

Role of Rac1/p38 and ERK-Dependent Cytosolic Phospholipase A₂ Activation in *Porphyromonas gingivalis*-Evoked Induction in Matrix Metalloproteinase-9 (MMP-9) Release by Salivary Gland Cells

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Abstract

Matrix metalloproteinase-9 (MMP-9) is a highly glycosylated endopeptidase implicated in a wide range of oral mucosal inflammatory and neoplastic diseases, including chronic periodontitis, a persistent mucosal inflammation attributed primarily to infection by oral anaerobe, *P. gingivalis*. In this study, we explored the role of Rac1 and mitogen-activated protein kinases (MAPKs) in the processes of MMP-9 release in sublingual salivary gland cells exposed to *P. gingivalis* key endotoxin, cell wall lipopolysaccharide (LPS). We demonstrate that the LPS-elicited induction in the acinar cell MMP-9 release is associated with MAPK, ERK and p38 activation, and occurs with the involvement of Rac1 and cytosolic phospholipase A₂ (cPLA₂). Further, we reveal that the LPS-induced MMP-9 release involves ERK-mediated phosphorylation of cPLA₂ on Ser⁵⁰⁵ that is essential for its membrane translocation with Rac1, and that this process requires p38 activation. Moreover, we show that phosphorylation and membrane localization of p38 with Rac1-GTP play a pivotal role in cPLA₂-dependent induction in MMP-9 release. Thus collectively, our findings infer that *P. gingivalis* LPS-induced up-regulation in the acinar cell MMP-9 release requires ERK-dependent recruitment of cPLA₂ to the membrane localized Rac1/p38 complex.

Keywords

P. gingivalis LPS, Oral Mucosa, Rac1, p38, ERK, cPLA₂ Activation MMP-9 Release

1. Introduction

Matrix metalloproteinase-9 (MMP-9), also known as gelatinase B, is a highly glycosylated zinc-dependent endopeptidase implicated in wound repair and a wide variety of inflammatory, degenerative and neoplastic diseases, including oral cancer, Sjogren's syndrome, rheumatoid arthritis, oral lichen planus, and chronic periodontitis [1]-[6]. An enhanced production of MMP-9, moreover, is associated with oral mucosal reaction to microbial and fungal infections [7] [8], as well as characterizes inflammatory response to lipopolysaccharide (LPS) of Gram-negative bacteria in several different cell systems [9]-[11]. Indeed, the elevated levels of MMP-9, along with the increased proinflammatory cytokine production, elicited in response to periodontopathic bacterium, *Porphyromonas gingivalis* and its key endotoxin, LPS, are directly responsible for persistent mucosal inflammation that leads to periodontal lesions and progressive destruction of teeth-supporting tissue, including bone loss [5] [8] [11]-[13].

Investigations into the events underlying the proinflammatory signal propagation indicate that oral mucosal responses to *P. gingivalis* LPS are mediated through the interaction with Toll-like receptor-4 (TLR4), stimulation of which leads to up-regulation in mitogen-activated protein kinases (MAPKs), and the activation of transcriptional factors that exert control over a wide range of proinflammatory mediators, including, MMP-9 [1] [14]. Although the rate-limiting step in MMP-9 regulation is gene transcription, the expression and the activity of MMP-9 remain also under the influence of posttranslational processing, proenzyme activation, and the inhibition by the family of endogenous tissue inhibitors of metalloproteinases (TIMPs) [1] [15]. This multifaceted control assures the low MMP-9 expression in normal tissue, and its rapid release and activation in response to inflammatory stimulus such as LPS [10]. Interestingly, we have linked recently the consequence of LPS stimulation to the involvement of JNK/p38 and ERK in the activation of transcriptional factors, AP-1 and NF- κ B [16] [17], and the relevant evidence suggests that MMP-9 expression is regulated at the transcriptional level by MAPK ERK, JNK, and p38 [1] [18] [19].

The literature data, moreover, suggest that MAPK cascade activation upon LPS stimulation plays an important role in the regulation of intracellular trafficking, membrane translocation, and the release of secretory products [10] [20] [21]. The LPS-induced p38 activation and its recruitment to the cytosolic aspect of the membrane-localized Rac1 have been linked to disintegrin-metalloprotease ADAM17 activation [14] [20], whilst up-regulation in MAPK and Rac activation is observed in association with LPS-induced pulmonary inflammation and TLR4-stimulated phagocytosis [22] [23]. Furthermore, there are indications that ERK activation upon LPS stimulation plays an important role in the phosphorylation of cytosolic phospholipase A₂ (cPLA₂) that facilitates the enzyme cytosol-to-membrane translocation, and that further up-regulation in cPLA₂ activation occurs with the involvement of Rac1/p38 complex [21] [24].

Interestingly, cPLA₂ activation through Rac1/p38 complex is associated with the enzyme targeting to intracellular membranes involved in secretory cargo processing such as the Golgi, vesicle formation, and vesicle fusion [25] [26]. Therefore, considering that MMP-9 undergoes extensive processing in the Golgi network and rapid release upon stimulation [27] [28], in this study we investigate the nature of factors associated with *P. gingivalis* LPS-induced up-regulation in MMP-9 release by salivary gland acinar cells.

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 μ g/ml), penicillin (50 U/ml), streptomycin (50 μ g/ml), and 10% fetal calf serum, and gently dispersed by trituration with a syringe and settled by centrifugation [29]. 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% O₂ and 5% CO₂ at 37°C for up to 16 h in the presence of 0 - 100 ng/ml *P. gingivalis* LPS [29]. *P. gingivalis* used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 33277 [30]. In the experiments evaluating the effect of ERK inhibitor, P98059, p38 MAPK inhibitor, SB202190, JNK inhibitor, SP600125, cPLA₂ inhibitor, MAFP (Calbiochem), and Rac1 inhibitor, NSC 23766 (Sigma), 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. Gelatin Zymography and Western Blot Analysis of MMP-9

The measurement of *P. gingivalis* LPS effect on the acinar cell MMP-9 activation was carried out by gelatin zymography [11] [27]. The spent acinar cell media, collected by centrifugation, were mixed with Laemmli buffer, lacking 2-mercaptoethanol, and subjected to electrophoresis using 8% SDS-PAGE containing 0.2% gelatin. Following, electrophoresis, the gels were washed three times for 20 min in zymogram wash buffer (2.5% Triton X-100, 50 mM Tris-HCl, pH 7.5), and incubated for 24 h at 37°C in a developing buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM CaCl₂, 5 μM ZnCl₂, and 150 mM NaCl. The Gels were then stained with 0.25% Coomassie Brilliant Blue solution [27], and the gelatinolytic activities were detected as transparent bands against the dark background. For Western blot analysis, the spent culture media and total cell lysates were boiled in SDS sample buffer for 5 min, separated on 8% SDS-PAGE, transferred to nitrocellulose membranes, and following blocking (5% skim milk), the membranes were incubated overnight at 4°C with the specific anti-MMP-9 antibody (Calbiochem).

2.3. Rac1-GTP Activation Assay

The measurement of Rac activation was conducted using Rac1 Activation Assay Kit (EMD Millipore). The salivary gland acinar 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 PAK1 PBD-agarose for 1 h at 4°C. The beads were washed three times in MLB, resuspended in Laemmli reducing sample buffer, resolved on SDS-PAGE, and immunoblotted for GTP-bound Rac1 using anti-Rac1 antibody.

2.4. cPLA₂ Activity Assay

The measurement of cPLA₂ activity in the acinar cells following various experimental conditions was carried out using cPLA₂ assay kit (Cayman). The cells were homogenized in 1 ml of 50 mM HEPES buffer, pH 7.4, containing 1 mM EDTA, and centrifuged at 10,000 × g for 15 min at 4°C [31]. The supernatants were then filtered through an Amicon YM30 filter concentrators (m.w. cut-off 30 kDa) to remove any contamination with secretory PLA₂, followed by 15 min incubation with 5 μM of calcium-independent PLA₂ inhibitor, bromoenol lactone, and the aliquots (10 μl) of such prepared cell lysates were subjected to cPLA₂ assay according to manufacturer's instruction.

2.5. Cell Membrane

To assess membrane translocation of cPLA₂, p38, and Rac1 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 [14]. 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 MgCl₂, 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. Immunoprecipitation and Immunoblotting

The acinar cells from various 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 [14]. 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 ERK, phospho-ERK, and p38, phospho-p38, (Calbiochem), cPLA₂ and pcPLA₂ (Ser⁵⁰⁵) (Cell Signaling), and Rac1 (EMD Millipore).

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

Considering the involvement of MMP-9 in a wide spectrum of inflammatory, autoimmune, degenerative and neoplastic diseases affecting the oral health [2]-[4], we employed rat sublingual salivary gland cells and investigated the factors involved in *P. gingivalis* LPS-induced enhancement in MMP-9 release. As shown in **Figure 1**, incubation of the acinar cells with the LPS elicited a time-dependent release of MMP-9 into the incubation medium, with the gelatinolytic activity readily apparent after 6 h of incubation, and the MMP-9 protein by Western blotting after 12 h. The activity of constitutively expressed MMP-2, however, remained unchanged with the LPS

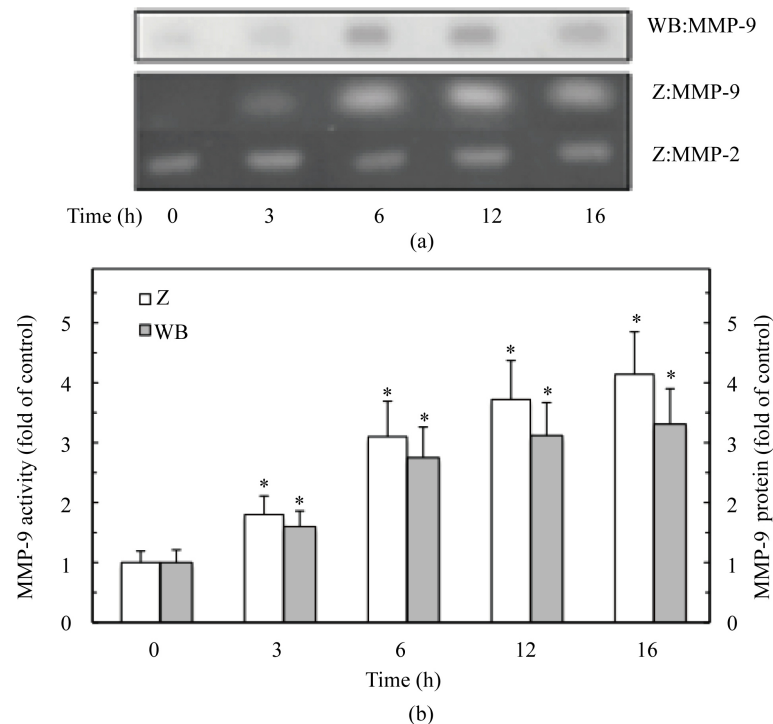


Figure 1. Effect of *P. gingivalis* LPS on the expression of MMP-9 in sublingual salivary gland acinar cells. The cells were treated with the LPS at 100 ng/ml and incubated for up to 16 h. At the indicated time periods the conditioned media were assayed for gelatinolytic activity of MMP-9 by zymography (Z), using MMP-2 as control (a), while the total cell lysates were analyzed for MMP-9 protein by Western blotting (WB). The relative level of MMP-9 protein and its gelatinolytic activity are expressed as fold of control (b). The data represent the mean ± SD of four experiments. * $P < 0.05$ compared with that of control (0).

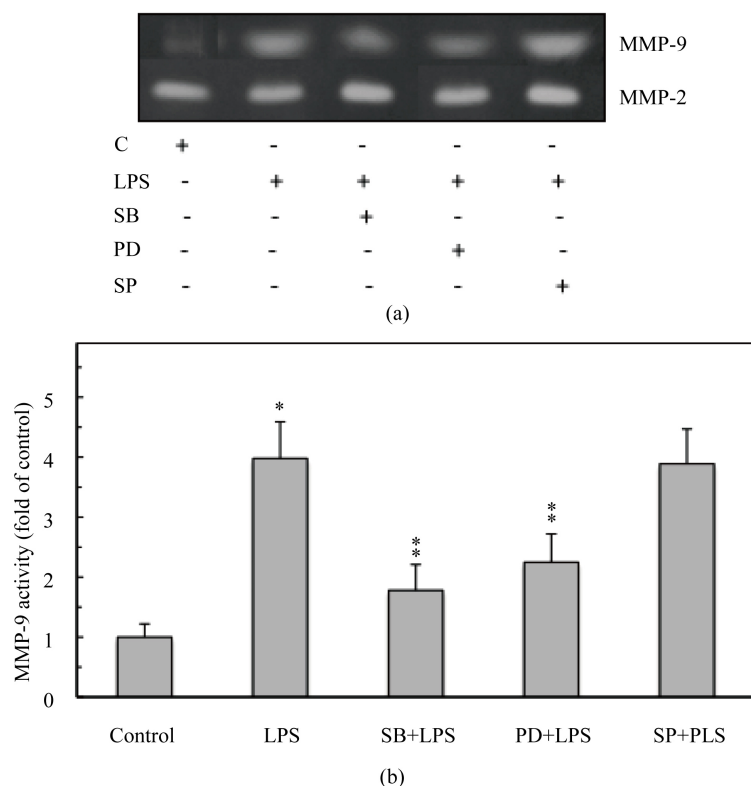


Figure 2. Effect of ERK, JNK, and p38 MAPK inhibitors on *P. gingivalis* LPS-induced MMP-9 activation in the acinar cells. The cells, preincubated with 30 μ M ERK inhibitor, PD98059 (PD), 10 μ M JKN inhibitor, SP600125 (SP), or 20 μ M p38 inhibitor, SB202190 (SB), were treated with the LPS at 100 ng/ml and incubated for 12 h. MMP-9 released into the medium was quantified by zymography using MMP-2 as control (a), and the effect of inhibitors on the activity MMP-9 is expressed as fold of control (b). Values represent the means \pm SD of four experiments. * P < 0.05 compared with that of control. ** P < 0.05 compared with that of LPS.

stimulation. Further, we found that the LPS-induced MMP-9 release and activation was susceptible to suppression by p38 MAPK inhibitor, SB202190 as well as the inhibitor of ERK, PD98059, but not the inhibitor of JNK, SP600125 (Figure 2), thus pointing to the role of p38 and ERK in *P. gingivalis* LPS-induced amplification in MMP-9 release.

In further assessment of factors that influence the process of the acinar cell MMP-9 release, we have revealed that the LPS-elicited induction in MMP-9 activation displayed also susceptibility to NSC23766, an inhibitor of Rac1 and the inhibitor of cPLA₂, MAFP (Figure 3). This suggests the involvement of Rac1 and cPLA₂ in the processes of MMP-9 release. Therefore, following on the above leads, we have evaluated the influence of the LPS on the acinar cell activity of cPLA₂ and Rac1 guanine nucleotide exchange factor (GEF), Dock 180. The results revealed that while the LPS-induced Dock 180 activation was not affected by the inhibitors of ERK (PD98059), p38 (SB202190), and cPLA₂ (MAFP), the activation of cPLA₂ by the LPS showed susceptibility to suppression by the inhibitors of ERK and p38, as well as the inhibitor of Rac1, NSC23766 (Figure 4). These findings, thus underscore the central role of Rac1 activation in the course of *P. gingivalis* LPS-induced up-regulation in MMP-9 release.

Hence, to address the character of the interaction between ERK, p38, cPLA₂, and Rac1 in mediation of MMP-9 release in response to the LPS stimulation, we assessed the requirements for membrane translocation of Rac1, p38 and cPLA₂. The results of Western blot analysis of whole cell lysates as well as the cell membrane fraction revealed that the incubation with the LPS resulted in recruitment of Rac1, p38, and cPLA₂ to the membrane, while the effect of Rac1 inhibitor, NSC23766, was reflected in the suppression in membrane translocation of all three proteins (Figure 5). Moreover, we found that blocking the p38 activation with SB202190, led to the suppression in membrane translocation of p38 as well as cPLA₂, while the inhibitor of ERK, PD98059, evoked a marked suppression in cPLA₂ translocation but had no effect on the LPS-induced membrane translocation of

Rac1 or p38. These data infer that the LPS-induced up-regulation in the acinar cell MMP-9 release requires ERK-dependent recruitment of cPLA₂ to the membrane localized Rac1/p38 complex.

Therefore, In further approach to ascertain the involvement of ERK in the signaling mechanism by which the Rac/p38/cPLA₂ cascade mediates the acinar cell MMP-9 release in response to *P. gingivalis* LPS, we examined the requirement and selectivity of the interaction between cPLA₂ and Rac1 in the presence of the inhibitors of ERK and p38 activation by co-immunoprecipitation. The results of Western blot analysis revealed that cPLA₂

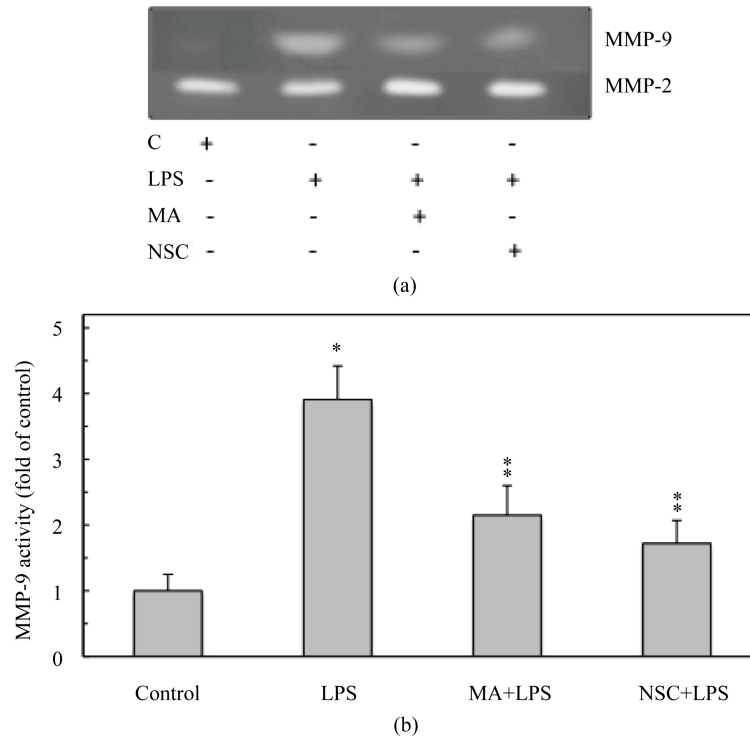


Figure 3. Effect of Rac1 and cPLA₂ inhibitors on *P. gingivalis* LPS-induced MMP-9 activation in the acinar cells. The cells, preincubated with 50 μM Rac1 inhibitor, NSC23766 (NSC), or 20 μM cPLA₂ inhibitor, MAFP (MA), were treated with the LPS at 100 ng/ml and incubated for 12 h. MMP-9 released into the medium was quantified by zymography using MMP-2 as control (a), and the effect of inhibitors on the activity of MMP-9 is expressed as fold of control (b). Values represent the means ± SD of four experiments. **P* < 0.05 compared with that of control. ***P* < 0.05 compared with that of LPS.

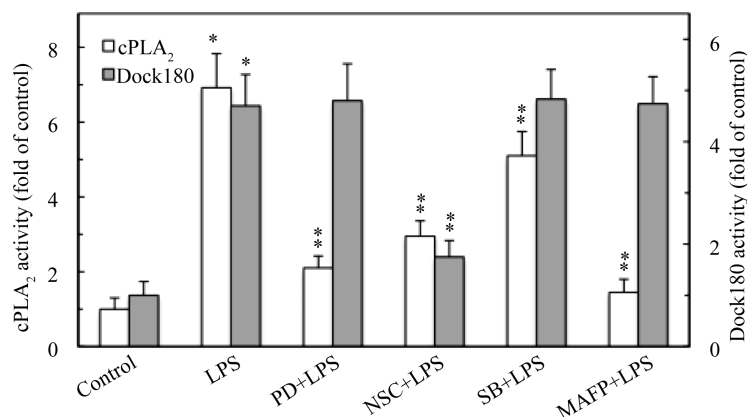


Figure 4. Effect of ERK, p38, Rac1, and cPLA₂ inhibitors on *P. gingivalis* LPS-induced changes in the expression cPLA₂ and Dock180 (GTP-Rac1) activities in salivary gland acinar cells. The cells, preincubated with 30 μM ERK inhibitor, PD98059 (PD), 50 μM Rac1 inhibitor, NSC23766 (NSC), 20 μM p38 inhibitor, SB02190 (SB), or 20 μM cPLA₂ inhibitor, MAFP, were treated with the LPS at 100 ng/ml and incubated for 1 h. Values represent the means ± SD of four experiments. **P* < 0.05 compared with that of control. ***P* < 0.05 compared with that of LPS.

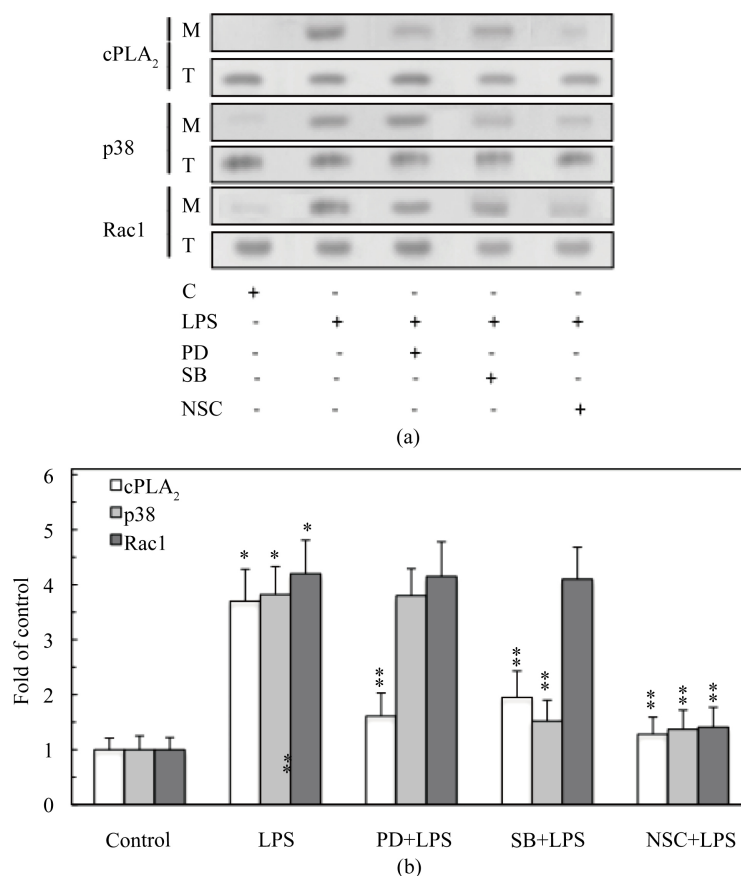


Figure 5. Effect of p38, ERK, and Rac1 inhibition on the changes induced by *P. gingivalis* LPS in membrane translocation of Rac1, cPLA₂ and p38 in salivary gland acinar cells. The cells, preincubated with 30 μ M ERK inhibitor, PD98059 (PD), 20 μ M p38 inhibitor, SB202190 (SB), or 50 μ M Rac1 inhibitor, NSC23766 (NSC), were treated with the LPS at 100 ng/ml, and incubated for 2 h. The lysates of whole cells (T) and the corresponding membrane (M) fractions were analyzed for cPLA₂ and p38 with specific antibodies (a). The relative densities of the membrane proteins are expressed as fold of control (b), and the total (T) Rac1, cPLA₂ and p38 were used as loading control. Values represent the means \pm SD of four experiments. * P < 0.05 compared with that of control. ** P < 0.05 compared with that of LPS.

found in association with Rac1-GTP following the LPS stimulation, also reacted with anti-pcPLA₂ (Ser⁵⁰⁵) antibody and was susceptible to inhibition not only by the inhibitor of Rac1, NSC23766, but also by the inhibitors of ERK (PD98059) and p38 (SB202190). The inhibitors of Rac1 and p38 however, while blocking the interaction between cPLA₂ and Rac1-GTP had no effect on the LPS-induced cPLA₂ phosphorylation on Ser⁵⁰⁵ (Figure 6). Further, by examining the results of co-immunoprecipitation experiments between p38 and cPLA₂ (Figure 7), we found that blocking the LPS-induced p38 phosphorylation with SB202190, led to the interference in the association of p38 with cPLA₂. Hence, we concluded that the LPS-induced pcPLA₂ localization with the Rac1-GTP for up-regulation in the acinar cell MMP-9 release requires activated (phosphorylated) p38 involvement.

4. Discussion

Porphyromonas gingivalis, a Gram-negative anaerobe colonizing the oral cavity, is recognized as a potent periodontopathic pathogen implicated in the etiology of periodontitis, a chronic inflammatory disease that leads to progressive destruction of teeth-supporting tissue and is the major cause of adult tooth loss [5] [8] [13]. Indeed, studies show that oral mucosal responses to *P. gingivalis* and its key endotoxin, LPS, are characterized by the disturbances in NO and prostaglandin production, increase in proinflammatory cytokine formation, and the elevation in MMP-9 release [5] [11] [14] [30]. Moreover, there are indications that *P. gingivalis* LPS and the increased levels of MMP-9 routinely detected in the circulation of periodontal disease patients may be responsible

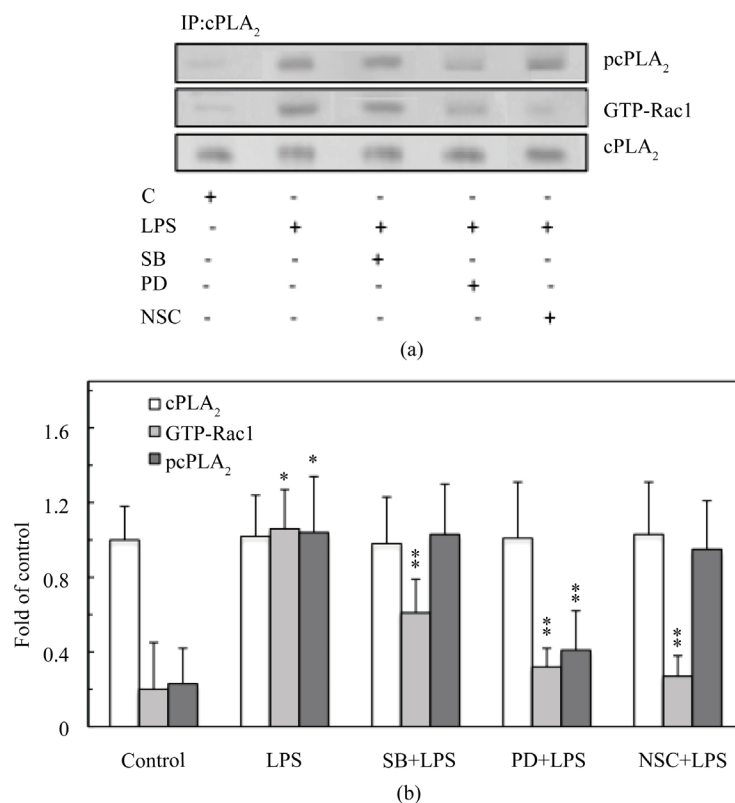


Figure 6. Effect of Rac1, p38, and ERK inhibition on the changes induced by *P. gingivalis* LPS in the acinar cell cPLA₂ phosphorylation and its association with GTP-Rac1. The cells, preincubated with 50 μ M NSC23766 (NSC), 20 μ M SB202190 (SB), or 30 μ M PD98059 (PD), were treated with the LPS at 100 ng/ml and incubated for 1 h. Cell lysates were immunoprecipitated (IP) with anti-cPLA₂ antibody and immunoblotted (WB) with anti-cPLA₂ antibody and anti-Rac1 (a). The cPLA₂ immunoblots were also reblotted with anti-phospho-cPLA₂ (pcPLA₂) antibody, and relative densities of proteins are expressed as fold of p38 control (b). The data represent the means \pm SD of four separate experiments. * P < 0.05 compared with that of control. ** P < 0.05 compared with that of LPS.

for a decrease in cardiac function and cardiovascular disease [13] [32]. Therefore, in the present study we investigated the nature of factors associated with *P. gingivalis* LPS-induced oral mucosal MMP-9 release.

Our results revealed that incubation of salivary gland acinar cells with *P. gingivalis* LPS, elicited a time-dependent increase in MMP-9 release into the incubation medium as judged by the rise in gelatinolytic activity and the MMP-9 protein level. Furthermore, we found that the LPS-elicited up-regulation in MMP-9 release and activation was susceptible to suppression by p38 inhibitor, SB202190, as well as the inhibitor of ERK, PD98059, whereas the inhibitor of JNK MAPK, SP600125, had no effect. These findings, thus support the data indicating that p38 along with ERK are involved in the regulation of MMP-9 expression at both the transcriptional and post-translational levels [1] [9] [18]. Indeed, stimulation of TLR4 with LPS is known to lead to the induction in MAPKs as well as transcriptional factors activation [1] [14] [17].

Further, we found that the LPS-elicited induction in MMP-9 release and activation displayed also susceptibility to suppression by Rac1 inhibitor, NSC23766, as well as the inhibitor of cPLA₂, MAFP, thus suggesting the involvement of Rac1 and cPLA₂ in the processes associated with MMP-9 processing. Moreover, by following the acinar cell activity of Rac1 GEF, Dock180 and cPLA₂, we revealed that while the LPS-induced activation of Dock180 was not affected by the inhibitors of ERK, p38 and cPLA₂, the activation of cPLA₂ showed susceptibility to suppression by the inhibitor of Rac1 (NSC23766) as well as the inhibitors of ERK (PD98059) and p38 (SB202190). Therefore, in the light of existing evidence as to the involvement of Rac1 GTPase in MAPK and cPLA₂ membrane recruitment and activation [22] [24] [33], the above findings attest to the involvement of Rac1 and cPLA₂ in mediation of oral mucosal MMP-9 release in response to *P. gingivalis* LPS challenge. In this connection, it is pertinent to note that the role of Rac1 in MAPKs activation has been also suggested in association with oral mucosal and pulmonary responses to LPS [9] [14] [19].

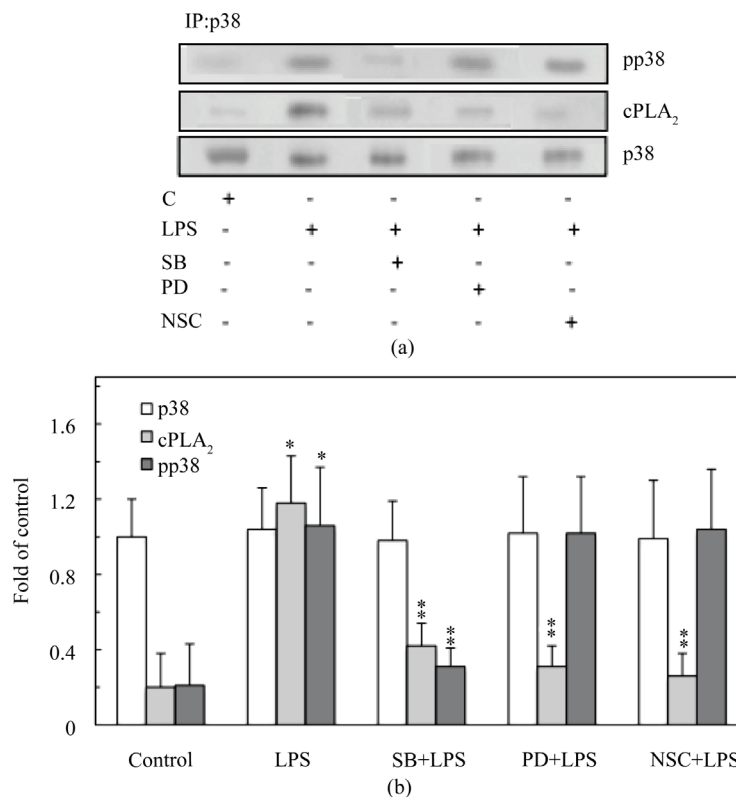


Figure 7. Effect of p38, Rac1, and ERK inhibition on the changes induced by *P. gingivalis* LPS in the acinar cell p38 phosphorylation and its association with cPLA₂. The cells, preincubated with 20 μM SB202190 (SB), 50 μM NSC23766 (NSC), or 30 μM PD98059 (PD), were treated with the LPS at 100 ng/ml and incubated for 1 h. Cell lysates were immunoprecipitated (IP) with anti-p38 and immunoblotted (WB) with anti-p38 and anti-cPLA₂ antibody (a). The p38 immunoblots were also reblotted with anti-phospho-p38 (pp38) antibody, and relative densities of proteins are expressed as fold of p38 control (b). 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.

Hence, to ascertain further the involvement of Rac1, p38, ERK, and cPLA₂ in the pathways leading to up-regulation in the acinar cell MMP-9 release in response to *P. gingivalis* LPS, we assessed the requirement and selectivity of the membrane translocation of these proteins. Our analyses revealed that incubation with the LPS elicited elevation in membrane translocation of p38, cPLA₂ and Rac1, while the effect of Rac1 inhibition with NSC23766 was reflected in the suppression in membrane translocation of all three proteins. Blocking the p38 activation with SB202190, led to the suppression in the membrane translocation of p38 as well cPLA₂, whereas the inhibition of ERK with PD98059, while affecting the translocation of cPLA₂ had no effect on the LPS-induced membrane recruitment of Rac1 or p38. Accordingly, we surmised that up-regulation by *P. gingivalis* LPS in the acinar cell membrane recruitment of cPLA₂ to Rac1/p38 requires ERK participation. This assertion is consistent with the literature data indicating that ERK-mediated cPLA₂ activation through phosphorylation on the critical Ser⁵⁰⁵ residue plays a crucial role in the enzyme translocation from cytosol to the membrane, and that the membrane-anchored Rac1/p38 complex may be involved in cPLA₂ activity regulation [12] [17] [21] [24] [34].

Indeed, examination of the requirement and selectivity of the interaction between cPLA₂ and Rac1 by co-immunoprecipitation revealed that cPLA₂ found in association with Rac1-GTP following the LPS stimulation, also reacted with anti-phospho-cPLA₂ (Ser⁵⁰⁵) antibody. Moreover, the association between cPLA₂ and Rac1 was not only subject to interference by the inhibitor of Rac1, NSC23766, but also by the inhibitors of p38 (SB202190) and ERK (PD98059). However, the inhibitors of Rac1 and p38, while blocking the interaction between Rac1 and cPLA₂, had no effect on the LPS-induced cPLA₂ phosphorylation on Ser⁵⁰⁵. Furthermore, by examining the LPS-induced immunoprecipitates of p38 with cPLA₂, we found that the association between the two proteins was not only subject to suppression by the inhibitors of Rac1 and ERK, but also by the inhibitor of

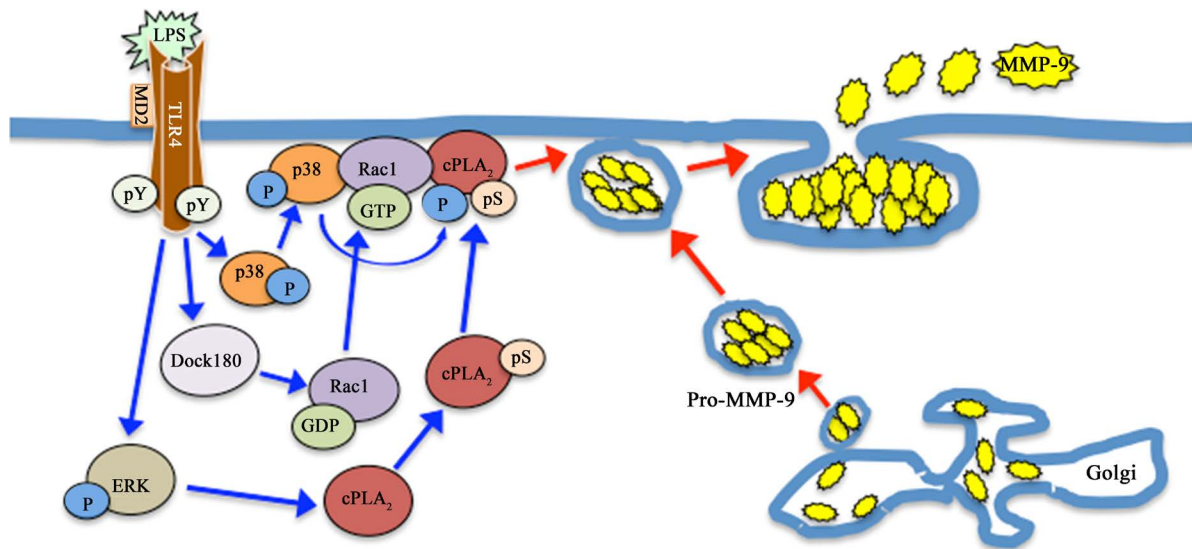


Figure 8. Schematic diagram of the pathways involved in *P. gingivalis* LPS-elicited induction in sublingual salivary gland acinar cell MMP-9 release. Engagement of TLR4 by the LPS triggers up-regulation in ERK and p38 MAPK phosphorylation, as well as induces the pathway of GEF, Dock180-mediated GTP-Rac1 formation and its membrane translocation. This in turn, promotes the membrane localization of pp38 with the Rac1-GTP, while pERK is involved in the phosphorylation of cPLA₂ on Ser⁵⁰⁵, which prompts its recruitment to the Rac1/p38 complex, and the induction in cPLA₂ activation. The pcPLA₂, by acting on membrane phospholipids, affects the membrane fusion events, and leads to the induction in MMP-9 release. P phosphate, pS phosphoserine, pY phosphotyrosine.

p38, SB202190, which blocked the LPS-induced p38 phosphorylation, thus pointing to the requirement of pp38 in the translocation of pcPLA₂ (Ser⁵⁰⁵) to the membrane-anchored Rac1/p38 complex for the enhancement in cPLA₂ activation. In this connection it is pertinent to note that the activated p38, in the absence of specific membrane localization partners, preferentially accumulates in the cytosol [19]. The above findings thus strongly suggest that the activation and translocation of pp38 to the membrane-localized Rac1-GTP plays a pivotal role in cPLA₂-dependent up-regulation in MMP-9 release. The fact that the activation of cPLA₂ by the LPS was susceptible to suppression by the inhibitor of p38 phosphorylation indicates that pp38 may be required for the enhancement in cPLA₂ activation of the membrane-recruited enzyme. Interestingly, studies show that the enhancement cPLA₂ activity by p38 may be either the result of p38-mediated phosphorylation of cPLA₂ on the additional three Ser residues (Ser⁴³⁷, Ser⁴⁵⁴ and Ser⁷²⁷), or just a consequence of cPLA₂ recruitment to the Rac/p38 complex [24] [35] [36].

Of particular importance to the interpretation of the results our study are the reports indicating that cPLA₂ activation through recruitment to the Rac1/p38 complex is associated with the enzyme targeting to intracellular membranes involved in secretory cargo processing such as the Golgi, vesicle formation, and vesicle fusion [24]-[26]. Apparently, by acting on membrane phospholipids, cPLA₂ is capable of affecting membrane curvature, vesicle budding, and the membrane fusion events [25] [37] [38]. As MMP-9 undergoes extensive processing in the Golgi network, vesicle trafficking, and rapid release upon stimulation [1] [27] [28], our findings as to the involvement of cPLA₂ in MMP-9 release provide an important insight into the factors responsible for up-regulation in MMP-9 secretion during oral mucosal inflammatory responses to *P. gingivalis*. Hence, therapeutic targeting cPLA₂ may prove to be useful approach for developing more effective treatments of chronic periodontitis.

5. Conclusion

The data presented in this report demonstrate that *P. gingivalis* LPS-induced up-regulation in salivary gland acinar cell MMP-9 release is associated with MAPK, ERK and p38 activation, and occurs with the involvement of Rac1 and cPLA₂. We further show that ERK-mediated phosphorylation of cPLA₂ on Ser⁵⁰⁵ plays an essential role for its membrane translocation with Rac1, and that this process requires membrane-localized p38 participation (Figure 8). Collectively, our findings provide a strong evidence for the role of ERK/cPLA₂ and Rac1/p38/pcPLA₂ cascade in *P. gingivalis* LPS-induced up-regulation in the acinar cell MMP-9 release.

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