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Role of Protein Kinase Cδ-Mediated Spleen Tyrosine Kinase (Syk) Phosphorylation on Ser in the Amplification of Oral Mucosal Inflammatory Responses to *Porphyromonas gingivalis*

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Abstract

The signaling events underlying oral mucosal inflammatory responses to *P. gingivalis* and its key endotoxin, lipopolysaccharide (LPS), relay primarily on the LPS engagement of Toll-like receptor-4 (TLR4), and the activation of IκB-kinase complex (IKK) and mitogen-activated protein kinases (MAPKs that exert their control over transcription factors implicated in the regulation of iNOS and COX-2 proinflammatory genes expression). Since spleen tyrosine kinase (Syk) has emerged recently as a major amplifier in the production of proinflammatory mediators, we investigated the process of recruitment and interaction of Syk with TLR4 in salivary gland acinar cells in response to *P. gingivalis* LPS. Our findings revealed that stimulation of the acinar cells with the LPS leads to protein kinase Cδ (PKCδ)-mediated phosphorylation of Syk on Ser which results in its localization with the membrane associated TLR4 complex and the activation through phosphorylation on Tyr. Further, our results support the involvement of Syk in the amplification of transcription factors involved in the assembly and expression of transcription complexes associated with the induction in COX-2 and iNOS genes. Therefore, our data suggest that PKCδ is a primary linchpin affecting the Syk recruitment to the membrane localized TLR4, and hence affects the efficiency of the kinase activation and the magnitude of oral mucosal inflammatory response to *P. gingivalis*.

Keywords

P. gingivalis, Oral Mucosa, PKCδ, Syk Activation, Ser/Tyr Phosphorylation

1. Introduction

Porphyromonas gingivalis, a prominent component of the oral microbiome, is a Gram-negative anaerobe found in periodontal pockets of people with gum disease where it plays a major role in the pathogenesis of periodontitis, a chronic inflammatory disease that is a primary cause of adult tooth loss [1] [2] [3] [4]. The extent of oral mucosal reaction to *P. gingivalis* invasion relays heavily on toll-like receptors (TLRs), a family of transmembrane pattern recognition receptors that recognize structurally common motifs of pathogens and initiate antibacterial responses [5] [6]. Among the virulence factors of *P. gingivalis* implicated in TLRs' activation and triggering vigorous inflammatory responses is the bacterium cell-wall lipopolysaccharide (LPS) [7] [8].

Indeed, studies indicate that *P. gingivalis* LPS, like LPS of other Gram-negative bacteria [9], is a potent activator of TLR4 leading to its dimerization at the several critical Tyr residues that are essential for the initiation of downstream signaling events [6]. The key element of this signaling is the activation of two sets of kinases, mitogen-activated protein kinase (MAPK) cascade and IκB-kinase complex (IKK) [5] [6] [9]. The activated MAPKs, including extracellular signal-regulated kinase (ERK), c-Jun terminal kinase (JNK), and p38 [10] [11], along with IKK, in turn, exert their control over transcription factors implicated in the induction of the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) genes that lead to up-regulation in the production of inflammatory mediators, PGE2 and NO [11] [12] [13].

While under normal physiological conditions, the transcription factors function at low or undetectable levels, their expression increases dramatically following transcriptional activation by inflammatory stimulus [14] [15] [16]. Indeed, evidence indicates that in response to stimulus, c-Jun, c-Fos, ATF2, and NF-κB factors undergo rapid phosphorylation by the specific upstream kinases that affect significantly their dimerization with different partners of transcription factor family and hence the transcriptional activity of NF-κB and AP1 complex [14] [15] [16]. Moreover, LPS-induced TLR4 activation and the ensuing phosphorylation of its tyrosine domain by Src-family kinases [6] [17], provide a convenient docking site for the recruitment of spleen tyrosine kinase (Syk), the activation of which is known to increase the expression of inflammatory genes [17] [18] [19].

Syk, initially found in hematopoietic cells and recognized for its role in adaptive immune responses, has emerged recently as a major effector in TLR4-mediated inflammatory reaction to LPS [18]. This 72 kDa non-receptor tyrosine kinase comprises of two tandem N-terminal Src homology 2 (SH2) domains, a linker region, and a C-terminal kinase domain [17] [18]. The first step in Syk activation is its binding through SH2 domains to the intracellular Toll-IL-1 receptor (TIR) domain of TLR4 or signaling proteins containing phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic regions [19]. This results in conformational changes in Syk and its activation through

phosphorylation on several tyrosine residues, which leads to the activation of the PLC, PI3K, MAPK and ERK signaling cascades, and amplification in the induction of inflammatory response [19] [20].

Although phosphorylation of Syk on multiple Tyr sites is the most apparent posttranslational modification affecting the kinase signaling potential, there are reports demonstrating that upon stimulation Syk also undergoes rapid phosphorylation on several residues of Ser [19] [21] [22]. One of the most prominent phosphorylation sites involves Ser^{297} within the linker region of interdomain B of Syk, which has been suggested to impact the efficiency of Syk activation through phosphorylation on Tyr [21] [22]. Therefore, in this study, we assessed the influence of Syk phosphorylation on Ser on its cell membrane recruitment, interaction with TLR4, and the activation through phosphorylation on Tyr in sublingual salivary gland acinar cells in response to stimulation by *P. gingivalis* LPS.

2. Materials and Methods

2.1. Salivary Gland Cell Incubation

The acinar cells of rat sublingual salivary gland were suspended in five volumes of ice-cold Dulbecco's modified (Gibco) Eagle's minimal essential medium (DMEM), supplemented with fungizone (50 μg/ml), penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% fetal calf serum, and gently dispersed by trituration with a syringe and settled by centrifugation [23] [24]. The cells were then resuspended in the medium to a concentration of 2×10^7 cell/ml, and transferred in 1 ml aliquots to DMEM in culture dishes and incubated under 95% O_2 and 5% CO_2 atmosphere at 37˚C in the presence of 0 - 100 ng/ml *P. gingivalis* LPS [24]. *P. gingivalis* used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 33277 [25]. In the experiments evaluating the effect of PKC inhibitors, classical PKC isoforms, Gö6976 and the inhibitor of classical and novel PKC isoforms, GF109203X (Sigma), as well as the inhibitors of JNK, SP600125, ERK, PD98059, and p38, SB202190 (Calbiochem), the cells were first preincubated for 30 min with the indicated dose of the agent or vehicle before the addition of the LPS. The viability of cell preparations before and during the experimentation, assessed by Trypan blue dye exclusion assay [2], was greater than 98%.

2.2. Cell Membrane Preparation

To assess membrane translocation of Syk as a function of kinase activation through phosphorylation on Ser and Tyr in response to *P. gingivalis* LPS, the sublingual salivary gland acinar cells from the control and experimental treatments were subjected to cell membrane preparation. The cells were homogenized for 10 s at 600 rpm in 3 volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 25 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol, 10 mM aprotinin, 10 mM leupeptin, 10 mM chymostatin, and 1 mM

PMSF [26]. The lysate was centrifuged at 5000 × *g* for 15 min, the supernatant was diluted with two volumes of cold homogenization buffer and centrifuged at 10,000 × *g* for 20 min. The resulting supernatant was then subjected to centrifugation at 100,000 × *g* for 1 h at 4˚C, and the obtained membrane pellet was suspended in the extraction buffer, containing 20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF. After 30 min of incubation at 4˚C, the suspension was centrifuged at 15,000 × *g* for 15 min, and the supernatant containing solubilized membrane fraction was collected and stored at −70˚C until use. Protein content of the prepared membrane fraction was analyzed using BCA protein assay kit (Pierce).

2.3. Immunoprecipitation and Immunoblotting

The acinar cells from the control and experimental treatments were collected by centrifugation and resuspended for 30 min in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 1 mM PMSF, and 1 mM NaF), containing 1 μg/ml leupeptin and 1 μg/ml pepstatin [26]. Following brief sonication, the lysates were centrifuged at 10,000 g for 10 min, and the supernatants were subjected to protein determination using BCA protein assay kit (Pierce). The lysates of whole cells as well as those of membrane preparations were then used either for immunoblots analysis, or proteins of interest were incubated with the respective primary antibodies for 2 h at 4˚C, followed by overnight incubation with protein G-Sepharose beads. The immune complexes were precipitated by centrifugation, washed with lysis buffer, boiled in SDS sample buffer for 5 min, and subjected to SDS-PAGE using 40 μg protein/lane. The separated proteins were transferred onto nitrocellulose membranes, blocked for 1 h with 5% skim milk in Tris-buffered Tween (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20), and probed with specific antibodies directed against PKC*δ*, Syk, phosphorylated Syk ($Tyr^{525/526}$) and phosphotyrosine (4G10) (EMD Millipore), phosphoserine (pSer) PKC substrate and phospho-c-Fos (Cell Signaling), and TLR4 (Sigma). Antibodies directed against ERK, phospho-ERK, p38, phospho-p38, JNK phospho-JNK, c-Jun, phospho-c-Jun, c-Fos, ATF2 and phospho-ATF2 were from Calbiochem.

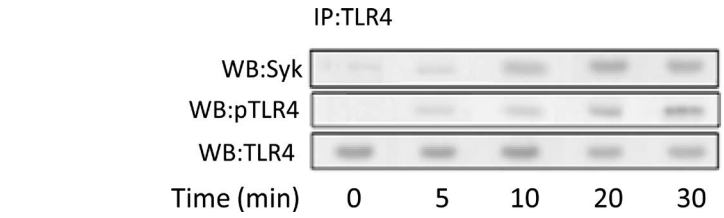
2.4. Data Analysis

All experiments were carried out using duplicate sampling, and the results are expressed as means ± SD. Analysis of variance (ANOVA) and nonparametric Kruskal-Wallis tests were used to determine significance. Any difference detected was evaluated by means of post hoc Bonferroni test, and the significance level was set at $p < 0.05$.

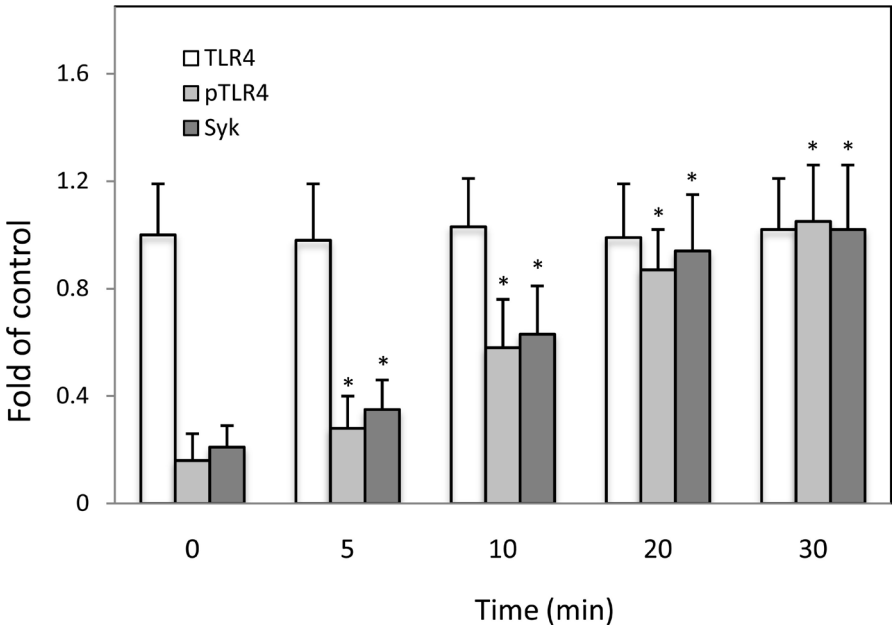
3. Results

Taking into account emerging evidence as to the role of Syk in the modulation of

bacterial endotoxin inflammatory signals associated with TLR4 activation and the secretion of various inflammatory mediators, including PGE2 and NO [18] [19], we investigated the nature of factors involved in the recruitment and interaction of Syk with TLR4 in sublingual salivary gland acinar cells in response to LPS of periodontopathic bacterium, *P. gingivalis.* By following the acinar cell TLR4 activation through phosphorylation on Tyr, and the extent of its interaction with Syk, we found that the effect of the LPS was manifested by a time-dependent induction in Syk association with TLR4 which paralleled that of the level of TLR4 phosphorylation (**Figure 1**). Moreover, by following the selectivity of interaction between the two proteins by co-immunoprecipitation we revealed that the association between TLR4 and Syk induced by the LPS required phosphorylation of both proteins on Tyr, as the two proteins were found in complex in both TLR4 (**Figure 1**) and Syk (**Figure 2**) immunoprecipitates. Therefore,



(a)



(b)

Figure 1. Induction by *P. gingivalis* LPS in sublingual salivary gland acinar cell phosphorylation of TRL4 and its association with Syk. The acinar cells were treated with 0 or 100 ng/ml of the LPS and incubated for up to 30 min. Cell lysates were immunoprecipitated (IP) with anti-TLR4 antibody and analyzed by Western blotting for total TLR4, phosphorylated TLR4 (pTLR4) with anti-pTyr (4G10), and anti-Syk, antibodies (a), and the relative densities of proteins are expressed as fold of total TLR4 control (b). Values represent the mean ± SD of four separate experiments. *P < 0.05 compared with that of control.

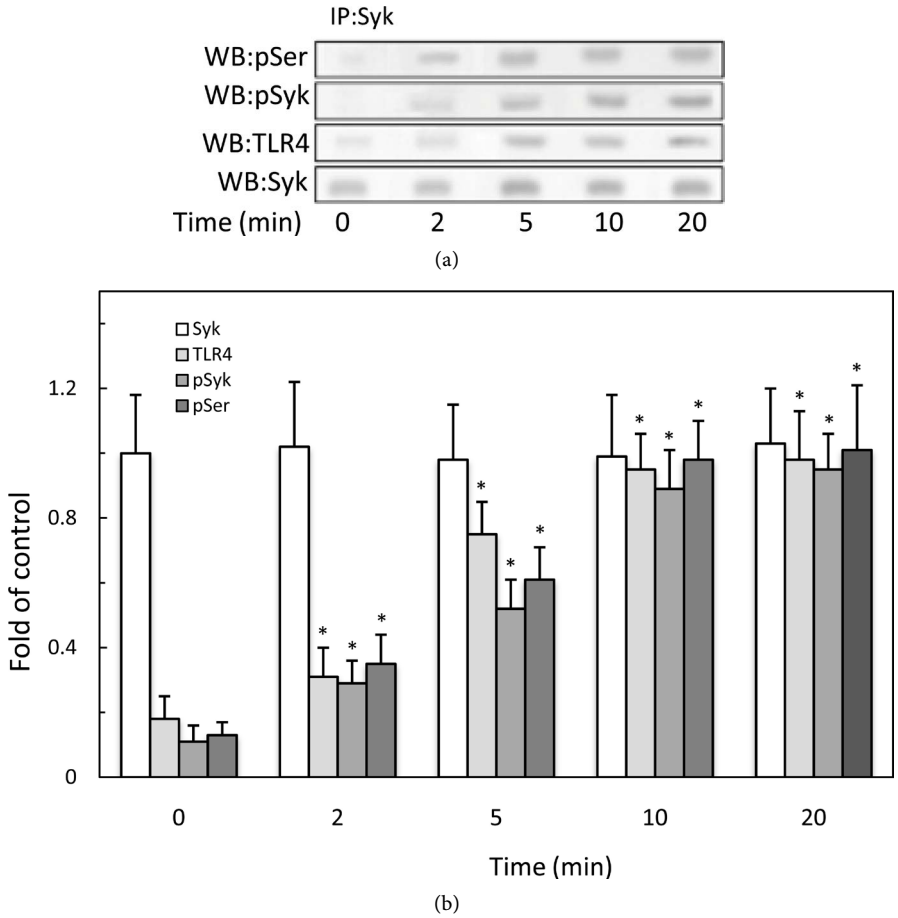


Figure 2. Impact of *P. gingivalis* LPS-induced acinar cell phosphorylation of Syk on Tyr and Ser on its association with TLR4. The acinar cells were treated with o or 100 ng/ml of the LPS and incubated for up to 20 min. Cell lysates were immunoprecipitated (IP) with anti-Syk antibody and immunoblotted (WB) with anti-Syk and anti-TLR4 antibody (a). The Syk immunoblots were reblotted with anti-pSyk ($Tyr^{522/526}$) (pSyk) and anti-phosphoserine (pSer) PKC substrate antibody, and the relative densities of proteins are expressed as fold of control (b). The data represent the mean ± SD of four separate experiments. *P < 0.05 compared with that of control.

we concluded that *P. gingivalis* LPS-induced Syk activation through phosphorylation on Tyr requires the involvement of the LPS-elicited TLR4 engagement.

Since in addition to Syk phosphorylation on Tyr sites, the kinase is also phosphorylated rapidly on Ser residues [19] [22], we next assessed the kinetics of Syk phosphorylation on Ser and Tyr in the acinar cells subjected to *P. gingivalis* LPS stimulation. The results revealed that the LPS-induced Syk phosphorylation on Ser precedes in time-dependent manner ahead of the kinase phosphorylation on Tyr (**Figure 2**). Moreover, we found that the LPS-induced phosphorylation of Syk on Ser was susceptible to suppression by the inhibitor of classical and novel PKC isoforms, GF109203X, but not the inhibitor of classical PKC isoforms, Gö6976 (**Figure 3**). These data, thus suggest the involvement of the novel PKC isozyme, identified earlier as PKCδ [27], in the processes of salivary gland acinar cell Syk phosphorylation on Ser.

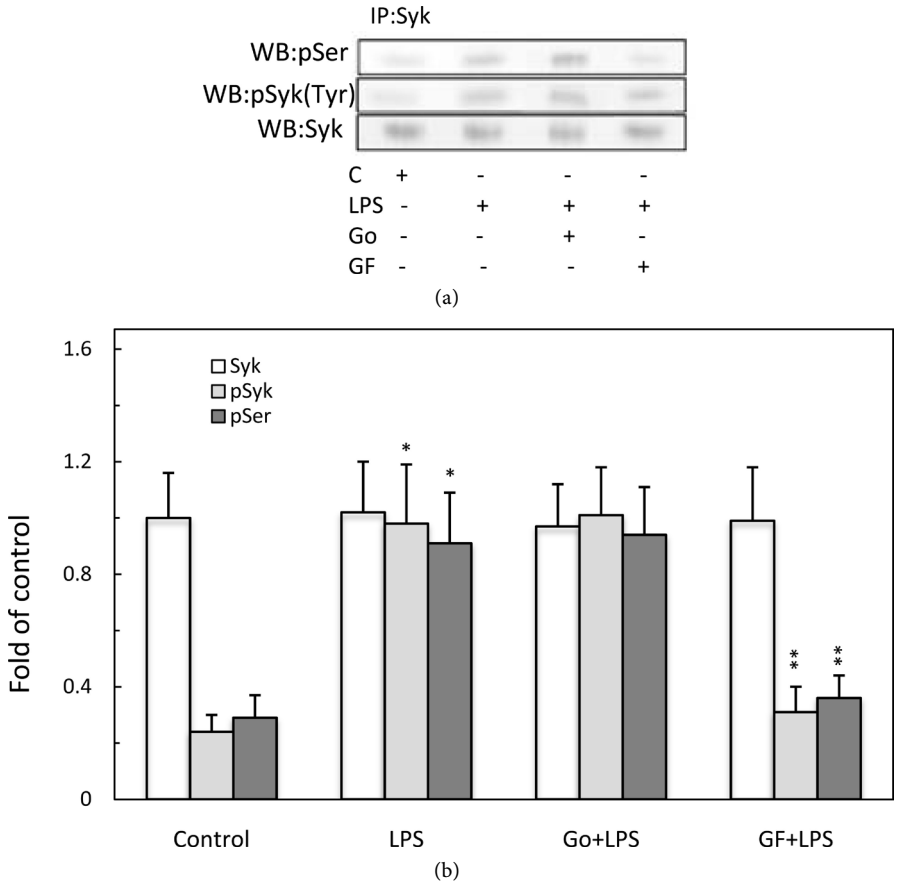


Figure 3. Role of protein kinase C (PKC) in *P. gingivalis* LPS-induced salivary gland acinar cell Syk phosphorylation. The acinar cells were preincubated with 5 μM of wide spectrum PKC inhibitor, GF109203X (GF) or 10 μM of classical PKC inhibitor, Gö6976 (Go) and incubated for 10 min in the presence of 100 ng/ml LPS. Cell lysates were immunoprecipitated (IP) with anti-Syk antibody, immunoblotted (WB) with anti-Syk, and reblotted with anti-phosphoserine (pSer) PKC substrate and anti-pSyk (Tyr) antibody (a). The relative densities of proteins are expressed as fold of control (b). The data represent the mean ± SD of four separate experiments. *P < 0.05 compared with that of control. **P < 0.5 compared with that of LPS.

Indeed, in further assessment of the role of PKCδ-mediated phosphorylation on Ser in *P. gingivalis* LPS-induced Syk activation by co-immunoprecipitation, we found that while PKCδ failed to co-precipitate with Syk in the absence of stimulation, the two kinases were found in complex following the acinar cell exposure to the LPS. Moreover, the association between the two kinases was dependent upon the activity of PKCδ, as pretreatment with PKC inhibitor, GF109203X, blocked the LPS-induced colocalization of PKCδ with Syk (**Figure 4**). Furthermore, analysis of Syk association with TLR4 demonstrated that the LPS-induced localization of Syk with the membrane anchored TLR4 was also susceptible to PKC□ inhibition by GF103203X.

As Syk localizes mainly in cytoplasm and its association with the membrane-bound TLR4 complex upon activation requires its membrane recruitment [19], we also examined the effect of *P. gingivalis* LPS on the requirement

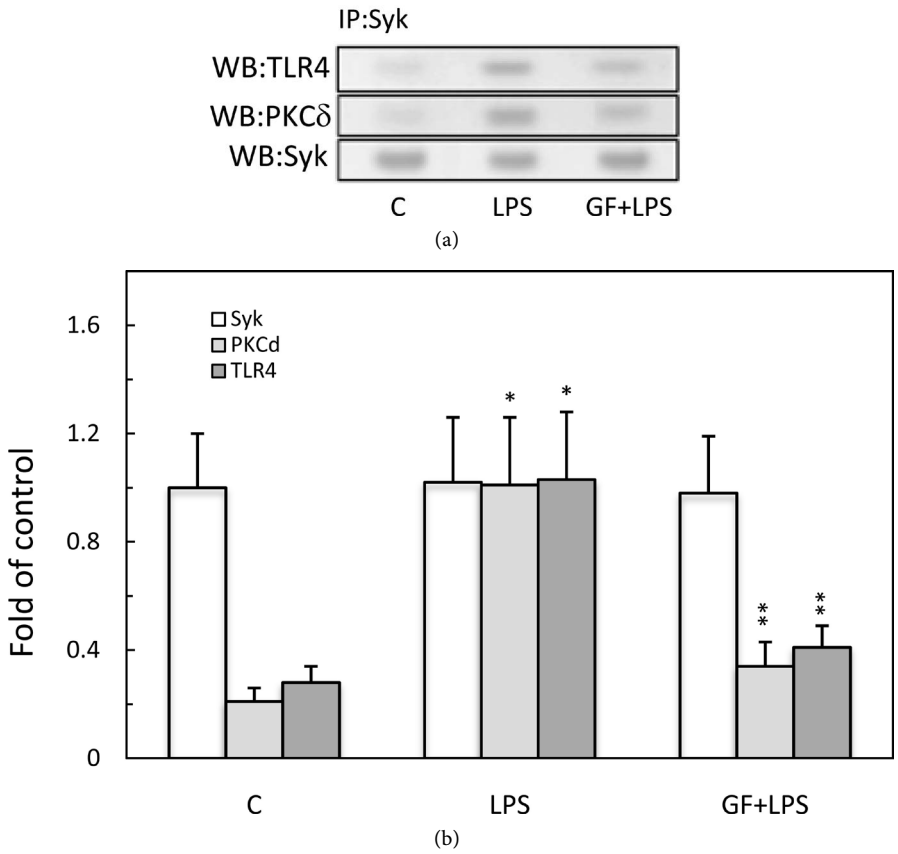


Figure 4. Effect of wide spectrum PKC inhibitor, GF109203X (GF), on *P. gingivalis* LPS-induced association of Syk with PKCδ and TLR4 in salivary gland acinar cells. The acinar cells were preincubated with 5 μM of GF and incubated for 10 min in the presence of 100 ng/ml LPS. Cell lysates were immunoprecipitated (IP) with anti-Syk antibody, immunoblotted (WB) with anti-Syk, and reblotted with anti-PKCδ and anti-TLR4 antibody (a). The relative densities of proteins are expressed as fold of control (b). The data represent the mean ± SD of four separate experiments. *P < 0.05 compared with that of control. **P < 0.5 compared with that of LPS.

and selectivity of Syk membrane translocation. The lysates of whole salivary gland cells as well as the membrane fraction were precipitated with anti-Syk antibody, and subjected to Western blot analysis using anti-pSyk (Tyr) and anti-pSer-PKC substrate antibody. The analyses revealed that the LPS caused a marked elevation in the membrane localization of Syk phosphorylated on both Ser and Tyr (**Figure 5**). Moreover, the LPS-induced membrane translocation of Syk as well as its phosphorylation on Ser and Tyr was blocked by the pretreatment of the acinar cells with PKC inhibitor, GF109203X. These findings thus suggest that the inhibition of the LPS-induced and PKCδ-mediated phosphorylation of Syk on Ser interferes with its membrane recruitment and activation through phosphorylation on Tyr. Accordingly, *P. gingivalis* LPS-induced Syk activation proceeds through the stage of PKCδ-mediated Syk phosphorylation on Ser, required for its recruitment to the membrane anchored TLR4, followed by the kinase activation through phosphorylation on Tyr.

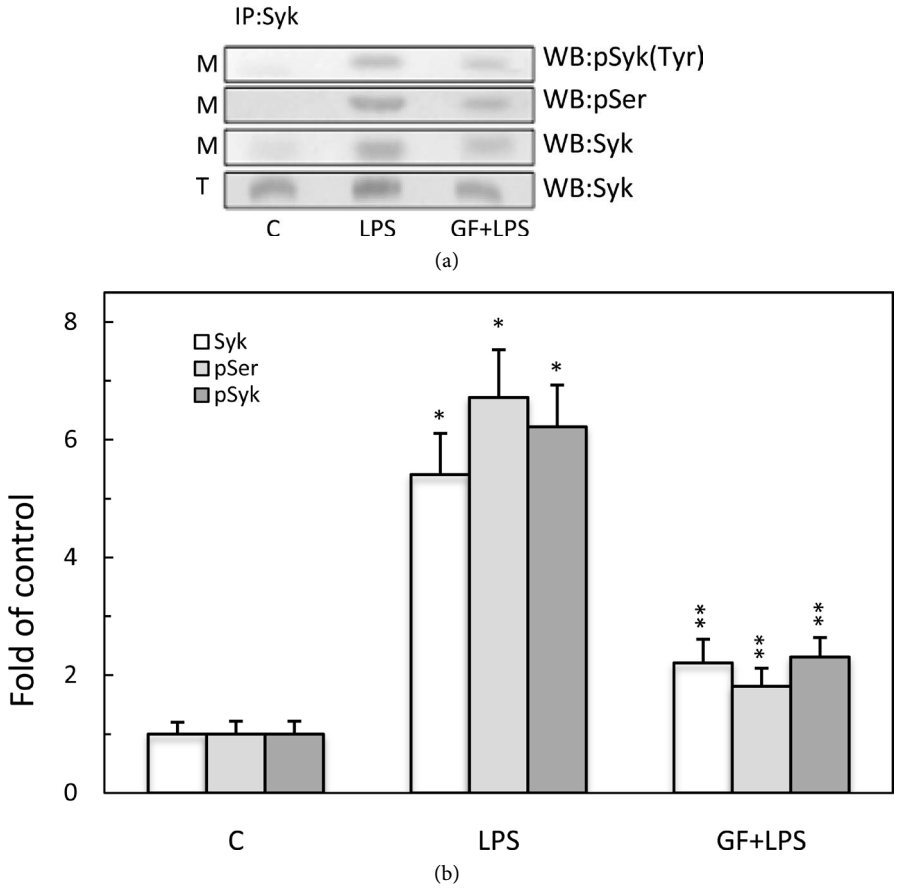


Figure 5. Impact of PKC inhibition on *P. gingivalis* LPS-induced salivary gland acinar cell Syk phosphorylation and membrane translocation. The acinar cells were preincubated with 5 μM of wide spectrum PKC inhibitor, GF109203X (GF) and incubated for 10 min in the presence of 100 ng/ml LPS. The lysates of whole cells (T) and the corresponding membrane (M) fractions were immunoprecipitated (IP) with anti-Syk antibody, immunoblotted (WB) with anti-Syk, and reblotted with anti-phosphoserine (pSer) PKC substrate and anti-pSyk (Tyr) antibody (a). The relative densities of proteins are expressed as fold of control (b). The data represent the mean ± SD of four separate experiments. *P < 0.05 compared with that of control. **P < 0.5 compared with that of LPS.

Moreover, as inflammatory response to LPS are associated with the induction in MAPKs signaling cascades that remain under regulatory influence of Syk [18], we next evaluated the influence of *P. gingivalis* LPS on the acinar cell MAPK/JNK, p38, and ERK activation. As illustrated in **Figure 6(a)**, the LPS effect was manifested by a marked increase in JNK, p38 and ERK activation through phosphorylation that showed susceptibility to the respective pharmacological inhibitors, SP600125, SB202190 and PD98059. Further, since among the downstream targets of Syk are the transcription factors implicated in the induction of genes involved in the production of inflammatory mediators, we then examined the LPS influence on transcription factors, c-Jun, c-Fos, and ATF2, activation through phosphorylation in the presence of MAPK inhibitors (**Figure 6(b)**, **Figure 6(c)**). The results of immunoblot analysis revealed that the LPS-induced acinar cell phosphorylation of c-Fos was subject to suppression by ERK inhibitor,

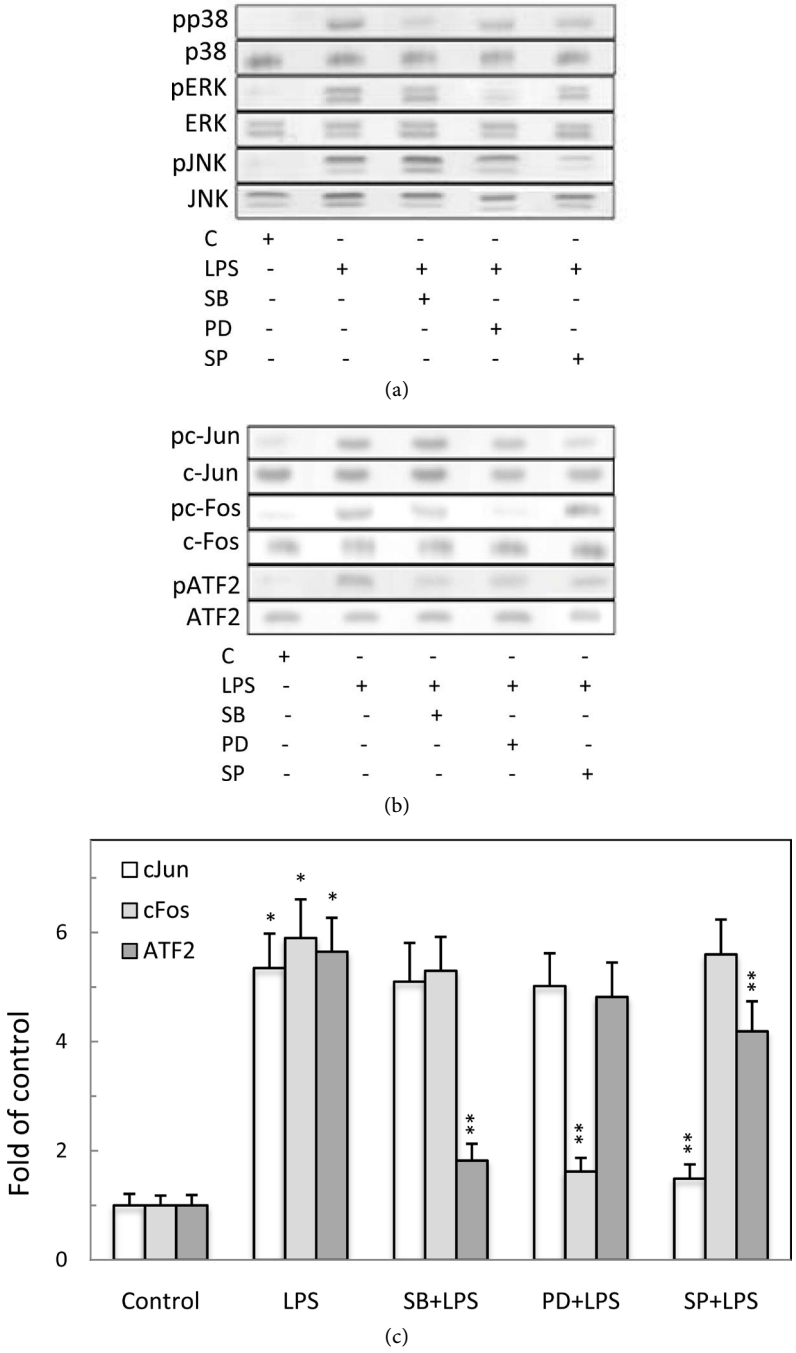


Figure 6. *P. gingivalis* LPS-induced salivary gland acinar cell MAPK p38, ERK and JNK activation, and their influence on transcription factors c-Jun, c-Fos and ATF2 phosphorylation. The acinar cells, preincubated for 30 min with 0 or 10 μM of JNK inhibitor, SP600125 (SB), 30 μM of ERK inhibitor, PD98059 (PD), or 20 μM p38 inhibitor, SB202190 (SB), were incubated for 1 h with the LPS at 100 ng/ml. (a), Cell lysates were analyzed by Western blotting with respective specific antibodies for total and phosphorylated MAPK p38, ERK and JNK. (b), Cell lysates were analyzed with specific antibodies for total and phosphorylated c-Jun, c-Fos and ATF2, and the relative densities of phosphorylated proteins are expressed as fold of control (c). The data represent the mean ± SD of four experiments. *P < 0.05 compared with that of control. **P < 0.5 compared with that of LPS.

PD98059, the ATF2 phosphorylation was inhibited by SB202190, an inhibitor of MAPK/p38, while the phosphorylation of c-Jun was susceptible to inhibition by JNK inhibitor, SP600125, as well as p38 inhibitor, SB202190. These results strongly attest to the role of MAPKs in the mediation of Syk regulatory effect on the transcription factors activation and therefore influence their assembly into an AP1 transcription complex.

4. Discussion

Oral mucosal reaction to periodontopathic bacterium, *P. gingivalis* a major component of oral microbiome is characterized by a massive rise in proinflammatory cytokine production, up-regulation in MAPK/JNK, p38 and ERK activation, and the induction in iNOS and COX-2 enzyme systems that lead to rapid induction in generation of inflammatory mediators, NO and PGE2 [11] [12] [13]. The signaling events underlying the inflammatory responses to *P. gingivalis* and its key endotoxin, LPS, relay primarily on the LPS engagement of TLR4, the activation of which initiates the signal transduction cascades converging on two sets of kinases, MAPK and IKK [5] [6] [9] [26]. While, the events associated with JNK and p38 kinase signal propagation result in the activation of transcription factors involved in the regulation of COX-2 gene expression, the ERK signals converge on IKK pathway and are associated with the induction of iNOS gene [5] [9] [12] [13]. Furthermore, dissection of the events following TLR4 ligation by LPS indicates that TLR4 activation and the ensuing phosphorylation of its intracellular tyrosine domain not only leads to recruitment to the cytoplasmic domain of the receptor of several different adaptor molecules involved in the propagation of signaling cascade, but also provides a docking site for Syk, a 72 kDa non-receptor tyrosine kinase implicated in the amplification in the induction of proinflammatory genes expression [17] [18] [19].

Indeed, studies show that upon stimulation with LPS Syk binds to TLR4 through its N-terminal SH2 domain and is activated through phosphorylation of its tyrosine residues [19] [20]. The activated Syk, in turn, binds to a number downstream signaling effectors and amplifies the inflammatory signal propagation [18]. Hence, to gain further leads into the pathways utilized by *P. gingivalis* endotoxin, LPS, in triggering up-regulation in oral mucosal inflammatory responses, we investigated the nature of factors involved in the recruitment and interaction of Syk with TLR4 in salivary gland acinar cells in response to stimulation by *P. gingivalis* LPS. By following the acinar cell TLR4 activation and its interaction with Syk, we found that stimulation with the LPS led to a rapid, time-dependent induction in the phosphorylation of TLR4 and Syk on Tyr, and that the association between Syk and TLR4 induced by the LPS required phosphorylation of both proteins on Tyr. These findings thus support the results obtained with neutrophils and macrophages demonstrating the increase in phosphotyrosine-dependent binding of Syk to the cytoplasmic region of TLR4 in response to stimulation by LPS [12].

Moreover, taking into consideration reports indicating that in addition to phosphorylation on Tyr, Syk is also phosphorylated on Ser residues, and since phosphorylation on Ser^{297} within the linker region of interdomain B of Syk has been suggested to affect the efficiency of Syk activation through phosphorylation on Tyr [21] [22], we further assessed the kinetics of Syk phosphorylation in the acinar cells exposed to the LPS stimulation. Our analyses revealed that the LPS-induced Syk phosphorylation on Ser occurs rapidly and proceeds in time-dependent manner ahead of the kinase phosphorylation on Tyr. Furthermore, we demonstrated that Syk phosphorylation on Ser was susceptible to suppression by the inhibitor of classical and novel PKC isoforms, GF109203X, thus supporting the involvement of the novel PKC isozyme, identified earlier as PKCδ in the processes of salivary gland acinar cell Syk phosphorylation on Ser [27].

Upon further analysis, moreover, we demonstrated that while PKCδ did not co-immunoprecipitate with Syk in the absence of stimulation, the two kinases were found in association following the acinar cell incubation with the LPS. The interaction between PKCδ and Syk, furthermore, was dependent on the activity of PKCδ, as PKC inhibitor, GF109203X, blocked the LPS-induced association of the two kinases. Taken together, these findings are in concert with several previous studies where PKCδ has been linked to functional association with non-receptor tyrosine kinases, including that of cSrc and Syk [18] [21] [27] [28] [29] [30].

Consequently, considering that Syk interacts with a wide variety of effector proteins located in plasma membrane or cytoplasm [19] [22], we next examined the effect of *P. gingivalis* LPS on the requirement and selectivity of Syk membrane localization. As Syk localizes mainly in cytoplasm and its association with TLR4 complex requires recruitment to the membrane [19] [31], we assessed the role of PKCδ-induced phosphorylation in membrane translocation of Syk. The analyses revealed that the LPS caused a marked elevation in membrane localization of Syk phosphorylated on both Ser and Tyr, and that the translocation of Syk as well as its phosphorylation on Ser and Tyr was blocked by the inhibition of PKCδ. Hence, we surmised that *P. gingivalis* LPS-induced activation of Syk proceeds through the stage of PKCδ-mediated phosphorylation of Syk on Ser, required for its recruitment to the membrane anchored TLR4, followed by the kinase activation through phosphorylation on Tyr. Interestingly, our assertion is in line with the literature data as to the involvement of PKCδ in Syk activation associated with Detectin-1 signaling and thrombin-induced NF-κB activity involved in ICAM-1 expression [29] [30] [32].

In further efforts to reveal the role of Syk in the amplification of inflammatory responses of the acinar cells to *P. gingivalis* LPS, we have turned our attention to the involvement of Syk in regulation of the expression of transcription factors affected byTLR4-mediated signaling cascades converging on IKK and MAPK kinase systems. The results of analyses revealed that the LPS-induced ERK activation, in addition to its well-defined involvement in the activation of IKK asso-

ciated with up-regulation in the expression of iNOS gene [9] [12] [33], also exerts its effect on the activation through phosphorylation on transcription factor, c-Fos, involved in the assembly of AP1 transcription complex associated with the induction in COX-2 gene expression. Furthermore, in agreement with the reported data [13] [19], we have found that MAPK/p38 is involved in phosphorylation of ATF2, while Syk-mediated activation of JNK along with p38 are involved in the phosphorylation of transcriptional factor, c-Jun. As phosphorylation of transcription factors alters their stability and the extent of dimerization with other members of AP1 complex, giving rise to complexes with different transcriptional potential [15], it is apparent that the Syk-induced changes in transcription factors activation plays a major role in the transcriptional outcome of proinflammatory genes expression (**Figure 7**). Hence, the pharmacological agents targeting Syk activation offer tempting alternative for the therapeutic intervention in the treatment of chronic periodontitis.

5. Conclusion

The findings presented herein, indicate that *P. gingivalis* LPS-induced upregulation in salivary gland acinar cell Syk activation proceeds through the pivotal

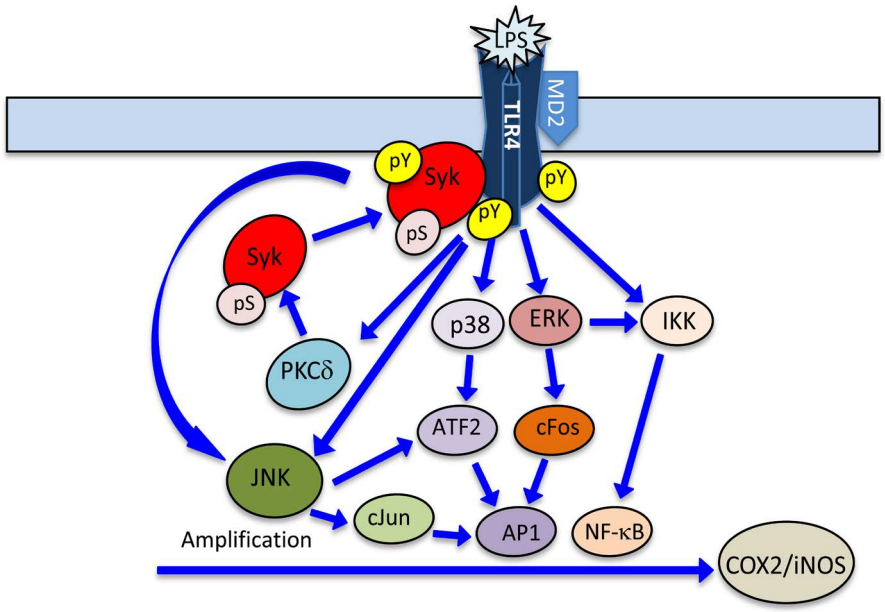


Figure 7. Schematic diagram illustrating the signaling mechanism involved Syk (spleen tyrosine kinase)-induced amplification of salivary gland acinar cell inflammatory responses to *P. gingivalis* LPS. Engagement of TLR4 by the LPS initiates the signal transduction cascades converging on PLC-dependent pathway of PKCδ activation, MAPKs, and IKK. The induction in PKCδ activation leads to Syk phosphorylation on Ser that results in up-regulation of its membrane colocalization with TLR4 and the activation through phosphorylation on Tyr. The activated Syk, in turn, amplifies the JNK, p38 and ERK cascades that lead to up-regulation in cJun, cFos and ATF2 transcription factors involved in the regulation of AP1 complex assembly and the induction of COX-2 gene, while the IKK signals converge on NFκB pathway involved in the induction of iNOS gene. pS phosphoserine, pY phosphotyrosine.

stage of PKCδ-mediated Syk phosphorylation on Ser required for its recruitment to the membrane-anchored TLR4, followed by the kinase activation through phosphorylation on Tyr. The activated Syk, in turn, binds to a number of downstream signaling effectors and amplifies the inflammatory signal propagation by affecting transcription factors activation and their assembly to transcriptional complexes involved in proinflammatory genes expression. Moreover, our data suggest that PKCδ is a primary linchpin affecting Syk recruitment to TLR4, and thus, influencing the efficiency of its activation and the magnitude of inflammatory responses.

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