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Porphyromonas gingivalis-Induced GEF Dock180 Activation by Src/PKCδ-Dependent Phosphorylation Mediates PLCγ2 Amplification in Salivary Gland Acinar Cells: Modulatory Effect of Ghrelin

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Abstract

Phospholipase Cγ2 (PLCγ2) plays a pivotal role in mediation of inflammatory reaction to bacterial lipopolysaccharide (LPS) as well as serves as a key target in modulatory influence of the hormone ghrelin. Here we explore the involvement of Rac1 and its activator, guanine nucleotide exchange factor (GEF), Dock180, in mediation of PLCγ2 activation in salivary gland acinar cells in response to *P. gingivalis* LPS and ghrelin. We show that stimulation of the acinar cells with the LPS leads to up-regulation in Dock and PLCγ2 activation, and is reflected in the membrane translocation of Rac1 and PLCγ2, while the effect of ghrelin is manifested by the suppression in Rac1 translocation. Further, we reveal that stimulation with the LPS leads to Dock180 phosphorylation on Tyr and Ser, while the modulatory influence of ghrelin, manifested by a drop in membrane Rac1-GTP, is associated with a distinct decrease in Dock180 phosphorylation on Ser. Moreover, we demonstrate that phosphorylation on Tyr remains under the control of Src kinase and is accompanied by Dock180 membrane translocation, while protein kinase Cδ(PKCδ) is involved in the LPS-induced phosphorylation of the membrane-recruited Dock180 on Ser. Thus, our findings underscore the role of Src/PKCδ-mediated GEF Dock180 phosphorylation on Tyr/Ser in modulation of salivary gland acinar cell PLCγ2 activation in response to *P. gingivalis* as well as ghrelin.

Keywords

***P. gingivalis*, Salivary Acinar Cell, Ghrelin, Dock180 Phosphorylation, Src, PKCδ, PLCγ2 Activation**

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1. Introduction

Porphyromonas gingivalis, a Gram-negative bacterium found in periodontal pockets of people with gum disease, is recognized as a major culprit in the etiology of periodontitis, a chronic inflammatory condition that affects about 15% of adult population and is a major cause of adult tooth loss [1] [2]. The oral mucosal responses to *P. gingivalis* and its key endotoxin, cell wall lipopolysaccharide (LPS), are characterized by the disturbances in NO signaling pathways, massive rise in epithelial cell apoptosis, and the increase in proinflammatory cytokine production [3]-[6]. Studies into the events underlying the proinflammatory signal regulation indicate that *P. gingivalis* LPS, like LPS of other Gram-negative bacteria, is capable of Toll-like receptor 4 (TLR4) ligation resulting in the receptor dimerization, followed by the TLR4 autophosphorylation at the several critical Tyr residues that are essential for initiation of downstream signaling events [3] [7]-[9]. The key element of this signaling is the receptor-mediated recruitment of phosphoinositide-specific phospholipase C (PLC) which catalyzes formation of the second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), from membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) [10]-[13].

Moreover, PLC activation not only plays a major role in defining the extent of inflammatory response to LPS, but is also considered as a primary target in modulatory influence of the hormone ghrelin on the mucosal responses to bacterial invasion [6] [9] [13]-[15]. This 28-amino acid peptide, initially isolated from the stomach [16] and subsequently identified in oral mucosa, saliva and the acinar cells of salivary glands [17], is commonly recognized as an important modulator of the processes of mucosal repair and the control of local inflammatory responses to bacterial infection. Indeed, engagement by ghrelin of the growth hormone secretagogue receptor type 1a (GHS-R1a), a G protein-coupled receptor (GPCR), leads to activation of heterotrimeric G protein-dependent signal transduction pathways, including PLC/PKC, PI3K, and Src/Akt implicated in signaling to NO-generating system [6] [13] [14] [18] [19].

The PLC generated second messengers have far-reaching regulatory and metabolic roles: DAG is known to stimulate the activity of a variety of enzymes, including PKC, while the IP_3 is recognized for its role in the regulation of the cytoplasmic calcium concentration [10] [11]. Other data point to the existence of cross talk between PLC and PKC, and indicate that while DAG causes stimulation of PKC activation, the PKC in turn may be involved in the reciprocal modulation of PLC as well as PI3K activation through phosphorylation on Ser [13] [20]-[22]. Furthermore, several PLC isozymes, including mucosal tissue PLCγ2, appear to be directly activated by Ras superfamily of small guanosine triphosphatases (GTPases), and many GPCR-initiated signaling pathways also involve Ras activation [11] [23]-[26]. The Ras superfamily of GTPases consist of over 150 small, 20 - 40 kDa, monomeric proteins, and are divided into five major families (Ras, Rho, Rab, Ran, and Arf) on the basis of sequence and functional similarities [24] [27] [28].

The small GTPases specifically implicated in the regulation of PLCγ2 activation are represented by the two members of Rho family, Rac1 and Rac2, and their activation status is controlled through the exchange of GDP for GTP, catalyzed by the guanine nucleotide exchange factors, also known as GEFs [23] [26] [27]. In mammals, the Rho GEFs comprise of 11 members and are referred to as Dock (dedicator of cytokinesis) 180-related family of GEFs [28]-[30]. The Dock180, facilitating GDP/GTP exchange in Rac1, responds to stimuli activating tyrosine kinase receptors (RTKs) by the Src kinase-mediated increase in Rac1-GTP formation, and up-regulation in Rac1 activation has been observed in association with LPS-induced gastric mucosal and pulmonary inflammation [26] [31] [32].

Taking into consideration a pivotal role of PLCγ2 in propagation of proinflammatory reaction to bacterial endotoxins being a primary target in modulatory influence of ghrelin on the extent of oral mucosal inflammatory reaction [5] [6], in this study, we examined the involvement of *P. gingivalis* LPS in the amplification of PLCγ2 activation associated with the salivary acinar cell Rac1 activation by GEF Dock180.

2. Materials and Methods

2.1. Salivary Gland Acinar Cell Incubation

The acinar cells of sublingual salivary gland, collected from freshly dissected rat (Sprague-Dawley) salivary glands, 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 (Sigma), and gently dispersed by trituration with a syringe [5]. After centrifugation, the cells were 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 at 37˚C for up to 2 h in the presence of 0 - 100 ng/ml *P. gingivalis* LPS [5]. *P. gingivalis* used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 33277 [33]. In the experiments evaluating the effect of ghrelin (rat), Rac1 inhibitor, NSC 23766, and wide spectrum PKC inhibitor, GF109203X (Sigma), PLC inhibitor, U73122, and Src family protein tyrosine kinases (SFK-PTKs) selective inhibitor, PP2 (Calbiochem), the cells were first preincubated for 30 min with the indicated dose of the agent or vehicle before the addition of the LPS.

2.2. Rac1-GTP Activation Assay

The measurements of Rac activation in the acinar cells were carried out with Rac1 Activation Assay Kit (EMD Millipore). The cells from the control and experimental treatments were lysed in magnesium lysis buffer (MLB), containing protease inhibitor cocktail (10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium orthovanadate, 1 mM PAF, and 1 mM NaF), at 4˚C for 30 min and centrifuged at 12,000 ×g for 10 min. The supernatants were precleared with GST beads and incubated with PAK-1 PBD-agarose for 1 h at 4˚C. The beads were washed three times in MLB, resuspended in Laemmle reducing sample buffer, resolved on SDS-PAGE, and immunoblotted for GTP-bound Rac1 using anti-Rac1 antibody.

2.3. PKC Activity Assay

Protein kinase C activity measurement in the acinar cells of sublingual salivary gland was conducted with ELISA PKC Activity Assay Kit (Stressgen). The cells were rinsed with 0.05 M phosphate buffer/saline, pH 7.4, settled by centrifugation, and suspended for 30 min at 4˚C in the lysis buffer consisting of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 4 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM PMSF, 1 mM sodium orthovanadate, and 10 µg/ml of leupeptin and aprotinin. Following sonication (3 × 10 sec pulses), the samples were centrifuged at 12,000 ×g for 10 min and the resulting supernatant was subjected to protein determination using BCA protein assay kit (Pierce). The samples from various experimental treatments were adjusted to 5 µg of crude protein/30 µl, and added to the wells for PKC activity measurement using peroxidase conjugated secondary antibody and TMB spectrophotometric quantification [22].

2.4. PLC Activity Assay

PLC activity in sublingual salivary gland acinar cells was measured by the production of inositol phosphates [34] [35]. Aliquots of the cell suspension (1 ml) were transferred to DMEM in cell culture dishes containing 2 µCi of myo-[2-^{3}H]inositol and incubated for 16 h under 95% O_2/5% CO_2 atmosphere at 37˚C. The cells were then centrifuged at 300 ×*g* for 5 min, washed three times with DMEM containing 5% albumin to remove the free radiolabel, and resuspended in a fresh DMEM free of albumin containing 10 mM LiCl. After 10 min equilibration period, the cells were transferred to a medium containing 0 or 100 ng/ml of H. pylori LPS and incubated for 1 h. In the experiments on the effect of ghrelin, and PLC, PKC and SFK-PTK Inhibitors**,** the cells prior to the addition of the LPS were first preincubated for 30 min with the indicated dose of the agent or the vehicle. At the end of the specified incubation period, the cells were treated for 30 min at 4˚C with 20 mM formic acid, and following neutralization with 20 mM ammonium hydroxide the lysates were centrifuged for 5 min at 12,000 × *g* to remove particulate material. The supernatants were applied to Dowex (AG1-X8 100-200 mesh) anion exchange (formate) columns, and following washing with 50 mM sodium formate/ 5 mM sodium tetraborate, the [^{3}H] inositol phosphates were eluted with 1 M ammonium formate/0.1 M formic acid [34]. The content of [^{3}H]inositol phosphates was measured by scintillation spectrometry and normalized against the protein content in the lysates determined by BCA protein assay kit (Pierce).

2.5. Cell Membrane Preparation

To assess membrane translocation of DOck180, Rac1 and PLCγ2 in response to the LPS and ghrelin, the sublingual salivary gland acinar cells from the control and experimental treatments were subjected to cell membrane preparation. The aliquots of the acinar cell suspension were settled by centrifugation at 1500 × *g* for 5 min, rinsed with phosphate-buffered saline, and 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 [36]. The cell lysates were then centri-

fuged at 5000 x *g* for 15 min, the supernatant was diluted with two volumes of cold homogenization buffer and centrifuged at 10,000 x *g* for 20 min. The resulting supernatant was then subjected to centrifugation at100,000 × *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).

2.6. Immunoblotting Analysis

The acinar cells of sublingual salivary gland 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 [5] [6]. 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 Rac1, Dock180, phosphotyrosine (4G10), and PKCδ (EMD Millipore), phosphoserine PKC substrate, phospho-Src (Tyr^{416}), and PLCγ2 (Cell Signaling), and cSrc (Sigma).

2.7. 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

In view of a central role of PLCγ2 in propagation of proinflammatory response to bacterial endotoxins as well as being a key target in modulatory influence of peptide hormone, ghrelin, we investigated the factors affecting the PLCγ2 activation in rat sublingual salivary gland acinar cells exposed to LPS of periodontopathic bacterium, *P. gingivalis.* As shown in **Figure 1**, incubation of the acinar cells with *P. gingivalis* LPS led to a marked increase in PLC activity, while preincubation with ghrelin elicited a significant reduction in the LPS effect. Moreover, as the literature evidence suggests that PLC activation shows dependence on Rac GTPases [25] [26], we have also assessed the influence of the LPS and ghrelin on the acinar cell activity of Rac1 GEF, Dock180. The results revealed that the effect of the LPS was manifested by a significant increase in Dock180 activity, whereas preincubation with ghrelin exerted the modulatory effect. Furthermore, the activation of PLC and Dock180 by the LPS was susceptible to suppression by PLC inhibitor, U73122, as well as the inhibitor of Rac1, NSC23766, thus pointing to existence of the cross talk between PLC and Rac1.

Since PLC as well Rac1 activation involves the membrane translocation, we next evaluated the influence of *P. gingivalis* LPS and ghrelin on the acinar cell membrane recruitment of PLC and Rac1. Western blot analysis of the whole cell lysates as well as the cell membrane fraction, using anti-PLCγ2 and anti-Rac1 antibody, revealed that incubation with the LPS resulted in translocation of both PLC and Rac1 to the membrane fraction, while the effect of ghrelin was manifested by the elevation in membrane-associated PLCγ2 and the suppression in the membrane translocation of Rac1 (**Figure 2**). Moreover, blocking the Rac1 activation with NSC23766 had no effect on the LPS and ghrelin-elicited membrane translocation of PLCγ2, while the inhibitor of PLC, U73122, appeared to exert less apparent effect on Rac1 translocation.

To gain further insights into the involvement of Rac1 in the regulation of PLC activation, we have followed the leads as to the role of Src/PKC in Rac1 GEF activation. Accordingly, the sublingual salivary gland cells prior to incubation with *P. gingivalis* LPS and ghrelin were pretreated with wide spectrum PKC inhibitor,

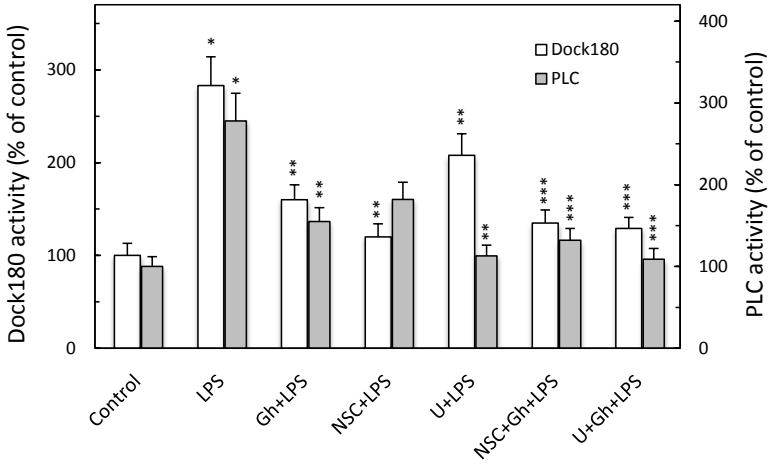

Figure 1. Impact of Rac and PLC inhibitors on the changes induced in sublingual salivary gland acinar cells by *P. gingivalis* LPS and ghrelin (Gh) in the expression of PLC and Dock180 (GTP-Rac1) activities. The cells, preincubated with 50 μM of Rac1 inhibitor, NSC23766, or 15 μM PLC inhibitor, U73122, were treated with 0.5 μg/ml Gh, and incubated for 1 h in the presence of100 ng/ml LPS. Values represent the means ± SD of five experiments. $^{*}p < 0.05$ compared with that of control, $^{**}p < 0.05$ compared with that of LPS, $^{***}p < 0.05$ compared with that of Gh + LPS.

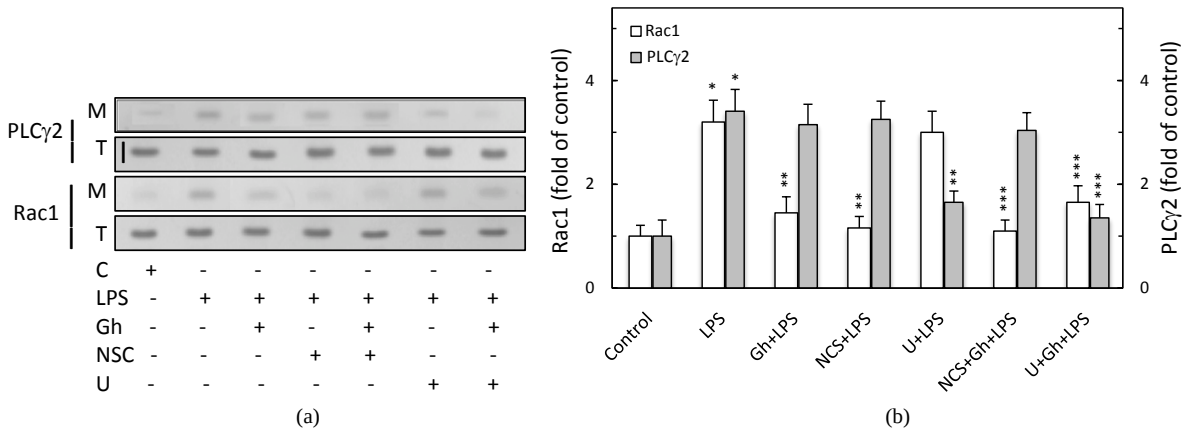

Figure 2. Impact of Rac and PLC inhibitors on the changes induced by *P. gingivalis* LPS and ghrelin (Gh) in membrane recruitment of Rac1 and PLC. The sublingual salivary gland acinar cells were treated with the LPS at 100 ng/ml or Gh at 0.5 μg/ml + LPS, in the presence of Rac1 inhibitor, NSC23766 (NSC) at 50 μM, or PLC inhibitor, U73122 (U), at 50 μM, and incubated for 2 h. The lysates of whole cells (T) and the corresponding membrane (M) fractions were analyzed for PLCγ2 and Rac1 with specific antibodies (A). The relative densities of proteins are expressed as fold of control (B), and the total (T) PLCγ2 and Rac1 were used as loading control. The data represent the means ± SD of four separate experiments. $^{*}p < 0.05$ compared with that of control, $^{**}p < 0.05$ compared with that of LPS, $^{***}p < 0.05$ compared with that of Gh + LPS.

GF109203X, or SFK-PTKs inhibitor, PP2, and assayed for PKC and GEF Dock180 activities. As illustrated in **Figure 3**, the effect of the LPS was associated with the elevation in the PKC and Dock180 activation, whereas the preincubation with ghrelin elicited further stimulation in PKC activity and the reduction in Dock180 activation. The activation of PKC and Rac1 by the LPS, moreover, was susceptible to suppression by PKC inhibitor, GF109203X as well as the inhibitor of SFK-PTKs, PP2, thus attesting to the involvement of PKC and Src in the processes of GEF Dock180 activation. Hence, to ascertain the nature of this involvement, the lysates of the acinar cells as well as the cell membrane fraction were immunoprecipitated with anti-Dock180 antibody, and subjected to Western blot analysis using anti-Dock180, anti-pSer-PKC substrate, anti-pTyr, and anti-Rac1 antibody (**Figure 4**). The results revealed that the effect of the LPS was manifested by the membrane elevation in Rac1 associated with Dock180 phosphorylated on Tyr as well as Ser, while the effect of ghrelin, characterized by a drop in membrane-associated Dock180 phosphorylation on Ser, was also reflected in a decrease in membrane translocation of Rac1. Further, we observed that PKC inhibitor, GF109203X, exerted the inhibitory effect not

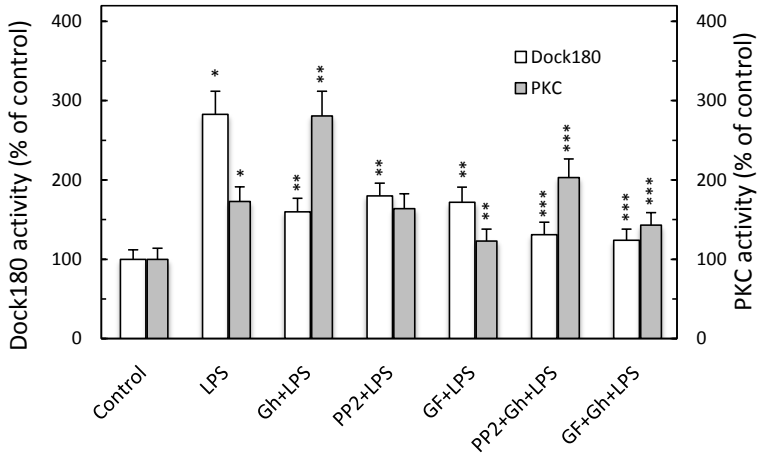

Figure 3. Effect of PKC and SFK-PTK inhibitors on ghrelin (Gh)-induced changes in the expression of PKC and Dock180 (GTP-Rac1) activities in salivary gland acinar cells exposed to *P. gingivalis* LPS. The cells, preincubated with 30 μM of SFK-PTKs inhibitor, PP2, or 5 μM of wide spectrum PKC inhibitor, GF109203X (GF), were treated with 0.5 μg/ml Gh, and incubated for 1 h in the presence of 100 ng/ml LPS. The data represent the means ±SD of four separate experiments. $^{*}p <$ 0.05 compared with that of control, $^{**}p < 0.5$ compared with that of LPS, $^{***}p < 0.5$ compared with that of Gh + LPS.

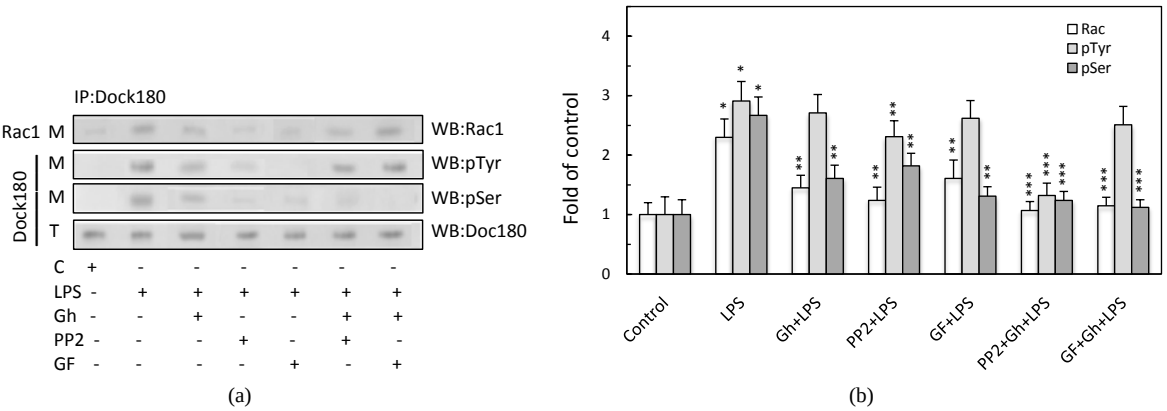

Figure 4. Impact of SFK-PTK and PKC inhibition on the changes induced by *P. gingivalis* LPS and ghrelin (Gh) in membrane translocation and phosphorylation of Dock180, and its association with Rac1. The acinar cells, preincubated with 30 μM SFK-PTKs inhibitor, PP2, or 5 μM of wide spectrum PKC inhibitor, GF109203X (GF), were treated with 0.5 μg/ml Gh, and incubated for 1 h in the presence of 100 ng/ml LPS. The lysates of whole cells (T) as well as the corresponding membrane (M) fractions were immunoprecipitated (IP) with anti-Dock180 antibody, and immunoblotted (WB) with anti-Dock180, anti-pTyr), anti-pSer-PKC substrate, and anti-Rac1 antibody (A). The relative densities of phosphorylated proteins are expressed as fold of control (B), and the total (T) Dock180 was used as loading control. The data represent the means ± SD of four separate experiments. $^{*}p < 0.05$ compared with that of control, $^{**}p < 0.05$ compared with that of LPS, $^{***}p < 0.05$ compared with that of Gh + LPS.

only on the LPS-induced membrane localization of Rac1 and Dock phosphorylation on Ser, but also caused further decrease in the effect of ghrelin on Rac1 translocation. We also noticed that that the effect of SFK-PTKs inhibitor, PP2, was associated with the suppression in the LPS and ghrelin-elicited phosphorylation of membrane-associated Dock180 on Tyr as well as Ser and a decrease in Rac1 membrane localization. These results suggest that Src kinase-mediated phosphorylation on Tyr may be required for the stimulus-induced membrane localization of Dock180, while the PKC isozyme, identified earlier as PKCδ [22], is involved in the phosphorylation of membrane-recruited Dock180 on Ser.

Therefore, to assess the extent of Src and PKCδ influence over the processes of Dock180 activation and their involvement in mediation of salivary gland acinar cell responses to *P. gingivalis* LPS and ghrelin, we examined the requirements and selectivity of the interactions by co-immunoprecipitation. The results, presented in **Figure 5**, demonstrated that PKCδ and Dock180 were present in association in both Dock180 and PKCδ immunopreci-

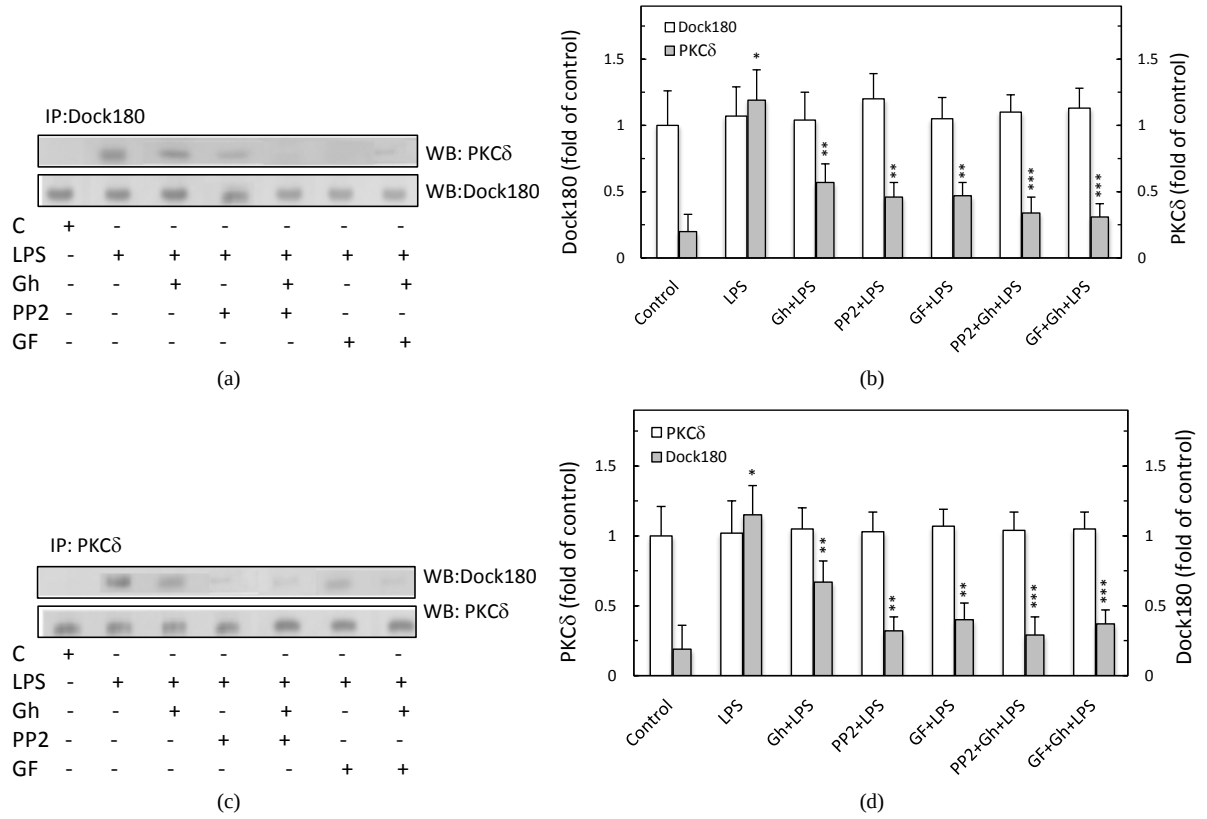

Figure 5. Impact of SFK-PTK and PKC inhibition on the changes induced by *P. gingivalis* LPS and ghrelin (Gh) in the association of Dock180 with PKC*δ* in sublingual salivary gland acinar cells. The cells, preincubated with 30 μM PP2, or 5 μM GF109203X (GF), were treated with 0.5 μg/ml Gh, and incubated for 1 h in the presence of 100 ng/ml LPS. (a) Cell lysates were immunoprecipitated (IP) with anti-Dock180 antibody and immunoblotted (WB) with anti-Dock180 and anti-PKC*δ* antibody, and the relative densities of proteins (b) are expressed as fold of Dock180 control; (c) Cell lysates were immunoprecipitated with anti-PKC*δ* antibody, immunoblotted with anti-PKC*δ* and anti-Dock180 antibodies, and the relative densities of proteins (d) are expressed as fold of PKC*δ* control. The data represent the means ± SD of four separate experiments. $^{*}P < 0.05$ compared with that of control. $^{**}P < 0.05$ compared with that of LPS. $^{***}P < 0.05$ compared with that of Gh + LPS.

pitates following the acinar cell stimulation with the LPS and ghrelin. Further, we found that the interaction between the two proteins was dependent on Dock180 phosphorylation on Tyr as well as the activity of PKC*δ*, since SFK-PTKs inhibitor, PP2, as well as wide spectrum PKC inhibitor, GF109203X, interfered with the co-immunoprecipitation. Moreover, examination of the interaction between Src kinase and Dock180 by co-immunoprecipitation revealed while the two proteins did not co-precipitate in the absence of stimulation, the Dock180 was found in association with Src following the acinar cell stimulation with *P. gingivalis* LPS and ghrelin (**Figure 6**). However, this association was subject to interference by SFK-PTK inhibitor, PP2. Upon further probing the salivary gland acinar cell Src immunoprecipitates with anti-pSrc(Tyr^{416}), we found that the effect of the LPS and ghrelin was manifested by the massive increase in Src phosphorylation on Tyr^{416}, which is the reflection of up-regulation in Src activation. Together, these data underscore the role of Dock180 phosphorylation on Tyr/Ser in modulation of salivary gland acinar cell PLC activation in response to *P. gingivalis* LPS.

4. Discussion

The mammalian phosphoinositide-specific PLC is a family of 13 isozymes divided into six subfamilies (PLC*β*, *γ*, *δ*, *ε*, *η*, and *ζ*) on the basis of their size, amino acid sequences, domain structure, and activation mechanisms [10] [11]. Perhaps the most ubiquitously expressed are the two isoforms of the PLC*γ* subfamily, PLC*γ*1 and PLC*γ*2, that appear to play a key role in regulation of cell growth, differentiation, and modulation of the immune and inflammatory responses [10] [13] [35]. Indeed, studies indicate that both PLC*γ* isoforms are activated by receptor and non-receptor tyrosine kinases, and PLC*γ*2 have been shown to undergo phosphorylation on Tyr and Ser fol-

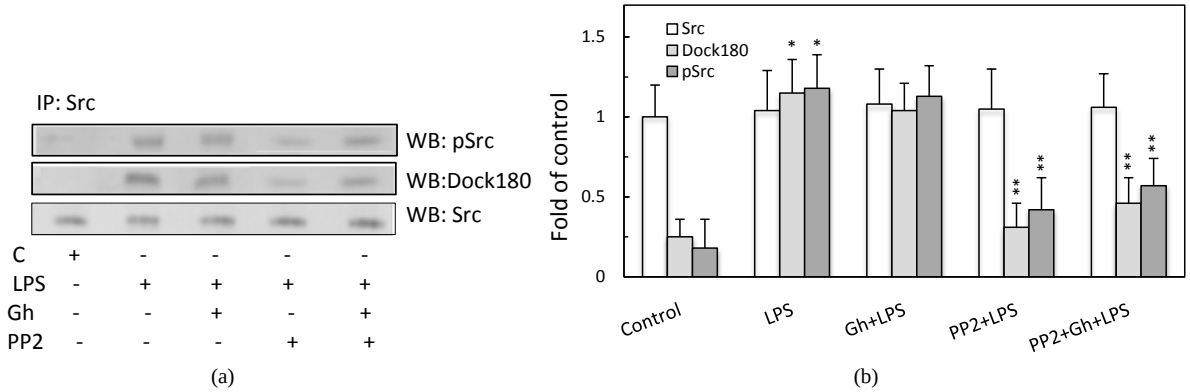

Figure 6. Impact of SFK-PTK inhibition on the changes induced by *P. gingivalis* LPS and ghrelin (Gh) in sublingual salivary gland acinar cell Src kinase phosphorylation and its association with Dock180. The cells, preincubated with 30 μM PP2 were treated with 0.5 μg/ml Gh, and incubated for 1 h in the presence of 100 ng/ml LPS. Cell lysates were immunoprecipitated (IP) with anti-Src antibody and immunoblotted (WB) with anti-Src and anti-Dock180 antibody (A). The Src immunoblots were also reblotted with anti-pSrc (Tyr^{416}) antibody, and the relative densities of proteins (B) are expressed as fold of Src control. $^{*}P < 0.05$ compared with that of control. $^{**}P < 0.05$ compared with that of LPS.

lowing TLR4 ligation by LPS resulting in the amplification in the enzyme activity [12] [13] [21]. Furthermore, recent evidence suggests that PLCγ2 is subject to modulatory influence by the members of Rho family of small GTPases, Rac1 and Rac2, the activation status of whose is controlled by the GEF Dock180 [11] [23] [26] [27] [35] [37]. Hence, considering that PLCγ2 is also the major cellular target of peptide hormone, ghrelin in controlling the extent of oral mucosal inflammatory responses to *P. gingivalis* [3]-[6], in the present study we explored the involvement Rac1 GTPase and its GEF, Dock180, in mediation of the amplification of PLCγ2 activation in response to *P. gingivalis* LPS, and explored the modulatory effect of ghrelin.

Relying on the literature evidence as to the involvement of Rac GTPases in PLC activation [23] [25] [26] [37], we have exposed rat sublingual salivary gland acinar cells to incubation with *P. gingivalis* LPS and ghrelin, in the presence of Rac1 and PLC inhibitors, NSC23766 and U73122, and followed their influence on the acinar cell activity of Rac1 GEF, DOck180 and PLC, as well as the membrane localization of Rac1 and PLCγ2. Results of analyses revealed that the effect of the LPS-induced a significant increase in Dock180 and PLC activity, while preincubation with ghrelin exerted the modulatory effect. Moreover, the LPS-induced activation of Dock180 and PLC was susceptible to suppression by Rac1 inhibitor, NSC23766, as well as the inhibitor of PLC, U73122, thus supporting the existence of the cross talk between PLC and Rac1. Indeed, up-regulation in PLC and Rac1-GTP formation (Dock180 activity) has been also observed in association with LPS-induced pulmonary inflammation in mice as well as gastric mucosal inflammatory response to *H. pylori* LPS [26] [32] [37]. Furthermore, considering that PLC enzymes are mainly cytosolic and translocate to the membrane upon activation [10] [11] [23], and that Rac proteins undergo regulatory control by GTP binding and membrane translocation for activation and hydrolysis to GDP for inactivation [24] [27] [29], we evaluated the influence of *P. gingivalis* LPS and ghrelin on membrane recruitment of PLCγ2 and Rac1. Western blot analysis revealed that the effect of the LPS was manifested by the elevation in membrane translocation of both PLCγ2 and Rac1, while the influence of ghrelin was reflected in the elevation in membrane-associated PLCγ2 and the suppression in the membrane translocation of Rac1. Moreover, we observed that blocking the Rac1 activation with NSC23766 had no effect on the LPS and ghrelin-elicited membrane translocation of PLCγ2, while the inhibitor of PLC, U73122, appeared to exert less apparent effect of Rac1 translocation. Hence, we concluded that up-regulation by *P. gingivalis* LPS in Rac1 membrane translocation plays a major role in PLCγ2 activation. This contention is consistent with the studies suggesting that the enhancement in PLCγ2 activation is a consequence of membrane proximity of Rac1 and the interaction with the split PH domain of PLCγ2 phosphorylated on Ser, which promotes its association withRac1 [13] [21] [25] [26] [37].

Next, to address the accumulating evidence as to the role Src and PKC in the regulation of PLC activation [10] [11] [13], we assessed the influence of SFK-PTK inhibitor, PP2 and PKC inhibitor, GF109203X on the activities of GEF Dock180 and PKC enzymes in the presence of the LPS and ghrelin. We noted that the effect of *P. gin-*

givalis LPS was associated with the elevation in the PKC and Dock180 activation, while ghrelin evoked further stimulation in PKC activity and the reduction in Dock180 activation. The LPS-induced activation of PKC and Rac1, moreover, was susceptible to suppression by PKC inhibitor, GF109203X, as well as the inhibitor of SFK-PTKs, PP2. Hence, we concluded that PKC and Src are active participants in GEF Dock180 activation. Our assertion is further supported by the results of Western blot analysis of Dck180, in which the lysates of the acinar cells as well as the cell membrane fraction were immunoprecipitated with anti-Dock180 antibody, and subjected to probing with anti-pSer and anti-pTyr antibody. The analyses revealed that incubation with the LPS elicited elevation in membrane Rac1 associated with Dock180, which was phosphorylated on Tyr as well as Ser. The effect of ghrelin, characterized by the presence of membrane-associated Dock180 phosphorylated on Tyr, and a drop in its phosphorylation on Ser, was also reflected in a decrease in the membrane translocation of Rac1. We have also observed that the LPS-induced membrane localization of Rac1 as well as Dock180 phosphorylation on Ser, were susceptible to suppression by PKC inhibitor, GF109203X, that also caused further decrease in the effect of ghrelin on Rac1 translocation. On the other hand, the effect of SFK-PTKs inhibitor, PP2, was reflected in the suppression of the LPS and ghrelin-elicited phosphorylation of membrane-associated Dock180 on Tyr and Ser as well as a decrease in Rac1 membrane localization. The fact that activation of Dock180 by the LPS, reported herein, was susceptible to suppression by both the wide spectrum PKC inhibitor, GF109203X, and the inhibitor of SFK-PTKs inhibitor, PP2, suggests that Src-kinase mediated phosphorylation on Tyr may be required for the stimulus-induced membrane localization of Dock180, while the PKC enzyme, identified earlier as PKCδ [22], is involved in the phosphorylation of membrane-recruited Dock180 on Ser.

The above findings, thus attest to the functional role of Dock180 phosphorylation on Tyr/Ser in the mediation of proinflammatory consequences of *P. gingivalis* LPS as well as the modulatory influence of ghrelin on the oral mucosal responses to this periodontopathic bacterium. Indeed, activation of Rac1 by Src-dependent phosphorylation of Dock180 on Tyr has been reported in association with PDGFRa-stimulated glioma tumorigenesis in mice and humans [31], and we have shown recently that the increase in *H. pylori* LPS-induced gastric mucosal Rac1-GTP generation occurs with the involvement of PKCδ [26] [37]. Therefore to add further credence to our assertion as to the role of Dock180 phosphorylation on Tyr/Ser in mediation of the signaling pathways triggered by *P. gingivalis* LPS as well as ghrelin, we investigated the hierarchy of the rapport between Src and PKCδ with respect to Dock180 phosphorylation by co-immunoprecipitation. Our analyses revealed that while PKCδ not co-precipitate with Dock180 in the absence of stimulation, the two proteins were found in association in both Dock180 and PKCδ immunoprecipitates following the acinar cell incubation with the LPS and ghrelin. Moreover, the association between Dock180 and PKCδ was dependent on the phosphorylation of Dock180 on Tyr as well as the activity of PKCδ, since pretreatment with SFK-PTKs inhibitor, PP2, as well as wide spectrum PKC inhibitor, GF109203X, interfered with the co-precipitation. Furthermore, we observed that following the acinar cell stimulation with *P. gingivalis* LPS and ghrelin, the Dock180 protein present in the Src immunoprecipitates, was found in association with Src phosphorylated on Tyr^{416}. As phosphorylation of Src on Tyr^{416} reflects the kinase activation state [6] [38], the findings provide a clear indication as to the involvement of Src in GEF Dock180 phosphorylation on Tyr.

Together, our data attest to the involvement of PKCδ and Src in modulation of Dock180 activation through phosphorylation on Tyr/Ser in response to *P. gingivalis* LPS. Although the full functional paradigm of GEF Dock180 phosphorylation has not yet been clearly defined, it is tempting to suggest that phosphorylation of Dock180 on Tyr could facilitate membrane anchoring and the interaction of Dock180 with nucleotide-free Rac1, thereby increasing Dock180 binding to Rac1 and its activation, while the LPS-induced phosphorylation of Dock180 on Ser may be responsible for further increase of Dock180 activation and, hence up-regulation in GTP loading to Rac1and the amplification in Rac1-GTP formation.

5. Conclusion

Our findings suggest that GEF Dock180 activation through Src/PKCδ mediated phosphorylation on Tyr/Ser plays a pivotal role in the salivary gland acinar cell PLCγ2 activation not only in response to proinflammatory *P. gingivalis* LPS signaling, but also in reaction to the modulatory action of ghrelin (**Figure 7**). Although the modulatory influence of ghrelin, signaling through GPCR activation, relies on Src-dependent Tyr phosphorylation of Dock180, and the propagation of proinflammatory events by *P. gingivalis* LPS relies on TLR4 ligation and sub-

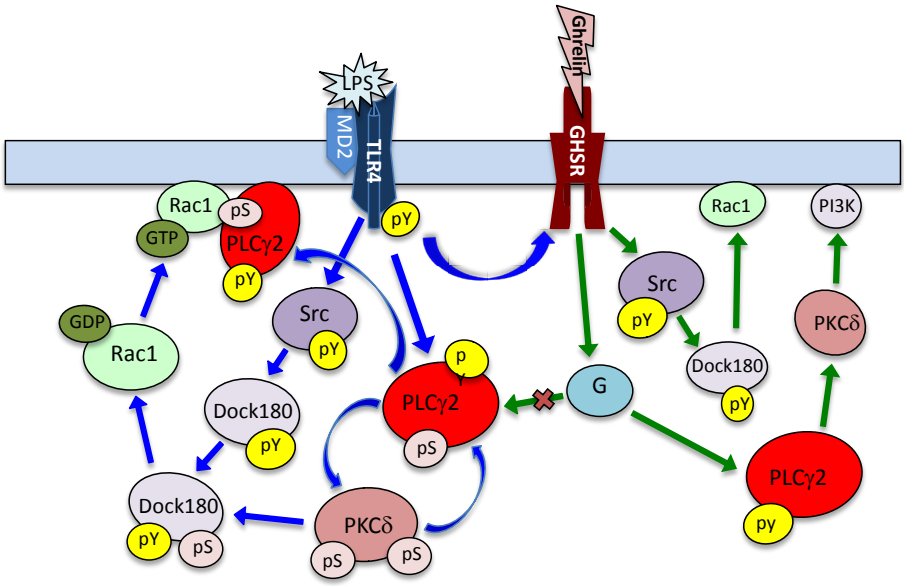

Figure 7. Schematic diagram of the regulatory role of GEF Dock180 phosphorylation on Tyr/Ser in modulation of Rac-1 activation in response to *P. gingivalis* LPS and ghrelin. Ligation by ghrelin of salivary gland acinar cell GHS-R1a activates several G protein-dependent signal transduction pathways, including that of Src kinase-dependent Dock180 phosphorylation on Tyr that maintains the regulatory level of Rac1-GTP formation. Binding of the LPS to TLR4 triggers Src kinase-dependent Dock180 phosphorylation on Tyr and the PLCγ2-mediated PKCδ activation that leads to the PKCδ-induced up-regulation in Dock180 and PLCγ2 activation through phosphorylation on Ser. The up-regulation in Dock180, in turn, stimulates the formation of Rac1-GTP and promotes its association with PLCγ2, thus resulting in the amplification in PLCγ2 activation. G heterotrimeric G-protein, pS phosphoserine, pY phosphotyrosine.

sequent amplification in Dock180 activation through Src/PKCδ-dependent Tyr/Ser phosphorylation, the major consequence of these seemingly opposing inputs is Rac1 activation and the amplification in PLCγ2.

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