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行政院國家科學委員會補助專題研究計畫 ■ 期中進度報告

（計畫名稱）

革蘭氏陽性菌細胞壁成分peptidoglycan刺激巨噬細胞前發炎物質釋放之訊息傳遞探討(2/3)

計畫類別：□ 個別型計畫　　■ 整合型計畫
計畫編號：NSC **93-2314-B-038-014**
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成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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執行單位:臺北醫學大學醫學研究所

中　華　民　國　　94 年 05 月 30 日

中英文摘要及關鍵詞(Keywords)

中文摘要及關鍵詞

關鍵字：peptidoglycan (PGN), 環氧化酶-2, Rac 1, phosphatidylinositiol 3-kinase (PI3K), Akt, NF-κB, 巨噬細胞

先前我們發現金黃色葡萄球菌細胞壁成份 peptidoglycan (PGN)可經由 Ras/Raf/ERK 訊息傳遞途徑，經由 IKKα/β及 NF-κB 的活化來誘導 RAW 264.7 巨噬細胞環氧化酶-2(COX-2)的表現。在本計劃中，我們將探討 Rac 1, phosphatidylinositiol 3-kinase (PI3K)及 Akt 在 PGN 誘導 RAW 264.7 巨噬細胞 NF-κB 活化及 COX-2 表現所扮演的角色。PGN 誘導 COX-2 表現可被 Rac1 dominant negative mutant (RacN17)，PI3K 抑制劑(wortamanin 及 LY294002)及 Akt 抑制劑(1L-6-Hydroxymethyl-chiro-inositol2-[(R)-2-O-methyl-3-O-octadecyl carbonate])所抑制。RAW 264.7 巨噬細胞給予 PGN 可以誘導 Rac1 及 Akt 的活化。PGN 誘導 Akt 的活化可被 RacN17，LY294002 及 Akt inhibitor 所抑制。PGN 增加 IKKα/β的磷酸化會被 RacN17，LY294002 及 Akt inhibitor 所抑制。PGN 增加 NF-κB 的活性同樣也會被 RacN17，wortmannin，LY294002，Akt inhibitor 及 AktDN 所抑制。巨噬細胞給予 PGN 可依時間依賴誘導 p85α及 Rac1 與 too-like receptor 2 (TLR2)結合在一起。經由以上的結果顯示，在 RAW 264.7 巨噬細胞中，PGN 可由 Rac1/PI3K/Akt 路徑，會使 IKKα/β之磷酸及 NF-κB 活化，最後誘導 COX-2 的表現。

英文摘要及(Keywords)

關鍵字：peptidoglycan (PGN), cyclooxygenase-2, Rac 1, phosphatidylinositiol 3-kinase (PI3K), Akt, NF-κB, macrophages

Previously, we found that peptidoglycan (PGN), a cell wall component of the gram-positive bacterium *Staphylococcus aureus*, may activate the Ras/Raf-1/extracellular signal-regulated kinase (ERK) pathway, which in turn initiates IκB kinases α/β (IKKα/β) and nuclear factor-κB (NF-κB) activation, and ultimately induces cyclooxygenase-2 (COX-2) expression in RAW 264.7 macrophages. In this study, we further investigated the role of Rac 1, phosphatidylinositiol 3-kinase (PI3K), and Akt in PGN-induced NF-κB activation and COX-2 expression in RAW 264.7 macrophages. PGN-induced COX-2 expression was attenuated by a Rac1 dominant negative mutant (Rac1N17), PI3K inhibitors (wortamanin and LY 294002), and the Akt inhibitor (1L-6-Hydroxymethyl-chiro-inositol2-[(R)-2-O-methyl-3-O-octadecyl carbonate]. Stimulation of RAW 264.7 macrophages with PGN caused the activation of Rac1 and Akt. The PGN-induced Akt activation was inhibited by Rac1N17, LY 294002, and the Akt inhibitor. The PGN-induced increases in IKKα/β phosphorylation was inhibited by Rac1N17, LY 294002, and the Akt inhibitor. The PGN-induced increases in κB-luciferase activity was also inhibited by Rac1N17, wortmannin, LY 294002, the Akt inhibitor, and an Akt dominant negative mutant (AktDN). Treatment of macrophages with PGN induced the recruitment of p85α and Rac1 to toll-like receptor 2 (TLR2) in a time-dependent manner. These results indicate that PGN may activate the

Rac1/PI3K/Akt pathway, which in turn initiates IKKα/β phosphorylation, and NF-κB activation, and ultimately induces COX-2 expression in RAW 264.7 macrophages.

報告內容[前言及文獻探討、研究目的、研究方法、結果與討論(含結論與建議)]

前言及文獻探討

Prostaglandins (PGs), lipid mediators, play important roles in many biological processes, including cell division, blood pressure regulation, immune responses, ovulation, bone development, wound healing, and water balance. Altered prostanoid production is associated with a variety of illnesses, including acute and chronic inflammation, cardiovascular disease, colon cancer, and allergic diseases (Smith *et al.*, 1991; DeWitt, 1991). Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to prostaglandin H_2 (PGH_2), which is then further metabolized to various PGs, prostacyclin, and thromboxane A_2 (Vane *et al.*, 1998). Two COX isoforms, COX-1 and COX-2, have been identified in humans (Mitchell *et al.*, 1995). COX-1 is generally thought to produce prostaglandins which serve to maintain cellular homeostasis and is known to be expressed constitutively in many cell types, including endothelial cells, platelets and gastric mucosa (Vane 1994), whereas COX-2 is inducibly expressed in most mammalian cells. COX-2 expression occurs rapidly by cytokines, growth factors, or bacterial endotoxin stimulation (Vane *et al.*, 1998; Lin *et al.*, 2001). COX-2 plays a major role in inflammatory processes, and its expression has been linked with several diseases associated with inflammation and colon cancer.

Peptidoglycan (PGN) is the major component of the cell wall of gram-positive bacteria. PGN is composed of alternating β-linked N-acetylmuramyl and N-acetylglucosaminyl glycan that are interlinked by peptide bridges resulting in a large complex macromolecular structure (Bone 1994; Ulevitch and Tobias, 1995). Like lipopolysaccharide (LPS) as a cell wall component of gram-negative bacteria, PGN induces most of the clinical manifestations of bacterial infections, including inflammation, fever, and septic shock etc (Schleifer and Kandler, 1972). Of importance, PGN can also induce production of proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis factor-α (TNF-α) (Wang *et al.*, 2001; Xu 2001; Wang *et al.*, 2000). PGN binds CD14 and Toll-like receptor 2 (TLR2) to trigger several crucial intracellular signaling responses including activation of transcription factor NF-κB and induction of proinflammatory cytokines (Wang *et al.*, 2001; Dziarski *et al.*, 1998). Previous work suggested that PGN-induced NF-κB activation is mediated through TLR2-dependent multiple signaling molecules including myeloid differentiation protein (MyD88), IL-1 receptor-associated kinase (IRAK), tumor necrosis factor receptor-associated factor 6 (TRAF6), NF-κB-inducing kinase (NIK), and the IκB kinase (IKK) signaling pathway (Wang *et al.*, 2001; Xu 2001). NF-κB is composed of Rel family homo- and heterodimers such as p50 and p65. This heterodimer is complexed to the inhibitory subunit IκBα that, upon stimulation, is phosphorylated and subsequently degraded. This process releases active NF-κB, which is then translocated from the cytosol to the nucleus, to bind specific DNA enhancer sequences, and to induce gene transcription (Baldwin 1996; Barnes and Karin, 1997).

The Rho family GTPase Rac1 participates in regulation of various cellular functions such as cytoskeletal reorganization, cellular growth and apoptosis (Van Aelst and D'Souza-Schorey,

1997). Rac1 is involved in different aspects of host defense against bacteria, including leukocyte chemotaxis (Van Aelst and D'Souza-Schorey, 1997) and production of oxygen radicals (Knaus *et al.*, 1991; Roberts *et al.*, 1999). It has been shown previously that Rac1 mediates a cytokine-stimulated, redox-dependent pathway necessary for NF-κB activation (Sulciner *et al.*, 1996) Toll-like receptor 2-mediated NF-κB activation also dependent on Rac1. More recently, Zampetaki *et al.* (2005) reported that biomechanical stress-induced NF-κB activation is mediated by Ras/Rac1.

Akt, a serine/threonine kinase, is a direct downstream effector of PI3K (Franke *et al.*, 1997). Akt can be modulated by multiple intracellular signaling pathways and acts as a transducer for many pathways initiated by growth factor receptor-activated PI3K. Akt can stimulate signaling pathways which upregulate the activity of the NF-κB in the Jurkat T-cell (Kane *et al.*, 1999). In addition, activation of phosphatidylinositol 3-kinase (PI3K) is involved in BK-stimulated NF-κB activation in human pulmonary epitheilial cells (A549) (Pan *et al.*, 1999). Where Rac1, PI3K, and Akt might participate in either pathway culminating in IκB kinase (IKK) activation or p65-mediated transactivation has not been investigated following PGN stimulation.

Recent studies from our laboratory showed that PGN induces TLR2, p85α, and Ras complex formation, and subsequently activates the Ras/Raf-1/extracellular signal-regulated kinase (ERK) pathway, which in turn initiates IKKα/β and NF-κB activation, and ultimately induces COX-2 expression in RAW 264.7 macrophages (Chen *et al.*, 2004). However, little information is available about the role of Rac1, phosphatidylinositiol 3-kinase (PI3K), and Akt to regulate NF-κB activation and COX-2 expression following PGN stimulation. This study was intended to identify the signaling pathway of PGN-induced PI3K/Akt activation and its roles in PGN-mediated NF-κB activation and COX-2 expression in RAW 264.7 macrophages. Our hypothesis is that PGN might activate the Rac/PI3K/Akt pathway through the recruitment of Rac and p85α to TLR2 to mediate IKKα/β activation and p65 phosphorylation, which in turn induces NF-κB transactivation, and finally to cause COX-2 expression in RAW 264.7 macrophages.

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研究方法

Cell Culture:The mouse macrophage cell line, RAW 264.7 maintained the cells in DMEM/Ham's F-12 nutrient mixture containing 10% FCS, 100 U/ml of penicillin G, and 100 μg/ml streptomycin. *Transfection and κB-Luciferase Assays*:κB-luciferase activity assay. *Immunoblot Analysis*: COX-2, α-tubulin, phospho-Akt (Ser473), Akt, phospho-IKKα (Ser180)/IKKβ (Ser181), and IKKα/β proteins assays. *Immunoprecipitation and Protein Kinase Assays*:Akt and IKKα/β kinase assays. *Rac Activity Assay*: Rac1 activity. *Co-immunoprecipitation of TLR2 complex formation*:Rac, TLR2, or p85α complex formation assays. *Statistical Analysis*:Results are presented as the mean ± S.E. from at least three independent experiments. One-way analysis of variance (ANOVA) followed by, when appropriate, Bonferroni's multiple range test was used to determine the statistical significance of the difference between means. A p value of less than 0.05 was considered statistically significant.

結果

Involvement of Rac1 in PGN-Induced COX-2 Expression

To explore whether Rac1 might mediate PGN-induced COX-2 expression, a Rac1 dominant negative mutant (RacN17) was used. As shown in Figure 1A, pretreatment of RAW 264.7 macrophages with RacN17 markedly inhibited PGN-induced COX-2 expression. However, RacN17 had no effect on the basal level of COX-2 expression (Figure 1A). Next, we directly measured the Rac1 activity in response to PGN. Figure 1B shows that treatment of RAW 264.7 cells with 30 μg/ml PGN induced an increase in Rac1 activity in a time-dependent manner. The response began at 1 min, peaked at 5 min, and declined at 10 min treatment (Figure 1B). Taken together, these results imply that Rac1 activation is involved in PGN-induced COX-2 expression.

Involvement of PI3K and Akt in PGN-Induced COX-2 Expression

To determine whether PI3K and its downstream main target, Akt, are involved in the signal transduction pathway leading to COX-2 expression caused by PGN, cells were treated with PI3K inhibitors (wortmannin (WT) and LY 294002 (LY)) and an Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol2-[(R)-2-O-methyl-3-O-octadecylcarbonate], Akt inh.). Pretreatment of cells for 30 min with wortmannin (30 nM) or LY 294002 (10 μM) significantly attenuated the PGN-induced COX-2 expression (Figure 2A). The PGN-induced COX-2

expression was also inhibited by an Akt inhibitor (3-100 nM) in a concentration-dependent manner. (Figure 2B). Since serine phosphorylation of residue 473 in Akt caused enzymatic activation, the antibody specific against phosphorylated Akt (Ser473) was used to examine Akt phosphorylation, an index of kinase activation. When cells were treated with 30 μg/ml PGN for various time intervals, Akt phosphorylation increased at 5 min, peaked at 30 min, and sustained to 120 min. (Figure 3A, upper panel). The protein level of Akt was not affected by PGN treatment (Figure 3A, bottom panel). We further investigated the relationships among Rac1, PI3K, and Akt in the PGN-mediated signaling pathway. As shown in Fig. 3B, transfection of RAW 264.7 cells for 24 h with RacDN (0.5 and 1 μg), or pretreatment of cells for 30 min with wortamanin (WT, 30 nM), LY 294002 (LY, 10 μM), or the Akt inhibitor (Akt nh., 100 nM) markedly attenuated the PGN-induced Akt phosphorylation. None of these treatments had any effect on Akt expression (Figure 3B). Based on these results, we suggest that activation of Rac1, PI3K, and Akt occurs upstream of Akt in the PGN-induced signaling pathway.

Rac, PI3K, and Akt Mediated PGN-Induced IKKα/β Activation

We further examined whether the IKKα/β activation occurred through the Rac1/PI3K/Akt signaling pathway. As shown in Figure 4A, stimulation of cells with 30 μg/ml PGN induced IKKα/β phosphorylation in a time-dependent manner. The response began at 5 min, peaked at 30 min, and declined at 60 min treatment. The protein level of IKKα/β was not affected by PGN treatment (Figure 4A, bottom panel). Transfection of cells with RacN17 (0.5 and 1 μg) for 24 h, or petreatment of cells with LY 294002 (LY, 10 μM) and the Akt inhibitor (Akt inh., 100 nM) for 30 min markedly attenuated the PGN-induced IKKα/β phosphorylation (Figure 4B). None of these treatments had any effect on IKKα/β expression (Figure 4B).

Rac, PI3K, and Akt Mediated PGN-Induced NF-κB activation

We further examined whether the activation of NF-κB occurs through the Rac1/PI3K/Akt signaling pathway. Using transient transfection with pGL2-ELAM-κB-luciferase as an indictor of NF-κB activity, we found that treatment of cells with 30 μg/ml PGN for 24 h caused an increase in κB-luciferase activity by 410 ± 45% (n=3). The PGN-induced increase in κB-luciferase activity was inhibited by transfection of cells for 24 h with RacN17 (1 μg) or AktDN (1 μg), or petreatment of cells for 30 min with wortamannin (30 nM), LY 294002 (10 μM), and the Akt inhibitor (100nM) by 45% ± 8%, 54% ± 7%, 33% ± 8%, 58% ± 9%, and 46% ± 7%, respectively (Figure 5). Taken together, these data suggest that activation of Rac1/PI3K/Akt pathway is required for PGN-induced NF-κB activation in RAW 264.7 macrophages.

Rac Is Associated with TLR2 by p85α upon PGN Stimulation

The rapid activation of Rac1 by PGN stimulation suggested that Rac1 activation might occur close to TLR2 in the PGN signal pathway. Therefore, we investigated whether PGN can induce the interaction between Rac1 and TLR2. As shown in Figure 6A, treatment of RAW 264.7 macrophages with 30 μg/ml PGN led to the rapid association of Rac and TLR2, as detected by immunoblotting using the antibody to Rac1 after immunoprecipitation of TLR2. The association

of TLR2 and Rac1 occurred at 1 min and peaked at 3~5 min (Figure 6A). Treatment of macrophages with PGN induced the association of p85α and Rac1 within 1 min, and this was sustained until at 5 min (Figure 6B). These results suggested that PGN induces Rac1 activation by interacting with TLR2 and p85α in RAW 264.7 macrophages.

討論(含結論與建議)

In conclusion, the findings of our study for the first time showed that PGN induced COX-2 transcription through the Rac/PI3K/Akt signaling pathway to increase in IKKα/β activity, p65 phosphorylation, NF-κB activation, which in turn induce COX-2 expression in RAW 264.7 macrophages. This is the first study showing PGN-induced Rac activation may occur through the recruitment of p85α and Rac to TLR2 in RAW 264.7 macrophages. The present study, together with our previous report (Chen *et al.*, 2004), delineates, in part, the signaling pathway of PGN-induced COX-2 expression in RAW 264.7 macrophages (Figure 7). With an understanding of these signal transduction pathways, we can design therapeutic strategies to reduce inflammation caused by gram-positive organisms.

附件

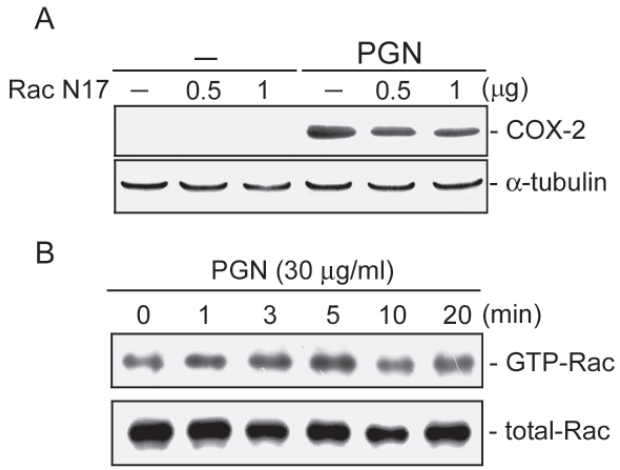

Fig. 1

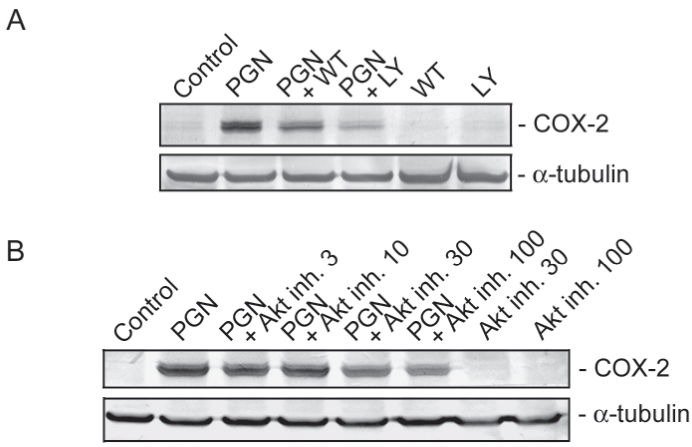

Fig. 2

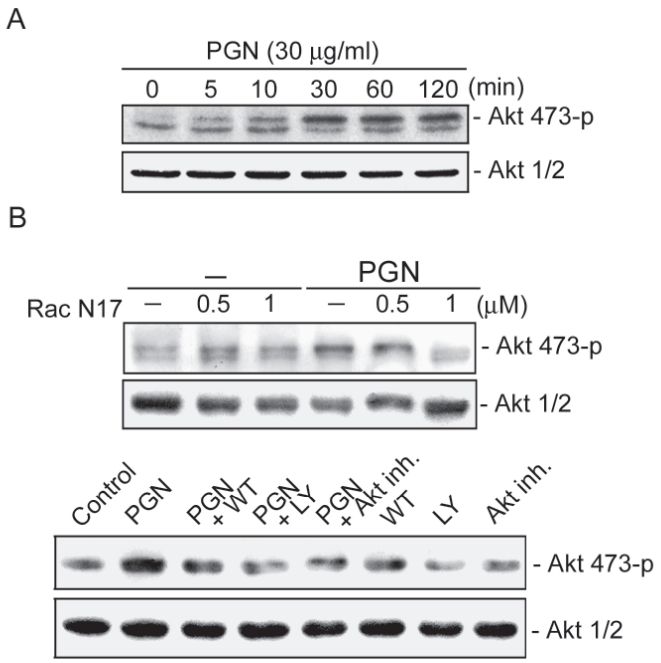

Fig. 3

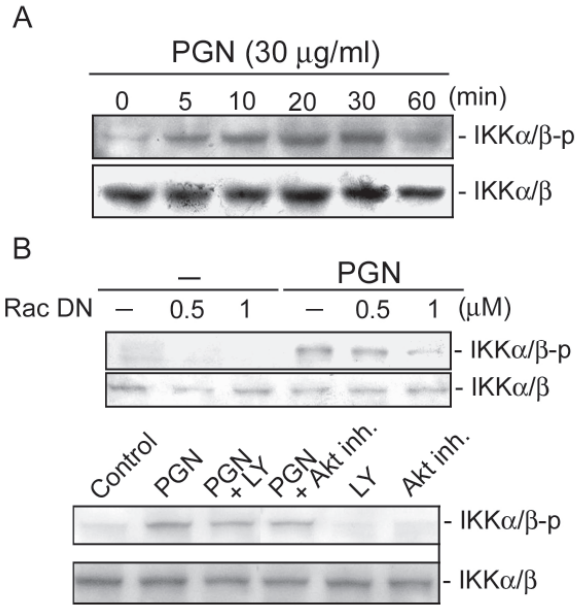

Fig. 4

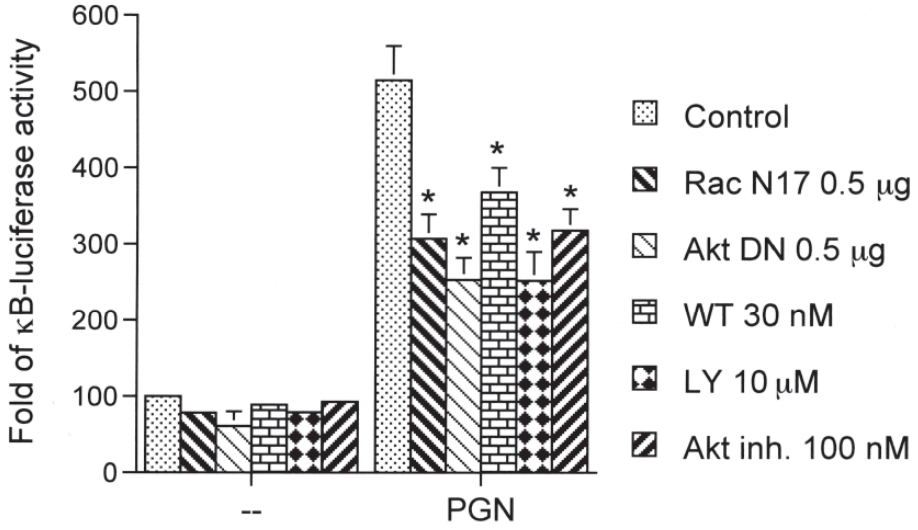

Fig. 5

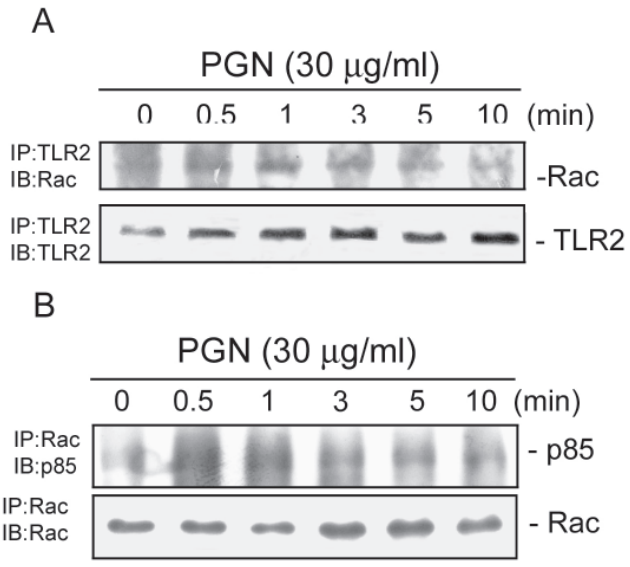
A
PGN (30 μg/ml)
0 0.5 1 3 5 10 (min)
IP:TLR2
IB:Rac
-Rac
IP:TLR2
IB:TLR2
- TLR2
B
PGN (30 μg/ml)
0 0.5 1 3 5 10 (min)
IP:Rac
IB:p85
- p85
IP:Rac
IB:Rac
- Rac

Fig. 6

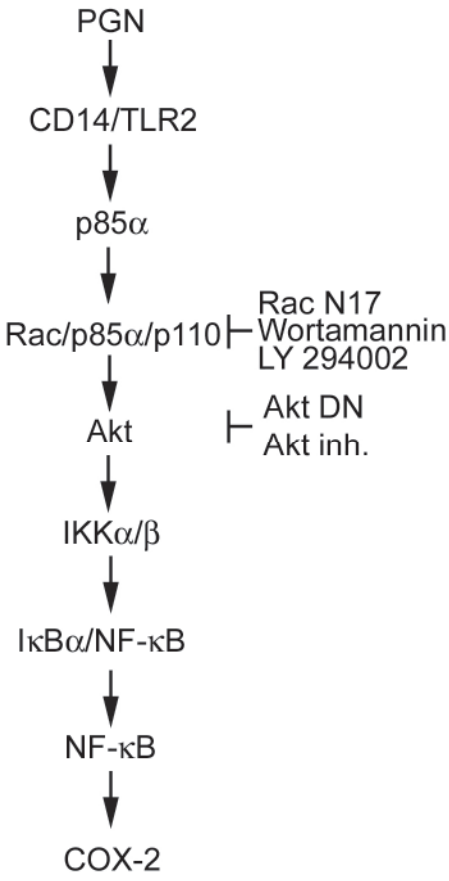
PGN
CD14/TLR2
p85α
Rac/p85α/p110
Rac N17
Wortamannin
LY 294002
Akt
Akt DN
Akt inh.
IKKα/β
IκBα/NF-κB
NF-κB
COX-2

Fig. 7