimuli. It leads to a steady state level of COX-1 responsible for the production of basal levels of PGs (137). Together, those results
**Fig. 1.8. Schematic comparison of COX-1 and COX-2 proteins.** A constitutive COX-1 and a mitogen-inducible COX-2 have been characterized. The COX-1 and COX-2 polypeptides are 61% identical. Two N-terminal N-linked glycosylation sites (circle N), the axial and distal heme-ligating histidines (circle H), active-site tyrosine (circle Y), and aspirin-modified serine (circle S) residues are conserved. In addition, both polypeptides end with STEL similar to the ER retention sequence KDEL. The 3' UTR of the COX-2 transcripts contains at least 12 copies of the AUUUA, “Shaw-Kamen” motif that is implicated in the rapid degradation of many cytokine and oncogene mRNAs. The constitutive COX-1 protein contains a 17 amino acid sequence near its amino terminus that is not present in COX-2 protein. In contrast, COX-2 contains an 18 amino acid sequence near its carboxyl terminus that is not present in the COX-1 molecule. The presence of this 18 amino acid epitope has been used to prepare antibodies that can distinguish the COX-2 protein from the COX-1 protein.
suggest that COX-2 is responsible for production of prostaglandins involved in inflammation.

Another distinction between COX-1 and COX-2 involves their differential expression. The two isoforms are encoded by genes located on separate chromosomes. COX-1 is located on human chromosome 9, while COX-2 is on human chromosome 1 (131,138). Similarly, in the case of mouse, COX-1 and COX-2 genes map to distinct chromosomes (Ch. 2 and Ch. 1, respectively). The COX-1 and COX-2 genes are similar in structure. The COX-2 gene contains ten exons and nine introns, but the exon encoding the hydrophobic 17 amino acid sequence in COX-1 is missing in the COX-2 gene. However, the COX-2 gene is substantially smaller that the COX-1 gene: COX-2 gene is approximately 8 kb, while the COX-1 gene is about 25 kb (139, 140).

COX-1 gene is a developmentally regulated “housekeeping” gene and lacks a TATA box. While it is not well known about the details of the regulation of COX-1 gene expression, COX-2 gene is characterized as an immediate-early gene (141). While Ristimaki et al suggested that post-transcriptional regulation contributes to the magnitude and duration of COX-2 mRNA expression in other systems (142), the cis-acting elements in 5'-flanking region of COX-2 gene are crucial for modulating COX-2 gene expression. The transcriptional activation of the COX-2 gene appears to be the major mechanism for increasing COX-2 expression. The COX-2 promoter contains a TATA box. Several relevant enhancer sequences, such as CRE, NFκB, NF-IL6, E-box, AP-2, SP-1, have been identified in the human and murine COX-2 gene promoter as shown in Fig.1.9 (143,144). In case of murine COX-2 promoter, there are sequences
Fig. 1.9. Organization of the putative cis-acting elements in the 5'-flanking region of the COX-2 gene from mouse and human.
that match binding sites for SP-1 and NF-IL6 within the first 370 bp. Between nucleotides -56 and -48, there are overlapping ‘CRE’ and ‘E-Box’ sequences (Fig. 1.9). The CRE sequence is associated with binding of transcription factors of the ATF family (CREB, ATF-2, ATF-3, etc), while the E-box is associated with the binding of members of the Myc, Max, MyoD, and USF transcription factor family. In bovine endothelial cells, a NF-IL6 (CEBPβ) site is responsible for induction of COX-2 by lipopolysaccharide and tumor-promoting phorbol ester while the same site is responsible for tumor necrosis factor α-mediated induction of COX-2 in MC3T3-E1 cells (145,146). An E-box sequence is essential for basal transcription and for luteinizing hormone- and gonadotrophin-releasing hormone stimulated transcription (147). A cAMP response element (CRE) mediates the effect of SRC protein on COX-2 expression in fibroblasts (148). COX-2 expression can be induced through multiple signaling pathways involving protein kinases A and C, tyrosine kinases, phosphatases, bacterial endotoxin (LPS), and Src. The crucial signaling pathway for COX-2 gene expression appears to be dependent on the cell type and stimulants (108,130,149). In addition, two putative NFκB binding elements were found in both human and mouse COX-2 promoters (Fig. 1.9 and 1.10). Recently, the requirement of NFκB binding element for COX-2 expression is controversial.

Prostanoid production by COX-i and COX-2 appears to be initiated through distinct signaling pathways that may rely on the activation of different phospholipases (122). While COX-1 is found in equivalent concentrations in both the nuclear envelope
Fig. 1.10. Putative NFκB binding elements in the 5'-flanking region of COX-2 gene. Two NFκB binding elements were found in human and mouse COX-2 promoter. The consensus sequence of NFκB binding site is 5'-GGGRNNYYCC-3', where R is A or G, Y is T or C, and N is any nucleotide (150, 151).
and the endoplasmic reticulum membrane, the concentration of COX-2 within the nuclear envelope is approximately twice the concentration in the endoplasmic reticulum membrane. In addition, products of COX-2, but not COX-1, is localized to the nucleus, which suggests that COX-2 may independently signal to the nucleus (152). Although COX-2 is usually not detectable during normal physiologic conditions, Cryer et al. reported that there is also some constitutive expression of this isoform in the rat kidney and brain, human prostate and lung, bone, and female reproductive system (267). COX-2 expression also occurs in transformed or cancerous cells. The first COX-2 cDNA was initially characterized on the basis of its elevated expression in Rous sarcoma virus transformed chicken embryo fibroblasts (153). Furthermore, Eberhart et al. and Kargman et al. showed that the constitutive overexpression of COX-2 has been detected in colon carcinomas (154,155). The two major substrates for both COX-1 and COX-2 are arachidonic acid and dihomo-\(\gamma\)-linolenic acid. They are also capable of catalyzing the oxygenation of eicosapentaenoic acid (EPA), \(\gamma\)-linolenic acid, \(\alpha\)-linolenic acid, and linoleic acid (156,157). EPA is converted to \(\text{PGH}_2\), whereas the 18-carbon fatty acids are converted to monohydroxy acids. There are subtle differences in substrate specificities between COX-1 and COX-2. In general, 18-carbon polyunsaturated fatty acids are more efficiently oxygenated by COX-2 than by COX-1. Docosahexaenoic acid (DHA, 22:6n-3) is a competitive inhibitor of both COX-1 and COX-2 without being a substrate for either enzyme (158).
1.10. The Signaling Pathways Leading to COX-2 Gene Expression

While the expression of COX1 is generally constitutive in most cells, COX-2 is rapidly and transiently induced by a variety of factors, such as cytokines, hormones, and tumor promoters, which activate various signaling pathways. Among various signaling pathways, G protein-mediated pathways, as well as the protein kinase C (PKC)-mediated pathway activated by phorbol 12-myristate 13-acetate (PMA) and the tyrosine kinase-mediated pathways activated by both growth factor receptors and Src kinase, can stimulate the expression of COX-2 gene (159,294,295). The 5'-flanking region of COX-2 was identified from human (160) and mouse (161). Regardless of the species, the common cis acting elements in COX-2 promoter region are canonical TATA box, CRE, NF-IL6 (C/EBPβ), AP2, SP1, NFκB, and GATA boxes. Various transacting elements should be activated via phosphorylation by a variety of signaling pathways so that those cis acting elements enhance the transcription of COX-2 gene. Therefore, it is quite important to understand various signaling pathways leading to activation of transcription factors for COX-2 gene expression. However, the signaling pathways leading to COX-2 gene expression depend on different cell types. But, several well-established signaling pathways for COX-2 gene expression were suggested, as described in Fig.1.11.
Fig. 1.11. The comprehensive signaling pathways leading to COX-2 gene expression. LPS induces not only NFκB activation, but also MAP kinases including ERK1/2, JNK, and p38. While TLR4 activation leads to activation of NFκB and JNK, it is not clear if the activated TLR4 induces ERK1/2 and p38. v-Src leads to the activation of Raf and MEKK1 and then activates ERKs and JNK, respectively. TF, transcription factor. ERK, extracellular-signal-regulated kinase; MEK, mitogen-activated protein kinase (MAPKK); MEKK, mitogen-activated protein kinase kinase kinase (MAPKKK); JNK, c-Jun N-terminal kinase; JNKK, JNK kinase; PPAR, peroxisome-proliferator activated receptor; RXR, retinoid X receptor; PUFA, polyunsaturated fatty acid; TF, transcription factor; PGs, prostaglandins.
First, it was reported that \( v\)-Src induces COX-2 gene expression in fibroblasts by the activation of JNK leading to activation of transcription factor, c-Jun, which binds to CRE site in COX-2 promoter (148,162). \( v\)-Src is a constitutively active form of c-Src kinase, and activates JNK by phosphorylation at serines 63 and 73. Xie et al. demonstrated that \( v\)-Src-induced COX-2 gene expression is mediated by Ras (162). There are several Ras effector proteins, such as Raf, PI3K, or MEKK, leading to phosphorylation of transcription factors (163). Among these effector proteins is MEKK that is downstream of Ras and activates JNK leading to phosphorylation of c-Jun (164). In turn, phosphorylated c-Jun can form heterodimers with c-Fos to bind to CRE sequence in COX-2 promoter (165). Therefore, it was suggested that \( v\)-Src/Ras/MEKK/JNK/JNK signaling pathway participates in \( v\)-Src-induced COX-2 gene expression. Indeed, it was demonstrated that a dominant negative JNK attenuated the \( v\)-Src-induced COX-2 promoter luciferase activity, but overexpression of wild-type JNK1 potentiates \( v\)-Src-medicated COX-2 promoter luciferase activity (148).

Additionally, \( v\)-Src also activates another MAP kinase enzymes, ERK-1 and ERK-2, via Raf kinase (166). Indeed, co-expression of ERK-1 or ERK-2 potentiates \( v\)-Src-induced COX-2 promoter activity and co-expression of dominant negative ERK-1 or ERK-2 substantially attenuate \( v\)-Src-induced COX-2 promoter activity. Moreover, a dominant-negative Raf-1 suppressed \( v\)-Src-mediated COX-2 promoter activity. Therefore, the \( v\)-Src/Ras/Raf/MEK/ERK pathway plays a role in \( v\)-Src-induced COX-2 gene expression (148). However, contrary to JNK that phosphorylates c-Jun, ERKs phosphorylate and activate TCF/Elk-1 that is responsible for increased expression from the c-Fos gene.
Thus, v-Src/Ras/Raf/MEK/ERK pathway may increase c-Fos and c-Jun protein abundance as a secondary response, and in turn, the activated c-Fos and c-Jun by v-Src/Ras/MEKK/JNKK/JNK pathway bind to CRE element to enhance COX-2 gene expression. Xie et al also showed that platelet-derived growth factor (PDGF) or serum strongly induces COX-2 gene expression and its receptor occupancy by such ligand is connected to Ras/MEKK/JNKK/JNK and Ras/Raf/MEK/ERK signaling pathways leading to the elevated COX-2 gene expression in fibroblast (169).

Second, Meade et al. demonstrated recently that fatty acids can induce COX-2 gene expression mediated through peroxisome proliferator activated receptors (PPARs) in epithelial cells and a putative PPAR response element (PPRE) was identified on COX-2 promoter (170). PPARs are members of the nuclear hormone receptor superfamily which regulate gene expression by binding to a recognition sequence termed PPRE and to date, three subtypes of PPAR (α, β/δ, and γ) have been identified (171). Transcriptional regulation by PPARs is achieved through PPAR-RXR (where RXR is the receptor for 9-cis retinoic acid) heterodimers (172). Fatty acids are ligands for PPARs, which implies that fatty acids are potential modulators for gene expression, such as COX-2, mediated through PPARs.

Third, LPS or endotoxin triggers very intricate signaling pathways leading to COX-2 gene expression as well as other cytokines in macrophage cells (173). One of LPS-induced signaling pathways is the mitogen-activated protein kinase (MAPK) pathway mediated through Ras protein. One potential effect of cells with LPS contamination is an activation of protein tyrosine kinase (PTK), such as Src family
proteins, although the precise mechanism has not been elucidated. Moreover LPS induced expression of proinflammatory cytokines was inhibited by various PTK inhibitors (174-177), and LPS treatment leads to the elevated expression of Lyn, Fgr, and Hck proteins. Furthermore, the Src tyrosine kinase family member, Lyn, was found to be activated and associated with LPS co-receptor, CD14 after LPS treatment (178). Therefore, LPS triggers the activation of Src-family kinases. It was shown that Ras is able to activate Raf-1 in macrophage cells (179). Additionally, Geppert et al. demonstrated that the dominant negative mutants of both Ras and Raf-1 suppressed LPS-induced activation of TNF promoter, although a constitutively active Raf-1 could not induce TNF promoter activity without LPS stimulation in macrophage cells, RAW264.7 (180). Indeed, LPS rapidly phosphorylates and activates Raf-1 and activated Raf/MEK/MAP-kinase (ERKs) pathway was inhibited by genistein, a tyrosine kinase inhibitor in macrophage cells, BAC-1.2F5 (181). Thus those results imply that Raf-1 must lie downstream of tyrosine kinase to constitute PTK/Ras/Raf/MEK/MAPK (ERK1/2) signal transduction pathway by LPS stimulation in macrophages.

However, such signaling pathway by LPS action may be challenged by several reports. First of all, the same pathway is activated by the growth factor CSF-1 (182) and by TNF-α (270,271). In addition, Lowell et al showed that Hck, Fgr, and Lyn are not obligatory for LPS-initiated signal transduction by using hck<sup>−/−</sup> fgr<sup>−/−</sup> lyn<sup>−/−</sup> triple mutant mice (185). Their analyses indicated that both elicited peritoneal (PEMs) and bone marrow-derived macrophages (BMDMs) from the triple mutant mice have no major defects in LPS-induced activation, although the total protein phosphotyrosine
level is greatly reduced in macrophages derived from the same mutant mice. They also showed that the activation of ERK1/2 is the same in normal and mutant macrophages after LPS stimulation. These data suggest that these Src-related kinases may not be absolutely required for LPS-induced macrophage activation. However, an important role for these kinases in LPS-mediated signal transduction can not be excluded in normal macrophages.

LPS receptor, Tlr4, was identified and a signaling pathway induced by LPS was established. LPS binds the soluble LPS binding protein (LBP) and the complex binds CD14, a monocyte/macrophage receptor molecule that is expressed in both soluble and membrane-associated forms. CD14 presents the LPS-LBP complex to the LPS receptor, Tlr4, a member of the Toll/IL-1R family proteins, which has TIR domain at cytoplasmic C-terminal end. The TIR domain of activated Tlr4 recruits an adaptor molecule, MyD88, which has DD domain at N-terminal end and TIR domain at C-terminal end. The TIR domains make a noncovalent interaction between Tlr4 and MyD88 molecules. In turn, another downstream signaling molecule IRAK, through DD domain interaction between MyD88 and IRAK, needs to be recruited to MyD88 bound to Tlr4 cytoplasmic domain. Recently, it was identified that IRAK is present in a complex with another adapter molecule in the absence of stimuli. Upon IL-1R activation, it was shown that Tollip-IRAK complex is recruited to IL-1AcP, not to IL-1R to transduce a signaling. While it still needs to identify that Tollip-IRAK complex participates in LPS-induced and Tlr4-mediated signaling pathway, IRAK interacts with MyD88 through their death domain and then IRAK autophosphorylation is triggered.
turn, the phosphorylated IRAK is dissociated from Tollip, leading to the association with TRAF6 for transducing the receptor mediated signal. TRAF6 is able to activate NIK and then leads to the activation of IKKs and consequently activation of NFκB through IκB degradation. O’Connell et al showed that the activation of IKK2 (IKKβ), not IKK1 (IKKα), is required for the LPS-induction of NFκB dependent gene expression (186). Recently, new adapter molecule, ECSIT, for TRAF6 was identified and appears to bridge TRAF6 to the MAP kinase MEKK1 by directly interacting with TRAF6 and MEKK1 (87). MEKK1 is a 195KDa protein that is believed to be activated upon proteolytic cleavage by a caspase into an active 80 KDa fragment (88). The activation of MEKK1 mediated by ECSIT leads to the activation of one of MAP kinases, JNK, which phosphorylates transcription factor, c-Jun. Therefore, the LPS-activated TLR4 signaling pathway provides the activation of both JNK and NFκB.

Besides activating ERKs and JNK, LPS also phosphorylates and activates another MAP kinase, p38, in RAW264.7 cells (173,180,187,188), although the mechanism for activating p38 is not elucidated to date. Meanwhile, several studies revealed that the overexpression of protooncogene Tpl2 that encodes a serine threonine protein kinase activates the ERK, JNK, and p38, as well as NFAT and NF-κB in a variety of cell types (189-191), as LPS activates the same pathways (173). Recently, Dumitru et al determined the MAP kinase activation with Tpl2-/- and Tpl2 +/- mice before and after stimulation with LPS. According to their results, the inactivation of Tpl2 specifically inhibited the activation of ERK1 and ERK2 upon LPS stimulation. Therefore, it implies that Tpl2 is required to activate ERK1 and ERK2 MAP kinases by LPS (192).
However, it should be still investigated how Tpl2 functions to result in activation of MAP kinase. Meanwhile, Yang et al showed the increased ERKs, JNK, and p38 MAP kinase phosphorylation in response to LPS, when human TLR4 and MD-2 were co-expressed in HEK293 cells (92).

As described above, three major signaling pathways participating in COX-2 gene expression. In nuclear level, c-Jun by MAP kinase pathway, PPAR as a nuclear hormone receptor, and NFκB by TLR4 mediated pathway are three major transcription factors for COX-2 gene expression. The 5' flanking region of COX-2 has various cis acting transcriptional elements including CRE, PPRE, NF-IL6, and NFκB binding elements. Herschman et al suggested that among these cis-acting elements, CRE and NF-IL6 are required for the optimal expression of COX-2 and NFκB may not be required for efficient COX-2 reporter transcription upon LPS stimulation (193). Meanwhile, Mestre et al suggested that NFκB, NF-IL6, and CER sites mediate COX-2 gene expression independently in response to LPS treatment and each cis acting element alone was less effective than the intact promoter in mediating COX-2 gene expression. Therefore, they suggested that there is redundancy between cis acting elements on COX-2 promoter region (194). However, our study strongly demonstrated that NFκB activation alone is sufficient and required for COX-2 gene expression by LPS (195).
2 INTRODUCTION

The pathogenesis of gram-negative septic shock is due to excess stimulation of host cells by bacterial lipopolysaccharide (LPS) endotoxin (1,2,12). Such stimulation leads to expression and release of a plethora of pro-inflammatory marker-gene products and lipid mediators, which in turn can initiate a chain of events leading to systemic toxicity (3,4). However, initial recognition of LPS by cells of the immune system is required to defend the host from a gram-negative infection before it becomes widely disseminated (5,6).

Identifying the downstream signaling pathways derived from LPS stimulation is of fundamental importance to understanding the cellular mechanism of gram-negative septic shock. Previously, CD14, a glycosylphosphatidylinositol (GPI)-linked membrane protein widely expressed in mononuclear cells, is considered a high-affinity receptor for LPS (26,27,28). However, CD14 lacks a cytoplasmic domain. Thus, there has been a puzzling question as to how CD14 transmits extra-cellular signals into downstream cytoplasmic signaling pathways. Recently, it was demonstrated that human toll-like receptor 2 (TLR2) mediates LPS-induced cellular signaling (60,61). Human TLR4 can constitutively activate NFκB, but fails to confer LPS-responsiveness (60). The human Toll protein is a trans-membrane protein with an extra-cellular domain consisting of leucine-rich repeats (LRR), and a cytoplasmic domain homologous to that of IL-1 receptor (198).

Genetic evidence indicating that murine TLR4 is the LPS receptor was demonstrated using two mouse strains [C3H/HeJ and C57BL/10ScCr including its
progenitor C57BL/10ScN (also known as C57BL/10ScNcr)] which are hyporesponsive to LPS (40,41,42). The C3H/HeJ strain has Tlr4 with a missense mutation to replace proline with histidine at position 712, whereas the C57BL/10ScCr strain is homozygous for a null mutation of Tlr4. It was also demonstrated that activation of NFκB and expression of certain NFκB-induced gene products in LPS-stimulated macrophages derived from the C3H/HeJ mouse strain were impaired (62,63). In addition, overexpression of the mutant Tlr4 derived from C3H/HeJ mice in human dermal endothelial cells results in the inhibition of LPS-induced NFκB activation (65). Arbour et al showed that the missense mutations (Asp299Gly and Thr399Ile) in extracellular domain of human Tlr4 causes a blunted response to LPS in humans (196). However, biochemical evidence that murine Tlr4 indeed confers LPS responsiveness has not been conclusively demonstrated. Thus, the first aim of our research is to demonstrate whether Tlr4 mediates LPS-induced signaling in murine macrophage RAW264.7 cells.

Activation of Toll proteins and IL-1 receptor induces the recruitment of the adapter molecule, MyD88 (49,67,70), and IRAK. The activation of IRAK by autophosphorylation results in an interaction with TRAF6 followed by the activation of NIK and IKKs, which in turn leads to the activation of NFκB and expression of NFκB-induced gene products (72,73,77). Results from our previous studies indicate that LPS induces the selective expression of the mitogen-induced cyclooxygenase (COX-2) in murine macrophages (22,175) and LPS activates NFκB in macrophages (24). Thus, it has been assumed that LPS-induced COX-2 gene expression would be mediated through the NFκB pathway. Recently Wadleigh et al suggested that the CRE and the
NF-IL6 cis acting elements, but not the E-box or the putative NFκB site (Fig.1.9), in the murine COX-2 promoter are important for efficient COX-2 transcriptional induction by LPS in murine macrophages, RAW264.7 (193). Their results contradicted our previous report regarding the role of NFκB in LPS-induced COX-2 expression in macrophages (24). Therefore, the role of NFκB in LPS-induced COX-2 expression in macrophages is not clearly established. The second aim of this research is to demonstrate whether the activation of NFκB is required and sufficient to induce COX-2 gene expression.

Because the COX-2 gene is over-expressed in sites of inflammation and tissues of many types of tumors (33,134,153-155), elucidating the signaling pathways is the key to understanding why COX-2 is over-expressed in such pathological states and can provide crucial information for identifying the potential targets for pharmacological and dietary modulation. In addition, while the genetic evidences were established that the null mutation and missense mutation of Lps locus causes the LPS-hyporesponsive mice, C3H/HeJ and C57B1/10ScCr (40-42), biochemically, it was not elucidated why such mutations result in the LPS-hyporesponsiveness phenotype. Our third goal is to determine how Tlr4 mutation impairs LPS-induced responses.

While Tlr4 and IL-1R elicits pleiotropic effects through activation of transcription factors, NFκB and AP-1 by activating JNK, it was already identified that LPS induces the activation of phosphotyrosine kinases and MAPKs including ERK1/ERK2, p38, and JNK. However, based on the current results on Tlr4 that mediate LPS-stimulating signaling, it is unable to elucidate how these kinases could be activated through the activation of Tlr4. It implies that there may be other signaling molecules for
transmitting LPS-induced signaling to the activation of PTKs or MAPKs through Tlr4. Thus the fourth aim of our research is to identify the molecules that are involved in the Tlr4 signaling pathway for the activation of MAPKs or PTKs.

We have addressed in this study three important issues in elucidating LPS-stimulated signaling pathways in murine macrophages. First, we determined whether the activation of NFκB is sufficient and required for LPS-induced COX-2 expression and second, we determined whether the activation of Tlr4 confers LPS-responsiveness as evaluated by the activation of NFκB and the expression of COX-2. Third, we determined why the Tlr4 mutant with a missense mutation at position 712 fails to transmit the LPS-induced signal to downstream signaling pathways.
3. MATERIALS AND METHODS

3.1 Reagents

Lipopolysaccharide (LPS) was purchased from DIFCO (Detroit, Michigan). Flag antibody M2 was purchased from Sigma (St Louis, MO) and HA antibody 12CA5 was from Roche (Indianapolis, IN). Goat anti-mouse immunoglobulin G (IgG) antibodies conjugated to horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Enhanced chemiluminescence (ECL) western blotting detection reagents were purchased from Amersham Corp (Piscataway, NJ). SuperFect Transfection Reagent was purchased from Qiagen (Valencia, CA). Luciferase Assay System and β-galactosidase Enzyme System were from Promega (Madison, WI). All other reagents were purchased from Sigma unless otherwise described.

3.2 Cell Culture

RAW264.7 cells (murine macrophage-like cell line, ATCC TIB-71) were cultured in LPS-free Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Intergen) and 10 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37 °C in 5% CO₂ air environment. Human embryonic kidney cells (293T cells) were provided by Sam Lee (Beth Israel Hospital, Boston, MA) and cultured in the same medium used for RAW264.7 cells. Mouse fibroblast cells, NIH3T3, was purchased from ATCC and cultivated in DMEM containing FBS and penicillin/streptomycin as mentioned above.
3.3 DNA Constructs

Flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Ly) tagged murine Tlr4 cDNA constructs [both wild type and mutant; pFlag-CMV1-TLR4 (wt), pFlag-CMV1-Tlr4 (P712H), respectively] were kindly provided by Bruce Beutler (Southwestern Medical Center, Dallas, TX). These constructs lack DNA sequences for the first 20 amino acids representing the signal peptide. The Tlr4 (P712H) mutant found originally in LPS-hyporesponsive mouse strain (C3H/HeJ) has a missense mutation at position 712 to replace proline with histidine.

The Flag-Tlr4 constructs were subcloned into pcDNA3.1/zeo (-) vector (Invitrogen) and the Flag epitope tag was replaced with the HA-tag sequence (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) by PCR cloning as depicted in Fig. 1.12. The first PCR reaction was performed with the primers, Xho-SS-FOR and SS-HA-REV. The second PCR reaction was performed with the primers, Xho-SS-FOR and Not-HA-REV, using the first PCR product as a template. The final PCR product was inserted in Xho1 and Not1 sites of pcDNA3.1/zeo (-)-Flag-Tlr4 (wt) and pcDNA3.1/zeo (-)-Flag-Tlr4 (P712H) plasmids, resulting in pcDNA3.1/zeo (-)-HA-Tlr4 (wt) and pcDNA3.1/zeo (-)-HA-Tlr4 (P712H) constructs, respectively. The primers used are as follows: Xho-SS-FOR, GTTAAACTCGAGCCACCATGTCTGCACTTCTGATCC; SS-HA-REV, AGCGT AGT CT GGG ACGTCGT ATGGGT AAGC AACT GC AGCTCC AAC AAG; NOT-HA-REV, GGGATTCGCGGCCGCAGCGTAGTCTGGGACGTCGTATGGG.

To prepare the truncated Tlr4 constructs [Flag/HA epitope tagged-ΔTlr4 (wt) and ΔTlr4 (P712H)] lacking extracellular LRR domain, DNA sequences spanning the epitope tag
plus N terminal 21 to 78 amino acids and transmembrane plus cytoplasmic region (amino acid 629 to 835) were amplified by PCR using Flag/HA-Tlr4 (wt) and Flag/HA-Tlr4 (P712H) plasmids, respectively, as templates. The primers used for the PCR cloning are as follows: Xho-Tlr-F208, GTTAAACTCGAGCCAGGATGATGCTCCCTGGCTCC; TlrR441Bam, ACACCAGATCCTTCTGAACATTTGGAGAAGCTATAGC for amplifying N terminal region, and BmTlrF2092, GAAGGGGATCCAA GACAATCATCAGTGTGTCAGTG, and Tlr-REV, CTCAGAGGATCCCTCCTCCCTCAGGTCCAAGTTGCCG, for the region spanning amino acid 629-835, respectively. The PCR products were subcloned into XhoI and BamH1 sites in pcDNA3.1/zeo (-) expression vector for Flag/HA-ΔTlr4 (wt) and Flag/HA-ΔTlr4 (P712H). The integrity of the sequences was confirmed by DNA sequencing. The expression of each construct was verified after transfection into 293T cells by immunoblot analysis using monoclonal Flag antibody (M2) (Sigma), monoclonal HA antibody (12CA5) (Boehringer Mannheim), and polyclonal anti-Tlr4 immune serum. Polyclonal anti-Tlr4 antibodies were prepared at the Core Laboratory, Louisiana State University Medical Center (New Orleans, LA) using a polypeptide containing 16 amino acids (NH2-Cys-Trp-Arg-Arg-Leu-Lys-Asn-Ala-Leu-Leu-Asp-Gly-Lys-Ala-Ser-Asn-COOH) located near the carboxylic terminus of human Tlr4. Expression plasmids for the wild type NFκB-inducing kinase (NIK), pRK-NIK (wt), and a dominant negative mutant NIK, pRK-NIK (KA), were gifts from Mike Rothe (Tularik, South San Francisco, CA). Wild type murine MyD88, [Flag-MyD88(wt)], dominant negative mutant [Flag-MyD88(ΔDD)], and constitutively active mutant [Flag-MyD88 (ΔToll)] were kindly
Fig. 1.2. Schematic illustration of different murine Toll-like receptor 4 (Tlr4) cDNA constructs used in these studies. Tlr4 (wt), HA or Flag-tagged full length wild type murine Tlr4; ΔTlr4 (wt), truncated HA- or Flag-tagged wild type Tlr4 lacking extracellular leucine rich repeat region (LRR) which is a constitutively active form of Tlr4; Tlr4 (P712H), HA or Flag-tagged full length mutant Tlr4 with the missense mutation (substitution of proline with histidine) at position 712. This mutant is found in LPS-hyporesponsive mouse strain (C3H/HeJ); ΔTlr4 (P712H), truncated mutant Tlr4 lacking the extracellular LRR. The non-LRR region (the region for amino acid 21 to 78) was depicted as a closed rectangular at N-terminus. Cloning strategy for these constructs is described in Materials and Methods. TIR domain, Toll/IL-1R homology domain.
provided by Jurg Tschopp (University of Lausanne, Switzerland) (70). The Flag-
MyD88 variant constructs were subcloned into pcDNA3 expression vector (Invitrogen).
Expression plasmids for wild type inhibitor kappa B, [pCMV4-1kBα(wt)], and a
dominant negative mutant, [pCMV4-1kBα(ΔN)], were provided by Dean Ballard
(Vanderbilt University, Nashville, TN). 2xNF-κB-Luciferase reporter construct was a
gift from Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). HSP70-β-
galactosidase reporter construct was from Robert Modlin (University of California, Los
Angeles, CA) (106). Mouse COX-2 promoter (-3,200bp) luciferase reporter construct
was from David DeWitt (Michigan State University, East Lansing, MI). All DNA
constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen) for
transfection.

3.4 Luciferase Reporter Gene Array

RAW264.7 cells were plated in six-well plates (5×10^5 cells/well) and transfected
with total 5 μg of DNA plasmids including HSP-70-β-gal plasmid as an internal control
using SuperFect transfect reagent (Qiagen) according to the manufacturer’s instruction.
Relative luciferase activity was determined by normalization with β-galactosidase
activity as described elsewhere (106).

3.5 Radioimmunoassay to Measure the Amount of PGE_2

RAW264.7 cells were plated at 0.5 x 10^6 per well of a 6-well plate one day before
transfection. Total 5 μg of plasmid DNA was transfected by using SuperFect transfect
reagent (Qiagen) according to the manufacturer’s instructions. The cell culture media
were collected 48 hours later for measuring accumulated prostaglandin E_2 that was
produced by cyclooxygenase. Levels of prostaglandin E2 in cell supernatants were determined by radioimmunoassay as an indication of COX enzyme activity. The aliquots of harvested media were transferred into plastic scintillation vials in triplicate. The volume was adjusted to 500 µl with PBS-gelatin (0.1%). A triplicate set of standards was run with each assay. The tritiated PGE2 (3H) was added to each vial containing natural PGE2 from sample, and rabbit antisera raised against PGE2 was added. The samples in vials were mixed thoroughly, covered with parafilm and incubated at room temperature. After 2 hours, secondary antisera (anti-rabbit gamma globulin) was added to precipitate the primary antisera, the vials were mixed again and in turn, were incubated at 4 °C for 48 hours. The vials were then centrifuged at 6,500g for 45 min, decanted quickly and the precipitate was dissolved by the addition of 0.05 N NaOH. Scintillation cocktail (EcoLume™, ICN, Costa Mesa, CA) was added to each vial, followed by vortexing. Radioactivity was determined by counting in a Beckman LS6800 scintillation counter. The data were analyzed and PGE2 levels were determined by using the computer program based on the logit-transformation.

3.6 Immunoprecipitation and Immunoblot Analysis

293T cells (1.2 x 10^6 cells/well) were seeded in 60 mm culture dishes and incubated for 24 hours. Cells were cotransfected with a total 5 µg of HA-ΔTlr4 (wt) or HA-ΔTlr4 (P712H) plasmid and Flag-MyD88 (wt) expression plasmids. After 48 hours, cells were washed with PBS (pH 7.5) and lysed for 30 min on ice in 0.5 ml of RIPA buffer [150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 5 mM EDTA, 1% Nonidet P-40, 0.5% Na-deoxycholate, 1 mM Na3VO4, 1 mM (4-(2-Aminoethyl) benzenesulfonyl]
fluoride hydrochloride, 10 μg/ml Aprotinin, 10 μg/ml leupeptin]. Cell lysates were clarified by centrifugation at 4 °C for 15 min at 12,000 g. Cell lysates were incubated with 1 μg of HA antibody (12CA5) for 4 hours and 70 μl of 50 % (v/v) protein A-agarose (Pierce) was added for overnight incubation at 4 °C with rocking. Immune complexes were solubilized with Laemmli sample buffer (90) after three washings with lysis buffer. The samples were fractionated by 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membranes were blocked with PBS – 0.1% Tween 20 containing 5% nonfat dry milk and blotted with the indicated antibodies. The reactive bands were visualized with the enhanced chemiluminescence system (Amersham) as described in our previous studies (22, 24, 175, 195).
4. RESULTS

4.1 Inhibition of NFκB Resulted in The Suppression of LPS-Stimulated COX-2 Gene Expression in RAW 264.7 Cells

Wadleigh et al. suggested that NFκB is dispensable for COX-2 gene expression by LSP stimulation in mouse macrophage cells, RAW264.7 (193). They measured murine COX-2 promoter activity by using -700 bp promoter reporter construct which has one NFκB element around -400 bp region. However their results contradicted our previous report regarding the role of NFκB in LPS-induced COX-2 expression in macrophages (24). Thus, this study was aimed to determine whether LPS-induced expression of COX-2 is mediated through activation of NFκB. In this study, it was investigated whether or not the inhibition of LPS-induced activation of NFκB leads to suppression of COX-2 expression. NFκB inducing kinase (NIK) specifically activates IKK leading to IκBα degradation and to NFκB activation. Therefore, cells were co-transfected with the luciferase reporter plasmid for NFκB or COX-2 promoter (-3.2kb) and an expression plasmid containing a dominant negative mutant of NFκB inducing kinase (NIK) or IκBα expression construct in order to specifically inhibit NFκB signaling pathway. The results show that LPS-induced COX-2 expression is significantly inhibited by co-transfection of cells with a dominant negative mutant of NIK or IκBα (Fig.1.13). These results indicate that the activation of NFκB is required for the full expression of COX-2 in LPS-stimulated RAW 264.7 cells.
Fig. 1.13. Suppression of LPS-induced activation of NFκB and COX-2 expression by a dominant negative mutant of NIK or IkBα. RAW 264.7 cells were co-transfected with 2 μg of luciferase reporter plasmid for NFκB response element (A and C) or COX-2 promoter (B and D), and 2 μg expression plasmid containing dominant negative mutant of NIK(KA) or IkBα(ΔN) cDNA. 1 μg of HSP70-β-gal construct was also included as a internal control. pRK and pCMV4 are the empty vectors for NIK and IkBα cDNA, respectively. Transfected cells were treated with LPS (20 ng/ml) for 24 hours. Luciferase activity was measured as described in Experimental Procedures. Panels are representative data from more than three different analyses. Values are mean ± SEM (n=3). RLA, relative luciferase activity (Fig. continued).
C

2x NF-κB $\rightarrow$ Luc

D

-3.2 kb COX-2 $\rightarrow$ Luc

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4.2 Activation of NFκB by NIK Leads to Expression of COX-2, and This Expression Is Suppressed by A Dominant Negative Mutant of IκBα in RAW 264.7 Cells

NFκB inducing kinase (NIK) is known to activate IKK which leads to NFκB after IκBα degradation. To date, NFκB activation is the only known biochemical function of NIK. Therefore, if direct activation of NFκB without involvement of LPS leads to the expression of COX-2, this result would support that the activation of NFκB alone is sufficient for the expression of COX-2. RAW264.7 cells were co-transfected with NIK and COX-2 reporter gene constructs. The results show that direct activation of NFκB by transfection of cells with the wild-type NIK plasmid leads to the expression of COX-2 (Fig. 1.14, lane 1).

Additionally, IκBα is the direct target protein by NIK-activated IKK and the degradation of IκBα releases a free NFκB leading to activation of NFκB. Therefore, if NIK-induced COX-2 gene expression is inhibited by IκBα, it implies that NFκB activation is required for COX-2 gene expression. As shown in Fig. 1.14, co-transfection of cells with NIK (wt) and a dominant negative mutant of IκBα significantly suppressed the NIK-induced COX-2 expression (Fig.1.14, lane 2). Together, these results indicate that the activation of NFκB is sufficient and required for the expression of COX-2 in LPS-stimulated macrophages. These data are also opposite to the recent report by Wadleigh et al. (193).
Fig.1.14. Activation of NFκB by NIK leads to expression of COX-2, which is significantly suppressed by a dominant negative mutant of IκBα. RAW 264.7 cells were co-transfected with 2 μg of luciferase reporter plasmid for NFκB (A) or COX-2 promoter (B), and 2 μg of expression plasmids containing NIK (wt) and a dominant negative mutant of IκBα (ΔN), respectively. 1 μg of HSP-β-gal construct was included as a internal control. Panels are representative data from more than three different analyses. Values are mean ± SEM (n=3).
4.3 Extracellular Domain Truncated Tlr4 (ΔTlr4) Functions As The Constitutively Active Form to Increase The Endogenous COX Enzymatic Activity Measured by The Amount of Accumulated PGE₂

In *Drosophila*, it was shown that the deletion of extracellular leucine-rich repeat (LRR) domain elicits the gain of function of *Toll* gene product (197). In addition, the gain of function by the truncated *toll* proteins lacking the extra-cellular domain was demonstrated in human Tlr4 (198). Based on the reports from *Drosophila Toll* and human Tlr4, extracellular domain truncated mouse Tlr4 (ΔTlr4 wt) and Tlr4 (P712H) [ΔTlr4 (P712H)] constructs were prepared as described in *Materials and Methods*. Mouse macrophage cell line, RAW264.7 cells, were transiently transfected with these Tlr4 constructs to demonstrate if ΔTlr4 elicits COX-2 enzymatic activity as a constitutively active form. After 48 hours of transfection, the culture media were collected for radioimmunoassay to measure the amount of accumulated PGE₂ that was produced by COX. As shown in Fig.1.15, ΔTlr4 transfection increased the endogenous level of PGE₂. This result implies that ΔTlr4 functions as the constitutively active form of Tlr4 and induces COX enzymatic activity in the absence of any other stimulation. In contrary, ΔTlr4 (P712H) did not increase the level of PGE₂, which means COX enzymatic activity was not affected by ΔTlr4 (P712H). Only one amino acid substitution within the cytoplasmic region of the protein causes these opposite results. Therefore, it is needed to investigate the biochemical mechanism to elucidate the discrepancy.
Fig. 1.15. ΔTlr4 transfection increased the endogenous cyclooxygenase (COX) enzymatic activity measured by radioimmunoassay. RAW264.7 cells were transfected with 5 μg of each type of Tlr4 expression construct as indicated. 48 hours after transfection, the culture media were collected to measure the accumulated PGE₂ which was produced by endogenous COX enzymes by radioimmunoassay as described in Materials and Methods. Values for lane 3, ΔTlr4 (wt) transfection, are significantly greater than those of other lanes (p<0.01). pcDNA3.1 is the empty vector for Tlr4 variants. Values are mean ± S.E. (n=3).
4.4 Constitutively Active Tlr4 (ΔTlr4), But Not The Truncated Mutant [ΔTlr4 (P712H)] of Tlr4 (P712H) Which Is Found in LPS-hyporesponsive Mouse Strain (C3H/HeJ), Activates NFκB And Induces COX-2 Expression in RAW 264.7 Cells

To determine whether or not activation of Tlr4 is sufficient to activate NFκB and to induce the expression of COX-2, cells were co-transfected with the truncated mouse Tlr4 construct (ΔTlr4) lacking the leucine-rich repeat extracellular domain and NFκB- or COX-2-luciferase reporter gene construct. The results show that transfection of cells with the constitutively active ΔTlr4 leads to activation of NFκB and COX-2 expression without LPS stimulation (Fig.1.16). However the same truncated ΔTlr4 (P712H) with the missense mutation at position 712 is unable to activate NFκB and COX-2 expression. Full length Tlr4 and full length Tlr4 (P712H) do not elicit NFκB activation and COX-2 reporter gene expression. These results indicate that the activation of Tlr4 is sufficient to activate NFκB and to induce COX-2 expression in RAW 264.7 cells.

4.5 Constitutively Active ΔTlr4-induced Activation of NFκB And COX-2 Expression Are Suppressed by Inhibition of NFκB with A Dominant Negative Mutant of NIK or IκBα in RAW 264.7 Cells

From the above results, we suggested that the LPS-stimulated COX-2 gene expression is mediated through NFκB activation and the activation of NFκB is not only sufficient, but required for LPS-induced COX-2 gene expression. In addition, the activation of Tlr4 itself by using ΔTlr4 causes the COX-2 gene expression.
Fig. 1.16. Constitutively active truncated Tlr4 (ΔTlr4), but not the mutant ΔTlr4 (P712H), activates NFκB and COX-2 expression. RAW 264.7 cells were co-transfected with 2 μg of luciferase reporter plasmid for NFκB (A) or COX-2 promoter (B), and 2 μg expression plasmid containing Flag-Tlr4 (wt), Flag-Tlr4 (P712H), Flag-ΔTlr4 (wt), Flag-ΔTlr4 (P712H), or pcDNA 3.1. 1 μg of HSP70-β-gal construct was also included. pcDNA3.1 is the empty vector for Tlr4 variants. Panels are representative data from more than three different analyses. Values are mean ± SEM (n=3). RLA, relative luciferase activity.
Then, it is needed to elucidate the signaling pathway by which the COX-2 is expressed by ΔTlr4. To determine whether the ΔTlr4-induced expression of COX-2 is mediated through activation of NFκB, we investigated whether ΔTlr4 transfection activates NFκB and in turn, the inhibition of NFκB by a dominant negative mutant of NIK or IκBα results in suppression of ΔTlr4-induced COX-2 expression. Because NIK and IκBα are specific upstream signaling molecules for activating NFκB, the specific inhibition by a dominant negative form of NIK or IκBα can be clear evidence to support that ΔTlr4-induced COX-2 gene expression is mediated through NFκB. Therefore, COX-2 or NFκB reporter plasmid was co-transfected with Tlr4 construct and a dominant negative mutant NIK (KA) or IκBα (AN) into RAW264.7 cells. The results showed that ΔTlr4 transfection activated NFκB as shown in Fig.1.7, A and C lane 2 and NIK (KA) or IκBα (AN) which are dominant negative forms of NIK and IκBα significantly suppressed the ΔTLR4-induced NFκB or COX-2 reporter gene expression (Fig.1.7). These results indicate that ΔTlr4-induced COX-2 expression is at least in part mediated through NFκB and the activation of NFκB is critical to induce COX-2 gene expression not only Tlr4-induced, but LPS stimulated COX-2 gene expression.

4.6 The Truncated Mutant [ΔTlr4 (P712H)] of Tlr4 (P712H) Inhibits LPS-induced Activation of NFκB And COX-2 Expression in RAW 264.7 Cells

Based on genetic evidence to date, Tlr4 is considered involved in LPS-stimulated signaling, although there are several reports that Tlr2 instead of Tlr4 is the LPS receptor. Thus, we need to establish whether Tlr4 is true LPS receptor. Meanwhile,
Vogel et al. showed *in vivo* genetic evidence that the missense mutation at *Lps* locus in LPS-hyporesponsive mice, C3H/HeJ, exerts a dominant negative effect on LPS sensitivity (40), although no functional and biochemical data for the mechanism by which this defect exerts its profound effect have been published to date. Thus, it is necessary to establish whether activation of Tlr4 confers LPS-responsiveness and how the missense mutation of Tlr4 [Tlr4 (P712H)] causes the LPS-hyporesponsiveness.

Therefore it was determined whether expressions of the mutant Tlr4 (P712H) found in C3H/HeJ mouse strain and its LRR domain truncated form, ΔTlr4 (P712H), inhibit NFκB activation and COX-2 gene expression in the presence of LPS treatment. The results show that the full length Tlr4 (P712H) and the truncated mutant ΔTlr4 (P712H) inhibit LPS-induced activation of NFκB and COX-2 gene expression (Fig.1.18. lane 2 and 4). However, the truncated wild type ΔTlr4 enhances LPS-induced activation of NFκB and COX-2 expression because it functions as a constitutively active form (Fig.1.18. lane 3). LPS-responsiveness is slightly enhanced in cells transfected with the full length Tlr4 as compared with vector transfected cells (Fig.1.18. lane 1). These results suggest that the full-length Tlr4 (P712H) and ΔTlr4 (P712H) act as the dominant negative mutant while ΔTlr4 is much more effective as a dominant negative form of Tlr4. Together, these results indicate that the activation of Tlr4 confers LPS-responsiveness in RAW 264.7 cells. However, our results do not permit us to rule out the possibility that other toll-like receptors also mediate LPS-responsiveness in RAW 264.7 cells.
Fig. 1.17. ΔTlr4 (wt)-induced activation of NFκB and COX-2 expression are inhibited by a dominant negative mutant of NIK (KA) or IκBα (ΔN). RAW 264.7 cells were co-transfected with 1.5 μg of luciferase reporter plasmid for NFκB (A and C) or COX-2 promoter (B and D) and 1.5 μg of expression plasmid containing constitutively active truncated Flag-ΔTlr4 (wt), truncated mutant Flag-ΔTlr4 (P712H), dominant negative mutant of NIK (KA) or IκBα (ΔN), or respective vector. 0.5 μg of HSP70-β-gal was also included as a internal control. pRK is the empty vector for NIK variants. Panels are representative data from more than three different analyses. Values are mean ± SEM (n=3). RLA, relative luciferase activity (Fig. continued).
Fig. 1. The truncated mutant ΔTlr4 (P712H) with a missense mutation at position 712 inhibits LPS-induced activation of NFκB and COX-2 expression. RAW264.7 cells were co-transfected with 2 μg of luciferase reporter plasmid for NFκB (A) or COX-2 promoter (B), and 2 μg of expression plasmid containing full length wild type Flag-Tlr4 (wt), mutant Flag-Tlr4 (wt), truncated wild type Flag-ΔTlr4 (wt), truncated mutant Flag-ΔTlr4 (P712H), or vector. 1 μg of HSP70-β-gal was also included as an internal control. Cells were treated with LPS (20 ng/ml) or vehicle for 24 hours. pcDNA3.1 is the empty vector for Tlr4 variants. Panels are representative data from more than three different analyses. Values are mean ± SEM (n=3). RLA, relative luciferase activity.
4.7 The Adaptor Protein MyD88 Is Co-immunoprecipitated with The Wild Type ΔTlr4 but Not with The Mutant ΔTlr4 (P712H) in 293T Cells

It remains unclear why the missense mutation at 712 residue in Tlr4 elicits the LPS-hyporesponsiveness. Thus, we investigated why the missense mutation to replace proline with histidine at position 712 of Tlr4 results in loss of function. In case of IL-R1 signaling pathway, the C-terminal TIR domain of MyD88 interacts with the TIR domain of cytoplasmic region of IL-1R by IL-1 stimulation and in turn, the N-terminal death domain of MyD88 associates with the death domain of IRAK to transmit the ligand-induced signaling. Since Tlr4 signaling pathway shares the same signaling molecules as the IL-R1 signaling pathway, MyD88 was the first candidate to elucidate the signaling pathway by Tlr4 activation. Medzhitov et al. demonstrated that MyD88 is an adaptor protein directly interacting with the cytoplasmic Toll/IL-1R homology (TIR) domain of human Tlr4 (49), and considered as one of the most upstream components of the human Tlr4-mediated signaling cascade. We find that proline at 712 lies within this TIR domain of murine Tlr4, which is critical for binding MyD88 adapter protein. Thus, we determined whether the mutation at position 712 interferes with binding of MyD88 to Tlr4 resulting in failure of the signal transmission.

Human embryonic kidney cells (293T cells) were co-transfected with an epitope (Flag) tagged MyD88 and HA-tagged wild type ΔTlr4 or the mutant ΔTlr4 (P712H) expression constructs. When cell lysates from these cells were immunoprecipitated with anti-HA antibody and immunoblotted using anti-Flag or Tlr4 antibodies, MyD88 was co-immunoprecipitated with the wild type HA-ΔTlr4 (Lane 1, Fig.1.19), but not
with the mutant HA-ΔTlr4 (P712H) (Lane 2, Fig.1.19). These results suggest that the mutant Tlr4 (P712H) is unable to interact with MyD88 and, thus fails in transmitting the signal to the downstream signaling molecules.

4.8 Constitutively Active Form of The Adapter Protein MyD88 Rescues Inability of The Dominant Negative Mutant Tlr4 to Activate The Downstream Signaling Pathway in RAW 264.7 Cells

The adaptor molecule MyD88 is known to be an immediate downstream signaling molecule interacting directly with the TIR domain of Tlr4 (49). Proline at 712 position is located in this TIR domain. Therefore, it would be interesting to determine whether substitution of this proline with histidine resulting from the missense mutation interferes with binding of MyD88 to the TIR domain of Tlr4. If the failure of the mutant Tlr4 to activate downstream signaling pathways is due to its inability to recruit the adapter molecule MyD88, transfecting cells with a constitutively active form of MyD88 should restore signal transmission. Indeed, co-transfection of RAW 264.7 cells with the dominant negative mutant [ΔTlr4 (P712H)] and a constitutively active form of MyD88 lacking Toll/IL-1R domain [MyD88 (ΔToll)] results in restoration of NFκB activation and COX-2 expression (Fig.1.20). Taken together these results suggest that hyporesponsiveness of the mouse strain (C3H/HeJ) to LPS is due to the disruption of Tlr4-mediated signaling pathways resulting from inability of the mutant [Tlr4 (P712H)] to recruit the downstream signaling molecule MyD88.
Fig 1.19. The adapter protein MyD88 is co-immunoprecipitated with the truncated wild type ΔTlr4 (WT), but not with the mutant ΔTlr4 (P712H), in 293T cells. The 293T cells were co-transfected with 2.5 μg of expression plasmids containing epitope-tagged full length murine Flag-MyD88 and 2.5 μg of epitope-tagged truncated wild type HA-ΔTlr4 (wt) cDNA, respectively, or the mutant HA-ΔTlr4 (P712H) cDNA. (A) Cell lysates were immunoprecipitated (IP) with monoclonal anti-HA antibody and immunoblotted (IB) using monoclonal anti-Flag antibody. (B) The membrane from panel A was reprobed with polyclonal Tlr4 antibodies, or (C) reprobed with monoclonal anti-HA antibody. (D) The same cell lysates were directly immunoblotted (IB) using monoclonal anti-Flag antibody. Failure to see a band in panel A, lane 2 indicates that MyD88 does not interact with Tlr4 (P712H). pcDNA3.1 is the empty vector for Tlr4 variants. Panels are representative pictures from three different analyses.
Fig. 1.20. The constitutively active form of the adapter protein MyD88 lacking Toll/IL-1R homology domain [MyD88 (Δ Toll)] can override the dominant negative effect of the mutant ΔTlr4 (P712H). RAW 264.7 cells were co-transfected with 1.5 μg of luciferase reporter plasmid for NFκB (A) or COX-2 promoter (B), and 1.5 μg of expression plasmids containing the truncated wild type Flag-ΔTlr4 (wt) or the truncated mutant Flag-ΔTlr4 (P712H), and constitutively active MyD88 (ΔToll), or respective vector. 0.5 μg of HSP70-β-gal constructs was also included as an internal control. pcDNA3 is the empty vector for MyD88 variants. Panels are representative data from more than three different analyses. Values are mean ± SEM (n=3). RLA, relative luciferase activity.
5. SUMMARY AND DISCUSSION

In this study, three important issues were presented concerning LPS-induced signaling pathways in the murine macrophage cell line, RAW264.7. First, it was established that the activation of NFκB is sufficient and required for LPS-induced COX-2 gene expression. These results contradict the results reported by Hershman's group who suggested that the CRE and the NF-IL6 cis acting elements in the murine COX-2 promoter are important for efficient COX-2 transcriptional induction by LPS in murine macrophages, RAW264.7. They further suggested that the putative NFκB site may not be required for the full-expression of COX-2 in LPS-stimulated macrophages (193). However, requirement of NFκB has been demonstrated in another study using pharmacological inhibitors of IκBα degradation or nuclear translocation of NFκB (24). It is not clear what causes this discrepancy. The murine COX-2 luciferase construct in this study contains 3.2 kb upstream promoter sequences, whereas, the COX-2 construct used in another study showing the results different from ours contains only 700 bp upstream promoter sequences (193). Although the κB binding site in murine COX-2 promoter is located within −400 kb, it is possible that other enhancer elements located further upstream of the 5' flanking region of the COX-2 gene are required for the full expression of COX-2 in LPS-stimulated RAW 264.7 cells. Indeed, two NFκB binding elements have been found at −400 bp and −1525 bp in mouse COX-2 promoter region and −261 and −555 bp in the human COX-2 promoter sequence, as described in Fig. 1.9 and Fig. 1.10. According to the results from Hershman's group, they used −700 bp of murine COX-2 promoter reporter construct which contains only one NFκB element...
around –400 bp and suggested that NFκB is dispensable for LPS-induced COX-2 gene expression. However, in this study, we used -3.2 kb of murine COX-2 promoter which contains both two known NFκB elements and any other cis acting elements. Additionally, there may be more putative NFκB elements in the longer 5’-flanking region of COX-2. Although Hershman’s group suggested that NFκB element is not required for COX-2 gene expression by LPS stimulation, contrarily, our results showed that NFκB is required for LPS-induced COX-2 gene expression and sufficient for COX-2 gene expression in RAW264.7 cells. Probably, more than two NFκB elements may need to harmoniously work together to induce COX-2 gene expression, because they used –700 bp COX-2 promoter containing only one NFκB element but our –3.2 kb COX-2 promoter include two NFκB elements or more putative elements.

While the results in this study support that a requirement of the activation of NFκB is critical for LPS-induced COX-2 gene expression in RAW264.7 cells, the cis acting elements which play a role in the modulation of COX-2 gene expression may depend on the type of cell and stimulant. For example, Yang et al suggested that the NF-1 element around –400 bp in the human COX-2 promoter region is a response region for TGF-β induced COX-2 gene expression (199).

Second, it was identified whether or not the activation of Tlr4 confers LPS responsiveness as evaluated by the activation of NFκB and the expression of COX-2. Although it was not clear how the signaling pathway is transduced by LPS stimulation due to the lack of transmembrane domain in GPI-linked membrane bound protein, CD14, until recently, CD14 was considered as the receptor for LPS. However,
Beutler's group identified the mutation of Tlr4 gene in LPS-hyporesponsive mice and these results imply that Tlr4 is involved in LPS signaling as a receptor molecule on cell surface. Meanwhile, Kirschning et al. suggested that human Tlr2, when cotransfected with CD14 into 293 cells, conferred LPS-inducibility of NFκB (61). However, overexpression of human Tlr4 constitutively activated the NFκB reporter gene and treatment of these cells with LPS did not enhance the reporter gene activity. Therefore, it was so controversial that it needs to be established whether or not Tlr4 is the LPS receptor.

The results from this study show that the transfection of RAW264.7 cells with the truncated ΔTlr4 as a constitutively active form of Tlr4 elicits LPS-responsiveness through NFκB activation, measured by the activation of NFκB and the expression of the COX-2 promoter reporter construct (Fig. 1.15). Additionally, a constitutively active form of Tlr4 also increased the enzymatic activity of the accumulated cyclooxygenases in RAW264.7 cells (Fig. 1.16). Moreover, the results in this study showed that the presence of a full-length of Tlr4 (P712H) or a ΔTlr4 (P712H) causes the attenuation of LPS-stimulated signaling, because both the full-length of Tlr4 (P712H) and the ΔTlr4 (P712H) function as the dominant negative mutant form of Tlr4. These results suggest that Tlr4 mediates the LPS-induced signaling leading to NFκB activation and COX-2 gene expression. Arbour et al. also showed that the missense mutations (Asp299Gly and Thr399Ile) in the extracellular domain of human Tlr4 causes a blunted response to LPS in humans (196). Together with the biochemical evidence and the genetic
evidence as mentioned previously, clearly indicate that Tlr4, but not Tlr2, confers LPS-stimulated responsiveness.

Meanwhile, it was not known why the missense mutation of P712H in mouse Tlr4 and the deletion mutation of the Tlr4 gene cause the LPS-hyporesponsive phenotype in C3H/HeJ and C57BL/10ScCr mouse strain, respectively. The third issue indicates that the failure in interaction of an adaptor protein, MyD88, with Tlr4 (P712H) leads to the disruption of LPS-stimulated signal transduction and eventually results in hyporesponsiveness to LPS. The cytoplasmic region of Tlr4 is TIR domain which interacts with a TIR domain of any other protein. The point mutation at P712H of Tlr4 is located in its cytoplasmic TIR domain. Therefore, such point mutation interferes with TIR domain mediated interaction between Tlr4 and MyD88 proteins.

Furthermore, Xu et al. recently showed that the mutation of P681H in Tlr2 which is equivalent residue in Tlr4 disrupts signal transduction in response to stimulation by gram-positive bacteria. They also showed that the P681H mutation in Tlr2 abolished interactions between Tlr2 and MyD88 (296). These data may also imply that MyD88 is the common immediate downstream adaptor molecule for other Tlr proteins, not restricted for Tlr2 and Tlr4.

Through the various signaling pathways including MAPK and NFκB activating pathways, LPS induces a diverse array of gene expression including inflammation marker gene products that are involved in innate immune responses and pathogenesis of gram negative septic shock. In this study, it was identified that Tlr4 works as a tollgate for LPS-induced gene expression through the activation of NFκB and other signaling
pathways and these results indicate that MyD88 is one of the immediate downstream signaling components for Tlr4-induced signaling pathways. In addition, it was demonstrated that the transcription factor NFκB is crucial in regulating LPS-stimulated gene expression. Therefore, our results underscore the importance of Tlr4-mediated signaling pathways in LPS-induced gene expression.

While LPS induces the activation of both NFκB and MAPKs including ERK1/2, JNK, and p38, to date, it was demonstrated that NFκB and JNK are activated by Tlr4 which is a known LPS receptor. It was shown that the co-transfection of Tlr4 and MD-2 induced the activation of ERKs, JNK, and p38 by LPS in HEK293 cells (92). However, as previously mentioned, MyD88 is the only adaptor protein that links transmembrane protein Tlr4 to the activation of NFκB and JNK. It is necessary to investigate the mechanism by which p38 and ERKs are activated upon LPS.
PART 2

THE INVOLVEMENT OF A SRC FAMILY PROTEIN, LYN, IN INDUCING COX-2 GENE EXPRESSION

1. REVIEW OF LITERATURE

1.1 Structure of The Src Family Protein

The Src family of protein tyrosine kinases is a member of a family of non-receptor tyrosine kinases and it consists of nine related proteins (Src, Yes, Lyn, Fyn, Lck, Blk, Fgr, Hck, and Yrk). The Src proteins are involved in cellular transformation and intracellular signal transduction. Src family members share the conserved domains: N-terminal acylation domain (SH4), unique domain, SH3, SH2, kinase domain (SH1), and C-terminal regulatory domain (Fig. 2.1).

The N-terminal acylation domain is responsible for targeting the Src proteins to the inner leaflet of the plasma membrane and is required for its own activation. N-terminal acylation of Src family protein is mediated through the co-translational myristylation or the post-translational palmitylation. All Src family members contain a consensus sequence of attachment of myristate: Met-Gly-X-X-Ser/Thr (Fig. 2.2). During translation, the initiator methionine residue is removed by methionine aminopeptidase and myristate co-translationally links to the N-terminal glycine residue via amide bond by the soluble enzyme N-myristyl transferase. Studies with pp60Src and p56Lck have shown that myristylation of Src-related proteins is required for membrane
association. Non-myristylated mutants do not bind efficiently to membrane and do not mediate cell signaling. However it was suggested that myristylation alone is not sufficient to stably anchor a protein to lipid bilayer and additional factors must therefore function to enhance membrane association of myristylated proteins (200).

Indeed, Silverman et al. has identified a motif of three alternating lysine residues within the N-terminal sequences of the Src family members that functions in conjunction with myristate to promote membrane binding (Fig.2.2) (201, 202). The cluster of basic amino acids in the Src family proteins forms electrostatic interactions with negatively charged phospholipids localized primarily to the inner leaflet of the membrane bilayer.

In addition, post-translational acylation of cystein residue 3 and/or 5/6 of p56\textsuperscript{lek} and p59\textsuperscript{fyn} with the 16 carbon fatty acid palmitate has been demonstrated. The consensus sequence for palmitylation is myr-Gly-Cys and 7 of the 9 Src family members contain this sequence (Fig. 2.2). Compared to amide-linked myristylation which is stable and irreversible, palmitylation is linked by a dynamic, reversible thioester bond and Camp et al. isolated palmityl thioesterases which break the thioester bond between palmitate and cysteine (203, 290). Therefore, it was suggested that such reversible palmitylation may be regulated by any signaling events.

Membrane targeting is considered as one of the cellular functions of those N-terminal acylation of Src family proteins and it is required for their activation (204).
SH4: myristylated/palmitylated N-terminus, required for membrane localization.
U (unique domain): varies between Src family members.
SH3: protein interaction domain, binds Proline-rich region
SH2: protein interaction domain, binds phosphorylated tyrosine residues, binds C-terminal phosphorylated tyrosine residue.
SH1: kinase domain, required for enzymatic activity, initial region of SH1 interacts with its own SH3, contains autophosphorylation site.
R (regulatory domain): phosphorylated C-terminal tyrosine residue interacts with its own SH2 domain for repression of Src kinase.

Fig. 2.1. Schematic structure of SRC family proteins. SH, src homology; myr, myristate; pal, palmitate
Fig. 2.2, Comparing N-terminal acylation motifs of the Src family proteins. A short motif (SH4) within the N-terminal unique region contains signals for myristylation and palmitylation, as well as other conserved amino acids. All Src family members contain a consensus sequence of attachment of myristate: Met-Gly-X-X-X-Ser/Thr. The initiator methionine residue is removed by methionine aminopeptidase and myristate co-translationally links to the N-terminal glycine residue during translation. The positively charged amino acids, Lysine (K) and arginine (R), are depicted in bold character and the negatively charged amino acid, aspartic acid (D), is circled. The cluster of basic amino acids in the Src family proteins forms electrostatic interactions with negatively charged phospholipids localized primarily to the inner leaflet of the bilayer. The consensus sequence for palmitylation is myr-Gly-Cys, and 7 of the 9 Src family members contain this sequence. N-terminal cysteine residues are indicated in a rectangular.
While the situation is probably more complex, it was suggested that palmitylation increases the efficiency of membrane binding from the study on Go protein which contains a myristylation and a palmitylation at N-terminal Gly and Cys residues, respectively, similar to Src family proteins (205). Furthermore, studies of p56\textsuperscript{ck} and p59\textsuperscript{lyn} indicate that palmitylation of Src family members enhances interactions with glycosyl phosphatidylinositol (GPI)-linked proteins and influences membrane localization (206,207).

Downstream of the acylation motif lies a short (50-80 amino acids) sequence, termed the unique domain, that varies highly among each Src family protein. Two distinct Src homology regions (SH3 and SH2) are followed. These regions are protein interaction domains. SH3 domain interacts with specific proline-rich sequences (208). The SH2 domain in turn binds to phosphorylated tyrosine residues in the context of specific amino acid sequences (209). SH1 kinase domain is responsible for enzymatic activity, and mutations within the kinase domain inactivate Src proteins. In addition, the major autophosphorylation site is also located in this kinase domain, and mutation of autophosphorylation sites leads to decreased kinase activity. Finally, near the end of the Src family proteins is a conserved tyrosine residue (e.g. tyrosine 527 in Src and tyrosine 508 in Lyn), that is critical for the regulation of the molecule. This tyrosine provides one of the key regulatory features for Src proteins and is a defining feature of the Src family kinases (210).
1.2 Regulation of The Src Family Proteins

*c-src* is the normal cellular proto-oncogene of *v-src* transforming gene. The study of the *v-src* oncogene demonstrated the active comparison of differences between the two molecules: *v-src* is the C-terminal truncated version of normal *c*-Src such that C-terminal tyrosine at 527 residue is deleted (211). Furthermore, the reports that either dephosphorylation or mutation of Y527 constitutively activates the *c*-Src kinase and enables it to transform cells, implicates this residue is a key regulatory element (291-293). Evidence suggests that the phosphorylated C-terminal tyrosine interacts intramolecularly with Src’s own SH2 domain, thereby inactivating the kinase, as depicted in Fig. 2.3. It has been postulated that the SH2-Y527 interaction would either distort or cover the kinase domain, leading to its inactivation in this ‘closed conformation’. The recent crystallographic studies shows that the kinase domain remains exposed, even in the ‘closed conformation’ when the SH2 domain binds phosphorylated C-terminal tyrosine, such as Y527 in Src. However, in addition to this interaction, the SH3 domain exhibits a separate intramolecular contact with the initial region of the kinase domain. Thus, inactivation of the kinase may result from torsional constraint where the dual binding of the SH2 and SH3 domains prevents free movement within the kinase domain (212-214). Mutation of either SH3 or SH2 frees this constraint and probably leads to decreased stability of the other inhibitory interactions. Indeed, mutations in the SH2 domain also activate the *c*-Src kinase, as would be expected by disrupting this intramolecular interaction (211). Therefore, both the SH2 and SH3 domains contribute to the regulation of the kinase via intramolecular
Fig. 2.3. Activation of the Src family proteins. The phosphorylated C-terminal tyrosine interacts intramolecularly with Src's own SH2 domain, thereby inactivating the kinase by distorting or covering the kinase domain, leading to 'closed conformation'. The SH3 domain exhibits a separate intramolecular contact with the initial region of the kinase domain. Thus, inactivation of the kinase may result from torsional constraint where the dual binding of the SH2 and SH3 domains prevents free movement within the kinase domain. Mutation of either SH3 or SH2 frees this constraint and leads to disrupting such intramolecular interaction. CSK is the cellular Src kinase.
Fig.2.4. The Src family proteins are phosphorylated by CSK and dephosphorylated by CD45. Phosphorylation of C-terminal Tyrosine residue is carried out by the cellular Src kinase (CSK). The receptor protein tyrosine phosphatase, CD45, is a candidate to dephosphorylate the C-terminal tyrosine residue of the Src family proteins.
interactions and disruption of these interactions may be a major mechanism for activation of Src. Phosphorylation of C-terminal Tyrosine residue is carried out by another kinase, the cellular Src kinase (CSK), and the targeted deletion of CSK in mice causes constitutive activation of the Src family kinase (Fig. 2.4) (215, 216). Despite considerable efforts, there is still uncertainty as to which protein tyrosine phosphatase (PTP) dephosphorylates the C-terminal tyrosine residue of the Src family proteins. In lymphocytes, the receptor protein tyrosine phosphatase, CD45, is a candidate for PTP and it is believed that CD45 is required for activation of Src family kinase. Absence of CD45 raises the steady-state level of Lck C-terminal phosphorylation and decreases the cellular response to stimulation through the antigen receptor (217, 218).

1.3 Functions of the Src family proteins

Until the past few years, hypotheses predicting the normal cellular functions of Src were based on studies of the biological effects of over-expressed, mutationally-activated forms. In fibroblasts, activated mutants of Src and its relatives are oncogenic, and in some other cell types, the same mutants induce differentiation. These biological effects are presumably the consequence of constitutive, high-level activation of signaling pathways, including various pathways involved in PI3 kinase, PLCγ, SHPT2, Ras, STATs, etc. Combined with the observations that endogenous Src is activated in growth factor-stimulated cells, the biological effects of activated Src led to the suggestion that its normal role might be to regulate cell proliferation and differentiation (219). Similarly, in various hematopoietic cell lines, Src relatives are activated in
response to antigen stimulation, and activated mutants can lead to the same responses as antigen stimulation, such as proliferation and cytokine production (220). However, direct attempts to identify the normal functions of Src family kinases, by blocking their function in living cells and organisms, have been hampered by problems of redundancy and specificity. To date, disruptions of the mouse Src, Fyn, Lck, Hck, Fgr, and Lyn genes have been reported (221-223). The most noticeable defect in Src− mice is osteopetrosis. Src is required for the normal differentiation and response of osteoclasts, a type of myeloid cell that absorbs bone. However, the biochemical explanation on such osteopetrosis in Src− mice is not clear (224). In addition, Fyn− mice showed alterations in the hippocampus. Two subregions of the hippocampus, the granule cell layer of the dentate gyrus and the pyramidal cell layer of the CA3 region, have 25% more neurons than in wild type mice. Such phenotype could be due to reduced cell death (225). Another Fyn− mouse strain shows neurological abnormality early in life: the neonates fail to suckle. This may be due to a 40-50% decrease in myelination in the brains of Fyn-deficient mice. However, the molecular explanations for such defects were not clear (226,227).

In a mutant Jurkat cell line, a lymphocyte cell line that lacks Lck failed to respond to antigen receptor stimulation with a Ca^{2+} flux. But re-expression of Lck in this cell line restores signaling. This proves the requirement for Lck for a Ca^{2+} response to antigen stimulation of Jurkat cells (228). In addition to Lck, another Src family proteins, Lyn, was suggested to be involved in the response to antigen receptor in B cells. Lyn disrupted chicken B cell line, Lyn− DT40, have delayed and reduced Ca^{2+}
mobilization in response to antigen receptor stimulation (229). In these cells, it was determined that both Lyn and another cytoplasmic kinase, Syk, associate with the tyrosine phosphorylated B cell antigen receptor (230,231). The studies with Lyn', Syk', and Csk' DT40 cells suggested a model: Lyn phosphorylates the antigen receptor to provide a binding site for Syk and then phosphorylates and activates Syk. According to those results, Csk' DT40 cells contain activated Lyn as expected, since Src family proteins are negatively regulated by Csk. In turn, the active Lyn activated Syk, indicating that activation of Lyn can cause activation of Syk (232). Furthermore, it was shown that in Lyn' DT40 cells, Syk tyrosine phosphorylation and activation in response to antigen is greatly reduced (233), but Lyn can still be activated in Syk' DT40 cells (234). Furthermore, since antigen-induced phosphorylation of Shc is decreased in both Lyn' and Syk' cells, it could be suggested that Shc may be phosphorylated by Syk not Lyn (235). Therefore, a model for the relationship between Csk, Lyn, Syk, and She with respect to the antigen receptor was provided in DT40 chicken B cells: Csk activated Lyn phosphorylates the antigen receptor, providing a binding site for Syk, and then phosphorylates Syk, and Syk in turn, phosphorylates She.

A growing number of extracellular agents that would stimulate Src family kinases were identified. In fibroblasts, Src, Fyn, and Yes are activated by platelet derived growth factor (PDGF), colony stimulating factor-1 (CSF1), epidermal growth factor (EGF), and fibroblast growth factor (FGF). They associate with the PDGF and CSF1 receptors through their SH2 domain (236-241). Various G-protein coupled receptors including those for thrombin, endothelin-1, and F-met-leu-phe, also activate Src family kinases.
kinases (242-245). In addition, oxidative or radiation stress appears to induce transcription via activation of Src family members (246, 247). Increased intracellular calcium may also stimulate Src proteins (248). In lymphocytes, Src relatives are activated by interleukin 2 (249).

As described above, Src family proteins participate in the broad range of signaling events and nine Src family proteins participate in various signaling pathways so that it may be hard to identify the essential functions that cannot be bypassed by other pathways. However, the challenge for the future will be to describe the full range of signaling pathways in which Src family kinases participate.

Among the cellular enzymes that are involved in signal transduction, the protein tyrosine kinases (PTKs) appear to play key roles in the initiation of various signaling cascades. The PTKs can be divided into two broad groups based on their predicted structures. One group, which possesses extracellular domains that generally bind polypeptide hormones, is the receptor PTKs, such as the platelet-derived growth factor receptor, the epidermal growth factor receptor, the nerve growth factor receptor, and the insulin receptor (250, 251). The second major group lacks extracellular sequences and is considered as the nonreceptor PTKs, even though many members of this group appear to be noncovalently associated with some type of cell surface ligand-binding protein.

There appear to be at least eight distinct families of nonreceptor PTKs. These include the previously identified Src, Abl, and Tes/Fps families as well as the recently described kinases exemplified by focal adhesion kinase (FAK) (251), cellular Src kinase
(CSK) (215), Janus kinases (JAK1, JAK2, and TYK2) (252, 253), spleen tyrosine kinase (SYK) (254), and the interleukin-2-inducible T cell kinase (ITK) (255).

Among the PTKs, the Src family is currently composed of nine members: Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk, and Yrk. The Src family protein has the characteristic structure: N-terminal acylation region, 'unique' domain, SH3, SH2, kinase domain, and C-terminal regulation domain. The sequences necessary for myristylation and stable membrane association are located at the N-terminus of the Src family protein. The myristate is covalently bound by amide linkage to a common glycine residue at position 2 (256) and the palmitate is posttranslationally linked to another common cysteine residue at position 3 (206). The 50- to 80-amino acid 'unique' domain is adjacent to the acylation sequence. The amino acid sequence of 'unique' domain is variable between Src family members. Followed are the roughly 50 amino acids SH3 (Src homology 3) and approximately 100 amino acid SH2 domain. These regions share identity with other non-receptor PTKs, as well as other classes of cellular regulatory and structural proteins. A major focus of research has highlighted a role for SH3 domain-mediated interactions in the regulation of signal transduction events. The SH2 domains of these signaling proteins bind tyrosine phosphorylated polypeptides. Tyrosine phosphorylation acts as a switch to induce the binding of SH2 domains, thereby mediating the formation of heteromeric protein complexes. The formation of these complexes is likely to control the activation of signal transduction pathways by tyrosine kinases (257,258). SH3 domains are small modules found in a diverse array of proteins. The presence of an SH3 domain confers the ability to interact
with specific proline-rich sequences in protein binding partners (210,219,259,260). The majority of the carboxyl-terminal half of the Src proteins represents the catalytic domain (this would be the SH1 domain), which is the region of highest Src family sequence identity. The catalytic domain includes the ATP binding site centered around a common lysine residue and the site of enzyme tyrosine autophosphorylation. The last 15 to 17 amino acids are termed the regulatory domain because deletion of these sequences or site-specific mutation of the terminal tyrosine to some other amino acid significantly activates the enzymatic function of Src PTKs (211). Biochemically, such alterations are related to the dephosphorylation status of the last tyrosine residue in the enzyme. In inactive and wild type Src proteins, the last tyrosine residue is phosphorylated by another cellular PTK, C-terminal Src kinase (CSK), and the phosphorylated carboxyl-terminal portion of the enzyme interacts with the enzyme’s own SH2 domain, thereby resulting in conformational change to hide the catalytic domain and keeping Src inactive. However, such alterations in C-terminal regulatory domain of Src proteins result in exposing the catalytic domain to be activated. Therefore, such alterations render Src PTKs constitutively active, and when expressed in an appropriate cell type, these mutants are highly oncogenic. Meanwhile, in order to activate a wild-type Src protein, the last C-terminal phosphorylated tyrosine should be dephosphorylated by a cellular phosphatase. It is believed that the phosphatase CD45 is a candidate to activate Src family kinases in T cells (261,262).
2. INTRODUCTION

Lyn is a member of the Src family of intracellular membrane-associated tyrosine kinases (263). Alternative splicing at the 5' end of the lyn gene results in the expression of p53 and p56 isoforms of the protein (264,268,269). Lyn is expressed mainly in hematopoietic cells of myeloid (macrophage, monocyte, and platelets) and B lymphoid origin (270). It is involved in the transmission of signals from a number of receptors such as the IL-2 receptor, SM-CSF receptor, high-affinity IgE receptor (FceR1), B-cell antigen receptor, and thrombin receptor (271-278). Furthermore, Lyn has been implicated in the phosphorylation of a number of signaling molecules, including phosphoinositol-3 kinase (PI-3K), ras GTPase activating protein (GAP), phospholipase C (PLC) γ2 and mitogen-activated protein (MAP) kinase (274,276,279,280).

Herbimycin A, an inhibitor of protein tyrosine kinases, blocked the LPS-induced protein tyrosine phosphorylations and LPS-induced biological response (17). Novogrodsky et al showed that tyrosine kinase inhibitors of the tyrophostin AG 126 family inhibited LPS-induced tyrosine phosphorylation of MAPK and production of TNFα, and prevented septic shock in mice (174). In addition, Orlicek et al demonstrated that the Src family kinase-selective inhibitor, PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), inhibits LPS- and IFN-γ-induced iNOS and TNF productions in the murine macrophage cell line RAW264.7 (176,177). Chanmugam et al reported that the Src family tyrosine kinases, Hck, Lyn, and Fgr, were the major tyrosine-phosphorylated proteins in LPS-stimulated macrophages and another protein tyrosine kinase inhibitor, Radicicol, suppressed the LPS-induced COX-2 gene
expression (175). Several publications suggest that three members of the Src-family of protein tyrosine kinases, Hck, Fgr, and Lyn, are strong candidates for the primary signal transducers of LPS responses (178,281-285). Moreover, expression in macrophages of a constitutively active mutant of Hck augments TNF-α production while antisense oligonucleotides to Hck inhibit LPS-induced responses (142). Chronic exposure (24-48 hours) of macrophages to LPS induces increased synthesis of Hck and Lyn, which correlates with the ability of LPS to prime macrophages for respiratory burst (285).

While all three of these kinases are rapidly activated after LSP treatment, it was shown that a portion of intracellular Lyn was co-immunoprecipitated with CD14 (178). All of these observations suggest that protein tyrosine kinases, especially Hck, Fgr, and Lyn play critical roles in LPS-initiated signaling pathways. However, Lowell et al showed by using hck<sup>−/−</sup> fgr<sup>−/−</sup> lyn<sup>−/−</sup> triple mutant mice that Hck, Fgr, and Lyn are not obligatory for LPS-initiated signal transduction (185). Their analyses indicated that both elicited peritoneal (PEMs) and bone marrow-derived macrophages (BMDMs) from the triple mutant mice have no major defects in LPS-induced activation, although the total protein phosphotyrosine level is greatly reduced in macrophages derived from the same mutant mice. According to their studies, the activation of the ERK1/2 and JNK kinases, as well as the transcription factor NFκB, is the same in normal and mutant macrophages after LPS stimulation. These data suggest that these Src-related kinases are not required for LPS-induced macrophage activation, but do not exclude an important role for these kinases in LPS-mediated signaling in normal macrophages. Other Src kinase family protein may play a role in LPS-induced signaling events.
The purpose of this study was to determine whether or not Lyn participates in LPS-induced signaling pathways leading to COX-2 gene expression that is induced by LPS stimulation by using the conditional alleles for cytoplasmic Src-like tyrosine kinases. Conditional alleles have proven essential for understanding the role of many genes because they add the dimension of time to genetic analyses and thereby avoid the myriad secondary effects and compensatory mechanisms brought into play when a gene is constitutively absent or active. Recently, Schreiber's group has developed a new inducible expression system using the cell-permeable chemical inducer of dimerization (CID) FK1012 (266). FK1012 is a dimer of FK506 and induces dimerization between two chimeric proteins containing the FK506-binding protein, FKBP (Fig. 2.5). The principle of this inducible expression system is to activate a certain signaling molecule by artificially inducing proximity with their effector targets. For the Src family kinases, their ability requires membrane localization by an N-terminal acylation (204). Additionally, all Src-family proteins have a conserved regulatory tyrosine residue at their C-terminal end, which must be dephosphorylated for maximum activity as previously described. For Lyn which is one of the Src-family kinases, Tilbrook et al already showed that a substitution of 508 tyrosine residue with phenylalanine at the C-terminus of Lyn (LynY508F) gives rise to a constitutively active Lyn protein. In addition to a constitutively active Lyn (Y508F), the tyrosine residue 397 within the kinase domain of Lyn is critical for maintaining kinase activity. Thus, another substitution of the tyrosine 397 residue with phenylalanine [Lyn (Y397F)] also results in a dominant negative mutant form of Lyn (286). Therefore, our conditional allele has
been designed to artificially localize a constitutively active Lyn chimera to the plasma membrane. By regulating the membrane localization of the active form of Lyn, it is possible to determine whether activation of Lyn can induce COX-2 gene expression. Besides a conditional allele, the tetracycline-controlled retroviral vector was used for the transfection and the expression of Lyn constructs (287, 288). The expression of endogenous COX-2 was then measured to determine whether or not the activation of Lyn can induce COX-2 gene expression and if the inhibition of Lyn by Lyn (Y397F) can suppress LPS-stimulated COX-2 gene expression.
Fig. 2.5. The principle of the conditional allele: inducible activation of Lyn protein. A membrane-bound docking protein of FKBP and a soluble targeting FKBP chimera were co-transfected and the interaction between the expressed docking and targeting proteins were induced by membrane permeable dimerizing agent, FK1012, which is a dimer form of FK506. FKBP is FK506 binding protein.
3. MATERIALS AND METHODS

3.1 Lyn Expression Constructs for Conditional Allele

The original constructs for the conditional Lyn allele were kindly provided by Dr. Schreiber in Harvard University, Cambridge, MA. pCMF2E expresses the membrane docking protein and it expresses 2 tandem repeat of FKBP (FK506 binding protein) fused with membrane binding motif. Due to the membrane binding motif, the expressed docking protein is localized to the cytoplasmic side of plasma membrane. As a targeting protein construct, human Lyn(Y508F) was modified to fuse 2 tandem repeat of FKBP with soluble Lyn whose first 10 amino acids were removed by PCR cloning with primers (Fig.2.6):

GCGGCACCTAGTAGCTTGAGTGACGATGGAGTAGATTTG and
GGTAAGGATCCCTATTATGCGTAGTCGTTACCGGATTAGCATTTT
GCTGGAATTGCCCTTC. The PCR products was subcloned into SpeI and BamHI restriction sites of pCF1E to produce pCF1E-2xFKBP-(A10)Lyn(Y508F) which is a soluble chimera of FKBP and constitutively active Lyn. In addition, the PCR products were inserted into the same restriction sites of pCMF2E to produce pCMF2E-2xFKBP-(A10)Lyn(Y508F) which has a membrane binding domain fused with FKBP and constitutively active form of Lyn. pCMF2E-2xFKBP-(A10)Lyn(Y508F) alone can be localized to plasma membrane without docking protein, because it already contains membrane targeting domain at N-terminal end.
Fig. 2.6. The schematic representation of the cytosolic targeting protein, 2xFKBP-(Δ10)Lyn(Y508F). C-terminal tyrosine was substituted with phenylalanine for the constitutively active Lyn, Lyn (Y508F). N-terminal acylation motif containing 10 amino acids was replaced with 2xFKBP in order to prevent a spontaneous membrane targeting, but FKBP motif in Lyn (Y508F) chimera is able to interact with another FKBP in membrane docking protein by the presence of dimerizing agent, FK1012.
Conversely, pCF1E-2xFKBP-(Δ10)Lyn(Y508F) does not contain the membrane binding motif. It therefore needs a docking protein for membrane targeting. Therefore, both of a membrane docking protein construct, pCMF2E, and a targeting protein construct, pCF1E-2xFKBP-(Δ10)Lyn(Y508F), were co-transfected in to NIH3T3 cell and the transfected cells were treated with dimerizing agent, FK1012, to get a artificial interaction between membrane bound 2xFKBP and cytosolic 2xFKBP-(Δ10)Lyn(Y508F). Transfection of these constructs into NIH3T3 cells was followed the detail procedures as it was described in Materials and Methods in the previous chapter.

3.2 Retroviral Expression Construct for Lyn

Mouse Lyn constructs including a wild type [Lyn(wt)], a dominant negative mutant [Lyn(Y397F)], and a constitutively active mutant [Lyn(Y508F)] was provided by Dr. Hibbs at the Ludwig institute in Melbourne, Australia (286). Flag epitope was fused at C-terminal end of each type of Lyn by PCR with primers:

CCATTGGTACCGAAAACCTTCCACCACGAGAGAAATATG.
TTTATCATCATCATTTTTATAATCCGGTTGCTGCTGATACTGC and
GTGCTTGCGGCCGCTATCATTTATCATATCATCATCATCTTTTATAA

TC. The PCR products were then subcloned into pcDNA3.1/zeo(-) expression vector. Each cDNA construct was subcloned into the retroviral vector, pLinx (Fig. 2.7) (287). The integrity of DNA sequence was confirmed by sequencing the constructs.
Fig. 2.7. The schematic representation of pLinx retroviral vector. The genetic organization of pLinx retroviral vector was from moloney murine leukemia virus (MMLV). LTR, long terminal repeat; tTA, tetracycline-regulated transactivator; IRES, internal ribosomal entry site; Neo, neomycin resistant gene; CMV, cytomegalovirus enhancer-promoter.
Fig. 2.8. The schematic procedure for transfection and infection with the retroviral vector, pLinx. The detail protocols were described in Materials and methods.
3.3 Transfection and viral infection for stably transfected colony

As described in Fig. 2.8, “phoenix” cells (1×10⁶ cells) which are the packaging cells were plated into a 60 mm dish one day before transfection. To prepare DNA/Superfect reagent complexes, 5 μg of DNA was diluted with serum free DMEM incomplete media in an eppendorf tube. Superfect reagent (30μl) was added to the same tube. The DNA/SuperFect reagent complexes were incubated for 10 minutes at room temperature. Then, 1 ml of complete media were added to the reaction tube. After several pipettings, the complexes were added to the phoenix cells. After 3 hours incubation, the media were removed and changed with DMEM complete media. After 24 hours post transfection, the culture media containing infectious viral particles produced by transfected phoenix cells were transferred to 15 ml culture tube and centrifuged at 1,500 rpm for 5 minutes at room temperature. Only the supernatant was added to the target cells, NIH3T3 cells, for the infection. Four μg/ml of Polybrene was added to increase the efficiency of infection. After 24 hours post-infection, the culture media was removed and changed with complete media and 48 hour after transfection, infected cells were harvested and plated with neomycin (800 ug/ml) containing media for single colony selection in the presence of 8 μg/ml if tetracycline. 1 or 2 weeks later, single colony could be visible under a microscope and then, each colony was transferred to the new culture dishes for propagation. Some of the propagated single colony cells were frozen for the aliquot and some of the selected cells were prepared for a cultivation with and without tetracycline to determine whether the expression of stably
transfected Lyn construct can be suppressed by the presence of tetracycline and induced by the absence of tetracycline through western blotting.

3.4 Lyn Constructs

Wild type murine Lyn cDNA was provided by Dr. Margaret L. Hibbs in Ludwig Institute for Cancer Research, Australia. Lyn (wt) cDNA was amplified by PCR with primers F1 and R1 and subcloned into Nol and BamH1 sites of p3xFlag-CMV14 expression vector (Sigma). Tyrosine at 508 residue was replaced with phenylalanine by PCR with specific primers, F2 and R2, and EcoR1/BamH1 fragment of PCR product replaced the C-terminal region of Lyn (wt)-3xFlag to give rise to Lyn (Y508F)-3xFlag. Although F2 primer contains a mutation at 397 residue as indicated below, subcloning by EcoR1/BamH1 sites result in only Y508F mutation because 397 residue is outside of the subcloning sites. Furthermore, the PCR product with primer F2 and R2 containing Y397F mutation was also used as a mega-reverse primer with a forward primer, F1, for PCR. The Xba1/EcoR1 fragment of the PCR product was inserted into the same restriction sites in Lyn (wt)-3xFlag to produce the Lyn (Y397F)-3xFlag construct. In addition, Xba1/EcoR1 fragment from Lyn (Y397F)-3xFlag replaced the region in Lyn (Y508F)-3xFlag to prepare Lyn (Y397F/Y508F)-3xFlag construct. A mutation of C3A in N-terminal region of Lyn was achieved by PCR of Lyn (wt) with a specific forward primer, F3, and R1. The PCR fragment containing C3A substitution was inserted into Not1/Xba1 sites of Lyn (wt)-3xFlag construct resulting in Lyn (C3A)-3x-Flag construct. Additionally, Lyn (C3A)-3xFlag was used as a template for another PCR with primers, F4 and R1, to prepare G2A/C3A mutations and the PCR product was
inserted into NotI/XbaI region of Lyn (Y397F/Y508F)-3xFlag cDNA. Consequently, it gives rise to Lyn (G2A/C3A/Y397F/Y508F)-3xFlag construct. The integrity of the complete DNA sequence was confirmed by sequencing all the constructs and the expression of Lyn constructs was verified by western blotting. All of Lyn constructs were described in Fig. 2.9 and the PCR primers for Lyn constructs were as follows: F1, AAAGCGGCCGCACCCATGGGATGTATTAAATCAAAAAGG; R1, GATGAAA GGATCCCCGTTGCTGCTGACTGCCC; F2, CGAAGATAACGAGTTTACA GCAAGGGAAGG (bold nucleotides stand for a mutated codon of Y397F); R2, GAT GAATGGATCCCGGTTGCTGCTGAAACTGCCC-3' (bold nucleotides stand for a mutated codon of Y508F); F3, GAGAAAGCGGCCCACCCCATGGGAGCTATTAAATCAAAAAGG (bold nucleotides stand for a mutated codon of C3A); F4, GAGAAA GCAGCCGCACCCATGGCCAGCTATTAAATCAAAAAGG-3' (bold nucleotides stand for a mutated codon of G2A).

3.5 Luciferase Reporter Gene Array

RAW264.7 cells were plated in six-well plates (5x10^5 cells/well) and transfected with total 5 µg of DNA plasmids including HSP-70-β-gal plasmid as an internal control using SuperFect transfect reagent (Qiagen) according to the manufacturer’s instruction. Relative luciferase activity was determined by normalization with β-galactosidase activity as described in our previous study (106).
**Fig. 2.9. The schematic illustration of murine Lyn constructs.** A kinase-defective Lyn (Y397F) is a kinase-defective form of Lyn due to a mutation of Y397F within a kinase domain of Lyn protein. Lyn (Y508F) is a constitutively active form, because Y508F in the regulatory domain leads to the open conformation of Lyn protein. Lyn (Y397F/Y508F) may also function as a kinase-defective form since it is still kinase-defective. The substitution of Cys with Ala at 3 residue for palmitylation inhibits the acylation at 3 residue in the protein. Gly at 2 is covalently linked with myristylate. Therefore, the point mutation of G2A blocks the myristylation of Lyn protein. Consequently, Lyn (G2A/C3A/Y397F/Y508F) is not available for the lipid-mediated membrane localization by both myristylation and palmitylation and also kinase-defective protein with the open-conformation. Therefore, Lyn (G2A/C3A/Y397F/Y508F) mutant may work as another inactive form of Lyn.
4. RESULTS

4.1 Conditional Allele of Lyn: Inducible Activation of Lyn

Both docking protein and targeting protein expression constructs for Lyn conditional allele were co-transfected into NIH3T3 cells. The expression of these constructs was verified by western blotting protocol (Fig.2.10). Twenty four hours after transfection, 1 μg/ml of dimerizing agent, FK1012, was added to the cells for 1 day in order to induce the interaction between the membrane bound docking protein and cytosolic Lyn (Y508F). In turn, cell lysates were prepared in RIPA buffer for the western blotting protocol with the COX-2 antibody to determine whether or not the activation of Lyn induces COX-2 gene expression. Although the docking protein and the targeting protein were expressed, the treatment of the dimerizing agent did not show the expression of COX-2.

4.2 Tetracycline-inducible Expression of Lyn Did Not Induce COX-2 Gene Expression in NIH3T3 Cells

As described in Materials and Methods, tetracycline-inducible Lyn (Y508F), a constitutively active mutant, was stably transfected into NIH3T3 cells. Each colony whose Lyn expression is regulated by the presence of tetracycline was isolated and its inducible expression of Lyn was verified by western blotting. As shown in Fig.2.11, three colonies show the inducible expression of Lyn (Y508F) by tetracycline. While those colonies induce the expression of a constitutively active Lyn [(Lyn (Y508F)], the expression of COX-2 was not induced by the inducible expression of Lyn (Y508F).
Fig. 2.10. The expression of a membrane docking protein and a targeting protein in NIH3T3 cells. The expression of pCSF1E-(Δ10)Lyn (Y508F)-HA (cytosolic targeting protein) and pCMF2E (membrane bound docking protein) was verified by western blotting using Lyn antibody. pCMF2E-(Δ10)Lyn (Y508F)-HA which has a membrane binding domain at N-terminal of the construct was also transfected. The endogenous expressions of Lyn were also detected at the points of 56 KDa and 53 KDa. Lane 1, No DNA transfection; Lane 2, pCMF2E-(Δ10)Lyn(Y508F)-HA; Lane 3, pCF1E-(Δ10)Lyn(Y508F)-HA (targeting protein); Lane 4, pCMF2E(docking protein).
4.3 ΔTlr4(wt)- or LPS-induced COX-2 Promoter Activity Was Suppressed by Herbimycin A in RAW264.7 Cells

It was already known that LPS responses could be suppressed by protein tyrosine kinase inhibitor such as herbimycin A (Sigma). In this study, it was determined that Tlr4 mediates LPS response leading to COX-2 gene expression. Therefore, ΔTlr4 (wt)-induced COX-2 gene expression should be suppressed in the presence of herbimycin A. As shown in Fig.2.12, ΔTlr4 (wt)-induced COX-2 promoter activity was suppressed by herbimycin A. This result may suggest that any protein tyrosine kinase, such as the Src family protein, may participate in Tlr4-induced signal transduction pathways. Since the chemical compound basically shows the unknown and nonspecific activity in addition to its specific biochemical activity, it is not sufficient to establish the involvement of the protein tyrosine kinase in Tlr4 signaling pathways on the basis of the result from herbimycin A. Therefore, a study with a dominant negative mutant and a constitutively active form of the Src family protein, especially Lyn, is needed to determine whether or not Lyn is involved in LPS- or Tlr4-induced signaling pathways.

4.4 Transfection of Lyn Variants Does Not Affect COX-2 Promoter Activity in RAW264.7 Cells

Several kinds of Lyn variants were constructed as described in Fig.2.9. Lyn (Y397F) is a kinase-defective mutant, because the mutated amino acid residue is in kinase domain. Lyn (Y508F) is a constitutively active form, because a Y508F mutation in C-terminal regulatory region results in an open conformation of Lyn. Furthermore, the acylation-defective Lyn mutants can not be localized to the cell membrane for its activation. Therefore, these Lyn variants were co-transfected with COX-2 reporter
construct into murine macrophage cell line, RAW264.7 cells, to investigate whether or not Lyn induces COX-2 gene expression. As shown in Fig. 2.13, any of Lyn constructs did not affect the expression of COX-2 reporter construct. While both Y397F and Y508F mutations were already verified as a kinase-defective and a constitutively active mutation, respectively (286), in this study, Lyn (Y508F) did not affect COX-2 promoter activity in RAW264.7 cells.
Fig. 2.11. Tetracycline regulated Lyn (Y508F) expression in NIH3T3 cells. Lyn (Y508F) construct in a retroviral vector was transfected and infected into NIH3T3 cells. After selecting the stably transfected clones, each clone was screened to isolate the tetracycline-regulated clones in which an expression of the transfected Lyn (Y508F) could be suppressed in the presence of tetracycline and induced by the absence of tetracycline. Cell lysate from each clone with or without tetracycline was used for immunoblot assay with Lyn antibody as shown in the upper panel. The expression of Lyn (Y508F) was regulated by tetracycline in Clone #1, 6, and 7. Clone #6 showed more efficient regulation of Lyn (Y508F) expression. The same blot was stripped and reprobed with COX antibody that recognizes the endogenous COX-1 and COX-2.
Fig.2.12. Suppression of ΔTlr4 (wt)- or LPS-induced COX-2 promoter activity by herbimycin A in RAW264.7 cells. RAW264.7 cells were transfected with a luciferase reporter plasmid for COX-2 promoter and a constitutively active form of Tlr4, ΔTlr4 (wt). (Lane 1 and 2) or the empty vector for ΔTlr4 (wt) (lane 3, 4, and 5). The transfected cells of lane 3 and 4 were treated with LPS (20 ng/ml) for 20 hours. At the same time, herbimycin A (0.5 μg/ml) (Sigma) was treated as indicated above and the final concentration of vehicle (DMSO) was 0.05%. Panels are representative data from more than three different analyses. Values are mean ± SEM (n=3).
Fig. 2.13. Transfection of Lyn variants does not affect COX-2 promoter activity in RAW264.7 cells. RAW264.7 cells were co-transfected with 2 µg of Lyn construct and 2 µg luciferase reporter plasmid for COX-2 promoter as indicated. 1 µg of HSP70-β-gal construct was also included as an internal control. A kinase-defective, Lyn (Y397F), and a constitutively active form of Lyn, Lyn (Y508F), (Lane 3 and 4, respectively) did not affect COX-2 promoter activity. The empty vector transfected cells of lane 8 were treated with LPS (10 ng/ml) for 12 hours. Panels are representative data from more than three different analyses. RLA, relative luciferase activity. Values are mean ± SEM (n=3).
5. SUMMARY AND DISCUSSION

The conditional allele of Lyn was designed to conditionally activate Lyn by regulating the membrane localization of Lyn protein. It is a good method to investigate the biochemical function of any membrane targeting protein, such as Src family protein. Thus, the conditional allele of Lyn was used to study its involvement in COX-2 gene expression. The co-transfection of a membrane docking protein and a Lyn targeting protein did not induced the expression of COX-2 in the presence of dimerizing protein, FK1026 (Fig. 2.10). However, it still need to be investigated under another modified conditions, such as time course treatment or increased concentrations of dimerizing agent.

The tetracycline-regulated expression of Lyn (Y508F) was shown in NIH3T3 cells (Fig. 2.11). The inducible expression of Lyn (Y508F) did not induce the expression of COX-2. Those results may imply that Lyn may not be involved in COX-2 gene expression. However, Xie et al. showed that pp60-src transfection induced COX-2 gene expression by activation of JNK in NIH3T3 cells (148). Furthermore, Yi et al. showed that a constitutively active Hck [Hck (Y501F)] augmented TNFα production in the murine macrophage cell line, BAC1.2F5 cells (142). They also showed that inhibition of endogenous Hck expression with anti-sense oligonucleotides diminished LPS-mediated TNFα production in BAC1.2F5 cells. Together, these observations suggest that a member of the Src family of tyrosine kinases is an important component of the signal transduction pathways leading to the pro-inflammatory cytokines. Indeed, a protein tyrosine kinase inhibitor, herbymycin A, suppressed LPS- or ΔTlr4-induced
COX-2 promoter activity in RAW265.7 cells, as shown in Fig. 2.12. Therefore, it may require further investigation on whether or not activation of Lyn is sufficient for COX-2 gene expression.

The Lyn variants including a kinase-defective and a constitutively active form were constructed and co-transfected with COX-2 promoter reporter construct into RAW264.7 cells. However, the transfection of Lyn variants did not affect the COX-2 promoter activity as shown in Fig. 2.13. The constitutively active form of Lyn, Lyn (Y508F), did not induced the reporter activity, either. These results imply that Lyn may not be involved in the signal transduction to COX-2 gene expression in RAW264.7 cells. The diminution of COX-2 reporter activity by herbimycin A might be due to its unknown specificity. Therefore, it is concluded that there may be no direct relevance between Lyn and COX-2 gene expression in RAW264.7 cells.
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APPENDIX 1

YEAST-TWO HYBRID SYSTEM WITH TLR4

Yeast-two hybrid protocol was performed by following DupLEX-A yeast two-hybrid system (OriGene Technologies, Inc). The protocol involves fusing the E. coli LexA protein with the cytoplasmic domain of human Tlr4 as the bait construct and the acid blob domain B42 with the human peripheral blood leukocyte cDNA library as the target construct. The cytoplasmic domain of human Tlr4 was subcloned into pEG202-NLS vector for the bait construct using PCR cloning method with primers: GCCGAGAATTCTCTCTGTTGTAGCAGTTCTGGTC and CCATGGGATCCCTTCAGATAGATGTTGCTCTT. The binding sites for LexA is placed upstream of a reporter gene (such as lacZ) in order to monitor the transcriptional activation by the interaction of bait and target proteins. Thus, the yeast EGY48 reporter strain was utilized for transformation with the bait and the target constructs and in turn, the colonies with the activation of reporter gene were selected for investigating the identity of the target cDNA by DNA sequencing. By following the manufacturer's protocol, three positive clones were isolated and they are going to be identified by further study.
## APPENDIX 2

### COMPONENTS FOR BUFFERS AND GELS

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x Laemmli Buffer</td>
<td>125mM Tris-Cl (pH 6.8), 20% (v/v) glycerol, 5% (w/v) SDS. 10% (v/v) β-mercaptoethanol, 0.016% (w/v) bromophenol blue.</td>
</tr>
<tr>
<td>RIPA Buffer</td>
<td>50mM Tris-Cl (pH 7.4), 1% (v/v) Nonidet P-40, 0.25% (w/v) Sodium deoxycholate, 150mM NaCl, 1mM EGTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM Na&lt;sub&gt;3&lt;/sub&gt;V&lt;sub&gt;4&lt;/sub&gt;, 10 μg/ml leupeptin, 10 μg/ml aprotinin.</td>
</tr>
<tr>
<td>Stripping Buffer</td>
<td>2% (w/v) SDS, 50mM Tris-Cl (pH 6.8), 100mM (pH 6.5) β-mercaptoethanol.</td>
</tr>
<tr>
<td>TBS-T (pH 7.6)</td>
<td>10mM Tris-Cl, 150mM NaCl, 0.05% (v/v) Tween 20.</td>
</tr>
<tr>
<td>Transfer Buffer</td>
<td>10mM NaHCO&lt;sub&gt;3&lt;/sub&gt;, 3mM Na&lt;sub&gt;2&lt;/sub&gt;CO&lt;sub&gt;3&lt;/sub&gt;, 20% (v/v) methanol (pH 9.9)</td>
</tr>
<tr>
<td>Polyacrylamide gel</td>
<td>8% Acrylamide/Bis solution, 400 mM Tris-HCl (pH 8.8). 10% glycerol, 1 mM EDTA, 0.2% SDS, 0.1% APS</td>
</tr>
</tbody>
</table>
APPENDIX 3

LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>ATF2</td>
<td>Activating transcription factor 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus enhancer-promoter</td>
</tr>
<tr>
<td>COX-1</td>
<td>Cyclooxygenase-1</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>cPLA2</td>
<td>cytosolic phospholipase A2</td>
</tr>
<tr>
<td>CRE</td>
<td>Cyclic AMP response element</td>
</tr>
<tr>
<td>CSF1</td>
<td>Colony Stimulating factor 1</td>
</tr>
<tr>
<td>CSK</td>
<td>Cellular Src kinase</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>Dif</td>
<td>Dorsal-like immunity factor</td>
</tr>
<tr>
<td>DLAK</td>
<td><em>Drosophila</em> LPS-activated kinase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ECSIT</td>
<td>Evolutionarily-conserved signaling intermediate in Toll pathways</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithermal growth factor</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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<tr>
<td>ERKs</td>
<td>Extracellular signal-regulated kinases</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth factor</td>
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<td>FKBP</td>
<td>FK506 binding protein</td>
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<tr>
<td>GPI</td>
<td>Glycosyl phosphatidylinositol</td>
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<td>Heat shock protein</td>
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<td>Immunoglobulin G</td>
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<td>IκBα</td>
<td>Inhibitory kappa B alpha</td>
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<td>IκB kinase</td>
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<td>IRES</td>
<td>Internal ribosomal entry site</td>
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<td>IL-1</td>
<td>Interleukin-1</td>
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<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
</tr>
<tr>
<td>IL-1RAcP</td>
<td>Interleukin-1 receptor accessory protein</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1R-associated kinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>JNKK</td>
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<td>KDO</td>
<td>3-deoxy-D-manno-octulosonic acid</td>
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<tr>
<td>LBP</td>
<td>LPS-Binding protein</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LRR</td>
<td>Leucine rich-repeat region</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
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<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
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<td>MAP kinase kinase</td>
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<td>MAPKKK</td>
<td>MAP kinase kinase kinase</td>
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<td>MEK-1</td>
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<td>MEK-2</td>
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<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
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<td>NIK</td>
<td>NF-κB-inducing kinase</td>
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<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>Platelet derived growth factor</td>
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<td>PGs</td>
<td>Prostaglandins</td>
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<td>Prostaglandin D₂</td>
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<td>PGH₂</td>
<td>Prostaglandin H₂</td>
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<td>PKC</td>
<td>Protein kinase C</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonicfluoride</td>
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<td>PPARs</td>
<td>Peroxisome proliferator-activated receptors</td>
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<td>PPRE</td>
<td>PPAR response element</td>
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<td>PTKs</td>
<td>Protein Tyrosine kinases</td>
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<td>Polyunsaturated fatty acid</td>
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<td>RIP</td>
<td>Receptor interacting protein</td>
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<td>Retinoid X receptor</td>
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<td>SAPKs</td>
<td>Stress-activated protein kinases</td>
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<td>Sodium dodecyl sulfate</td>
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<td>SH</td>
<td>Src homology</td>
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<td>Transcription factor</td>
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<td>TNFα</td>
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<td>Toll-interacting protein</td>
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<td>TNF-receptor associated factors</td>
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<td>TRADD</td>
<td>TNF receptor associated death domain</td>
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<td>Description</td>
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<tr>
<td>tTA</td>
<td>Tetracycline-regulated transactivater</td>
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<td>Thromboxane A&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>UTR</td>
<td>3′-untranslated region</td>
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THE AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY
VITA

The author was born in the beautiful rural area of Young Chun in the Republic of Korea on Jan. 25th, 1968. He was graduated from Pusan National University with a bachelor's degree of science in the Department of Molecular Biology in February 1991 and did military army service until September 1992.

In March 1993, he enrolled in graduate school to pursue a master of science degree in the Department of Molecular Biology at Pusan National University. His research focus was the differential expression of PCNA by natural compounds and he graduated with the degree of Master of Science in molecular biology in February 1995.

He worked as a teaching assistant in the Department of Molecular Biology at Pusan National University for one year beginning in November 1994. In August 1996, he was accepted by the graduate school of Louisiana State University and pursued his doctoral studies in the Department of Biochemistry. He worked as a graduate research assistant for Dr. Daniel Hwang.

The author is currently a candidate for the degree of Doctor of Philosophy in biochemistry, which will be conferred in August 2001.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Sang Hoon Rhee

Major Field: Biochemistry

Title of Dissertation: Lipopolysaccharide Receptor, Toll-like Receptor 4, Mediated Signaling Pathways Leading to Cyclooxygenase-2 Expression in Murine Macrophages

Approved:

[Signature]

Major Professor and Chairman

[Signature]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

Date of Examination:

25 April 2001