

***Porphyromonas gingivalis*-Stimulated TACE Activation for TGF- α Ectodomain Shedding and EGFR Transactivation in Salivary Gland Cells Requires Rac1-Dependent p38 MAPK Membrane Localization**

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Abstract

Oral mucosal inflammatory responses to *P. gingivalis* and its key virulence factor, lipopolysaccharide (LPS), are characterized by a massive rise in proinflammatory cytokine production, up-regulation in mitogen-activated protein kinase (MAPK) cascade, and the induction in epidermal growth factor receptor (EGFR) activation. In this study, we report that stimulation of salivary gland acinar cells with *P. gingivalis* LPS leads to p38 MAPK-dependent release of soluble TGF- α ligand and the increase in EGFR phosphorylation. Further, we show that the LPS-induced TGF- α shedding and EGFR transactivation involve the activation of membrane-associated metalloprotease, TACE also known as ADAM17, through phosphorylation by p38 MAPK, and require Rac1 participation. Moreover, we demonstrate that blocking the Rac1 activation leads to the suppression in the membrane translocation of Rac1 as well as p38, thus indicating that the LPS-elicited p38 membrane recruitment for TACE phosphorylation requires colocalization with Rac1. Hence, our findings imply that Rac1 membrane translocation serves as an essential platform for the localization of p38 with TACE, TGF- α ectodomain shedding, and the EGFR activation.

Keywords

P. gingivalis LPS, Oral Mucosa, p38 MAPK, TGF- α , TACE Activation, Rac1, EGFR Transactivation

1. Introduction

Lipopolysaccharide (LPS), a component of the outer membrane of *P. gingivalis* bacterium colonizing the oral cavity, is recognized as a potent endotoxin responsible for eliciting persistent mucosal inflammation that leads to periodontal lesions and progressive destruction of teeth-supporting tissue, including bone loss [1]-[4]. The oral mucosal responses to *P. gingivalis* LPS are mediated through the interaction with Toll-like receptor-4 (TLR4), stimulation of which leads to the activation of transcriptional factors that exert control over a wide range of proinflammatory mediators [2] [5]-[7]. The engagement of TLR4 by LPS, moreover, is known to elicit signaling events leading to the activation of mitogen-activated protein kinase (MAPK) cascade, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase [8]-[12]. The activated MAPKs, in turn, exert their control over transcriptional factors activation through phosphorylation on the critical Ser/Thr residues [8]-[11]. Moreover, the recent evidence indicates that LPS-induced proinflammatory signal propagation is associated with epidermal growth factor receptor (EGFR) activation [13]-[16].

The EGFR is a transmembrane glycosylated glycoprotein, consisting of an extracellular ligand binding domain, a single membrane-spanning region and a cytoplasmic tyrosine kinase domain, implicated in growth and repair processes of a variety of epithelial cells, including those of oral mucosa and salivary glands [17]-[19]. Upon ligand binding to the extracellular domain, EGFR undergoes dimerization, and the intrinsic tyrosine kinase activation through phosphorylation of key tyrosine residues within the cytoplasmic region of the receptor [18] [20]. Subsequently, these phosphorylated tyrosine residues serve as docking sites for a variety of the recruited signal transducer molecules [18]. Studies indicate that in addition to its cognate epidermal growth factor (EGF) ligand, the EGFR activation occurs in response to transforming growth factor- α (TGF- α), heparin binding EGF-like growth factor (HB-EGF), epiregulin, amphiregulin, and epigen [18] [20]. All these ligands are expressed as inactive membrane-anchored proteins that in response to a specific cellular stimulus undergo proteolytic cleavage, termed ectodomain shedding, to release the particular mature growth factor [18] [21].

The cleavage of EGFR ligands occurs with the involvement of membrane-anchored family of disintegrin-metalloproteases (ADAMs), which show preference with respect to stimulus response and exhibit various degree of substrate specificity [18]. Among the metalloproteinases that respond to LPS stimulation, the primary role is assigned to TNF- α converting enzyme (TACE), also known as ADAM17 [12] [16] [22]. Indeed, studies show that LPS-induced TACE activation leads to the enhancement in TGF- α shedding and EGFR transactivation [12] [16]. Furthermore, EGFR transactivation by TACE-dependent TGF- α shedding has been linked to LPS-induced TLR4 signaling through p38 MAPK [12] [21], and there are strong indications for the involvement of Rac1 GTPase in the activation process [23] [24].

As oral mucosal inflammatory responses to periodontopathic bacterium, *P. gingivalis*, are characterized by a massive rise in proinflammatory cytokine production, up-regulation in MAPK and Rac1 activation, and the induction in EGFR phosphorylation [2] [11] [19] [25], in this study, we examine the nature of factors associated with *P. gingivalis* LPS-induced amplification in salivary gland acinar cell EGFR transactivation. Our results demonstrate that stimulation of the acinar cells with the LPS leads to the enhancement in TACE-dependent shedding of TGF- α and the increase in EGFR activation. We also show that the LPS-induced TACE activation occurs through phosphorylation by p38 MAPK and requires Rac1 participation.

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 [25]. 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 4 h in the presence of 0 - 100 ng/ml *P. gingivalis* LPS [25]. *P. gingivalis* used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 33277 [6]. In the experiments evaluating the effect of p38 MAPK inhibitor, SB202190, ERK inhibitor, P98059, JNK inhibitor, SP600125, and TACE (TNF- α converting enzyme) inhibitor, TAPI-1 (Calbiochem), Rac1 inhibitor, NSC 23766, and EGFR inhibitor, AG1478 (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. M. TGF- α Shedding Assay

The quantification of TGF- α release by the acinar cells were carried out with TGF- α ELISA Kit (EMD Millipore). The cells were incubated with *P. gingivalis* LPS at 100 ng/ml for 4 h, and the supernatant was collected and concentrated. In the experiments on the effect of MAPK and TACE inhibitors, the cells prior to the addition of the LPS were first preincubated with the indicated dose of the agent or the vehicle. To prevent soluble TGF- α binding to EGFR, the cells before the addition of the LPS were also incubated with 2 μ g/ml anti-EGFR neutralizing antibody. Following centrifugation at 5000 rpm for 10 min, the level of TGF- α in concentrated cell supernatants was measured by following the manufacturer's (EMD Millipore) instructions.

2.3. TACE Activity Assay

TACE activity measurement in the acinar cells of sublingual salivary gland was conducted with InnoZyme TACE Activity Kit (EMD Millipore). The cells from the control and experimental treatments were rinsed with 0.05 M phosphate buffer/saline, pH 7.4, settled by centrifugation, and suspended for 30 min at 4°C in magnesium lysis buffer (MLB). Following sonication (3 \times 10 sec pulses), the samples were centrifuged at 12,000 \times 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 added to the wells pre-coated with anti-TACE antibody, and the activity of captured TACE enzyme was measured using an internally quenched fluorescent peptide substrate after 10 min of incubation at room temperature.

2.4. Cell Membrane Preparation

To assess membrane translocation of Rac1 and p38 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 \times g for 15 min, the supernatant was diluted with two volumes of cold homogenization buffer and centrifuged at 10,000 \times g for 20 min. The resulting supernatant was then subjected to centrifugation at 100,000 \times 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 \times 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).

2.5. 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 μ g/ml leupeptin and 1 μ g/ml pepstatin [6] [25]. 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 μ g 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, p38, phospho-p38, and JNK, phospho-JNK (Calbiochem), Rac1, EGFR, phospho-EGFR, EGF, and TGF- α (EMD Millipore), TACE, phospho-TACE (Thr⁷³⁵), and β -actin (Sigma).

2.6. Data Analysis

All experiments were carried out using duplicate sampling, and the results are expressed as means \pm SD. Analy-

sis 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 recent evidence as to the role of LPS in pro-inflammatory stimulus propagation through the processes associated with EGFR transactivation [12] [15] [16], we investigated the factors involved in the release of EGFR ligand, TGF- α , by salivary gland acinar cells in response to LPS of periodontopathic bacterium, *P. gingivalis*. The results revealed that exposure of rat sublingual salivary gland cells to incubation with the LPS elicited a significant induction in MAPKs activation, as evidence by a marked increase in p38, ERK, and JNK phosphorylation (Figure 1). Moreover, we found that the effect of the LPS was also reflected in a massive induction in the release of soluble TGF- α ligand (Figure 2). Furthermore, following the literature indication as to the involvement of MAPK in EGFR transactivation [21], we examined the effect of EGFR kinase inhibitor, AG1478, on the LPS-induced MAPK phosphorylation. As shown in Figure 1, pretreatment of the acinar cells with AG1478 led to a substantial suppression of the LPS-induced ERK phosphorylation, while the extent of p38 and JNK phosphorylation remained essentially unaffected. Further, we found that the LPS-elicited induction in TGF- α release was susceptible to suppression by p38 inhibitor, SB202190, as well as the inhibitor of TACE,

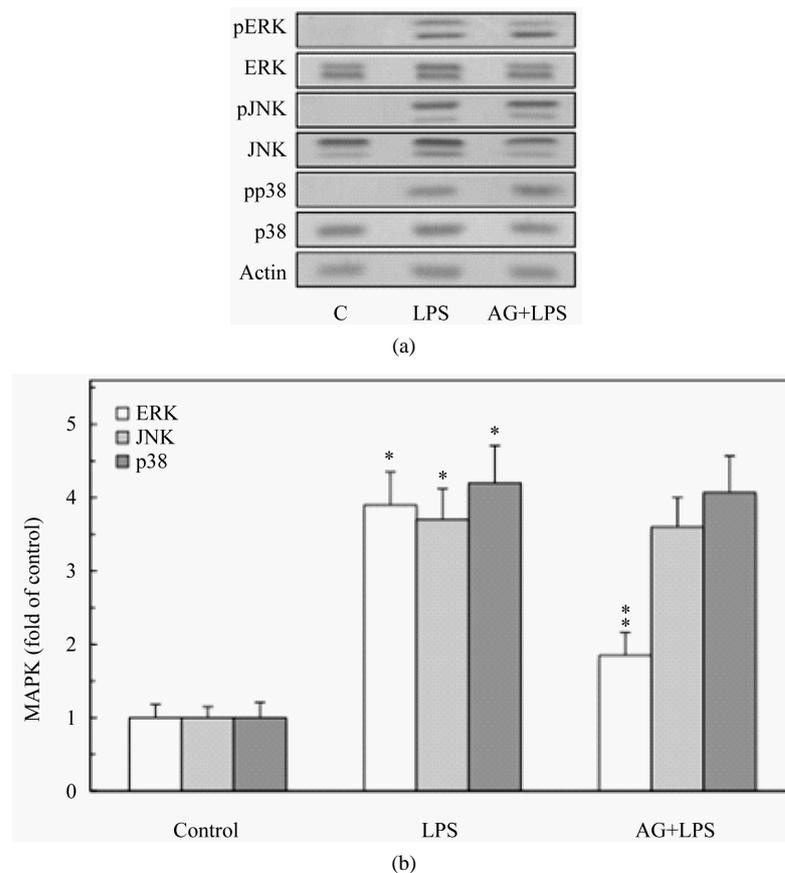


Figure 1. Effect of EGFR tyrosine kinase inhibitor, AG1478 (AG), on *P. gingivalis* LPS-induced changes in sublingual salivary gland acinar cell MAPK phosphorylation. The cells were preincubated with 0 or 5 μM of AG and incubated for 30 min with the LPS at 100 ng/ml. Cell lysates were analyzed by Western blotting for total and phosphorylated MAPKs (a), and the relative densities of phosphorylated MAPK proteins (b) are expressed as fold of control. Actin blot shows equal lane load. The data represent the mean \pm SD of four experiments. * $P < 0.05$ compared with that of control. ** $P < 0.05$ compared with that of LPS.

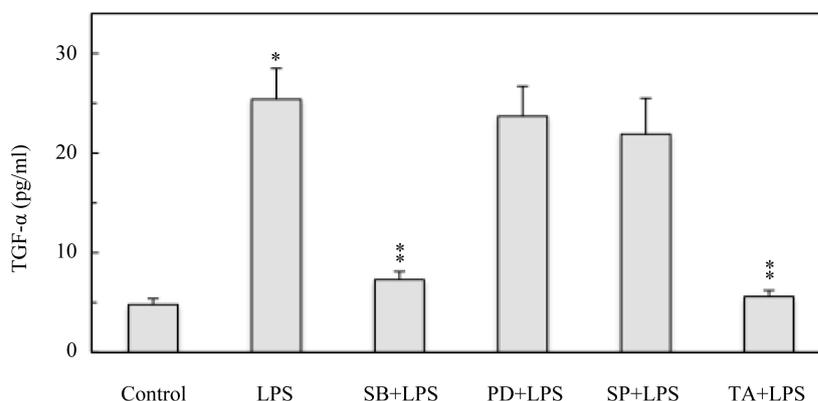


Figure 2. Effect of MAPK and TACE inhibitors on *P. gingivalis* LPS-induced changes in TGF- α release by sublingual salivary gland acinar cells. The cells, preincubated with 20 μ M p38 inhibitor, SB202190 (SB), or 30 μ M ERK inhibitor, PD98059 (PD), or 10 μ M JKN inhibitor, SP600125 (SP), or 10 μ M TACE inhibitor, TAPI-1 (TA), were treated with the LPS at 100 ng/ml and incubated for 4 h. TGF- α released into the medium was quantified by ELISA. Values represent the means \pm SD of five experiments. * $P < 0.05$ compared with that of control. ** $P < 0.05$ compared with that of LPS.

TAPI-1, while the inhibitors of ERK (PD98059) and JNK (SP600125) had no effect (Figure 2). These results, while attesting to the role of EGFR transactivation in the mediation of LPS-induced ERK activation, strongly suggest that p38 along with TACE are involved in TGF- α ectodomain shedding induced by *P. gingivalis* LPS.

In further assessment of the pathways involved in *P. gingivalis* LPS-induced EGFR activation via TGF- α ligand shedding, we examined the acinar cell EGFR tyrosine phosphorylation in the presence of neutralizing antibodies to the EGF and TGF- α , as well as the inhibitors of p38 and TACE (Figure 3). Western blot analysis revealed that pretreatment of the cells with an antibody to TGF- α led to a profound inhibition in the LPS-induced EGFR phosphorylation, whereas preincubation with an EGF-neutralizing antibody produced less apparent effect on the LPS-induced EGFR phosphorylation. This indicates that TGF- α plays a major role in the acinar cell EGFR activation in response to *P. gingivalis* LPS. The phosphorylation of EGFR by the LPS, moreover, was susceptible to suppression by p38 inhibitor, SB202190, as well as the inhibitor of TACE, TAPI-1 (Figure 3), thus attesting to the involvement of p38 and TACE in the TGF- α ligand release.

Accordingly, to ascertain the nature of the relationship between p38 and TACE, we examined the impact of the LPS on TACE activity in the presence of p38 MAPK inhibition. The results revealed that the effect of the LPS was manifested by a significant increase in the acinar cell TACE activity, whereas preincubation with p38 inhibitor, SB202190, elicited a marked reduction in the LPS-elicited effect (Figure 4). Moreover, the LPS-induced TACE activity was also subject to suppression by Rac1 inhibitor, NSC23766, thus suggesting that TACE activation shows dependence on Rac1 GTPase. Hence, to assess the character of the cross talk between p38 MAPK and Rac1 leading to the LPS-induced TACE activation, we investigated the requirement and selectivity of the interactions by co-immunoprecipitation. The results revealed that while TACE did not co-precipitate with p38 in the absence of the LPS stimulation, the two proteins were found in complex in both p38 (Figure 5) and TACE (Figure 6) immunoprecipitates following the acinar cell incubation with the LPS. Moreover, we found that association between the p38 and TACE was subject to interference by p38 inhibitor, SB202190, which not only blocked the LPS-induced p38 phosphorylation (Figure 5), but also interfered with the LPS-induced phosphorylation of TACE on Thr⁷³⁵, which is the reflection of TACE activation (Figure 6). Furthermore, the LPS-induced association between p38 and TACE was blocked by the inhibitor of Rac1, NSC23766, which interfered with the interaction between phosphorylated p38 and the TACE (Figure 5), as well as TACE phosphorylation (Figure 6). Together, these data suggest that the LPS-induced process of TACE activation by p38 MAPK requires Rac1 GTPase participation.

In further assessment of the role of Rac1 in *P. gingivalis* LPS-induced TACE activation in the acinar cells, we examined the requirement and selectivity of the membrane translocation of p38 MAPK and Rac1. The results of Western blot analysis of the whole cell lysates as well as the cell membrane fraction, using anti-p38 and anti-Rac1 antibody, revealed that incubation with the LPS resulted in recruitment of both p38 and Rac1 to the

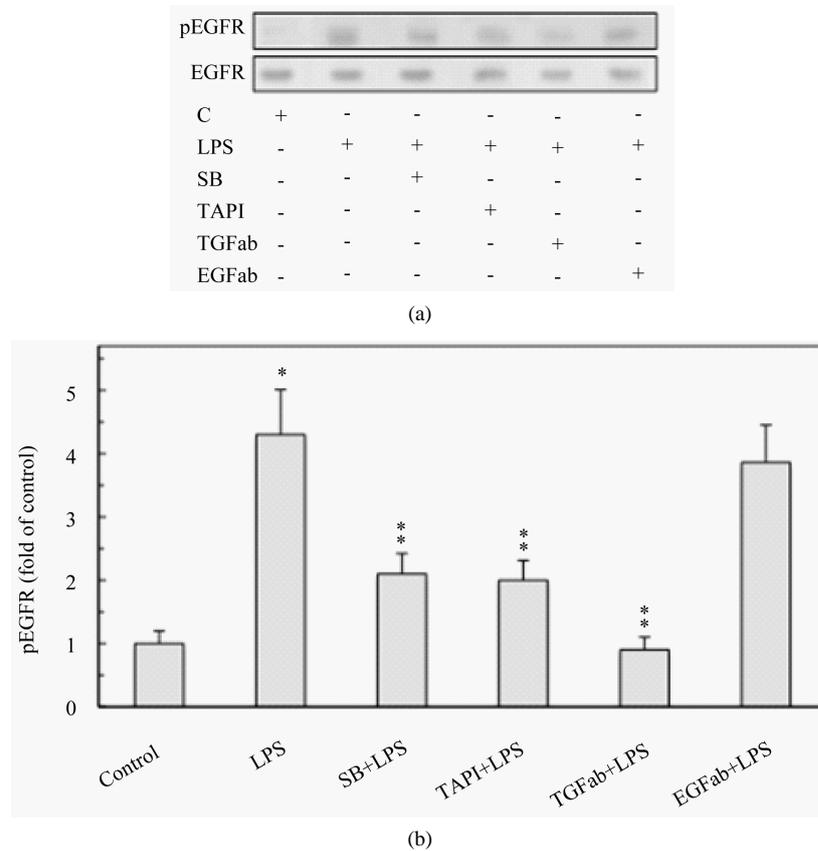


Figure 3. Effect of p38 and TACE inhibitors on *P. gingivalis* LPS-induced EGFR activation in sublingual salivary gland acinar cells. The cells, preincubated with 20 μ M of p38 inhibitor, SB202190 (SB), or 10 μ M TACE inhibitor, TAPI-1 (TAPI), or 4 μ g/ml neutralizing antibody against TGF- α (TGFab), or 4 μ g/ml neutralizing antibody against EGF (EGFab), were treated with the LPS at 100 ng/ml and incubated 10 min. Cell lysates were analyzed by Western blotting for total and phosphorylated EGFR (a). The relative density of pEGFR protein is expressed as fold of control (b), and the total EGFR was used as loading control. 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.

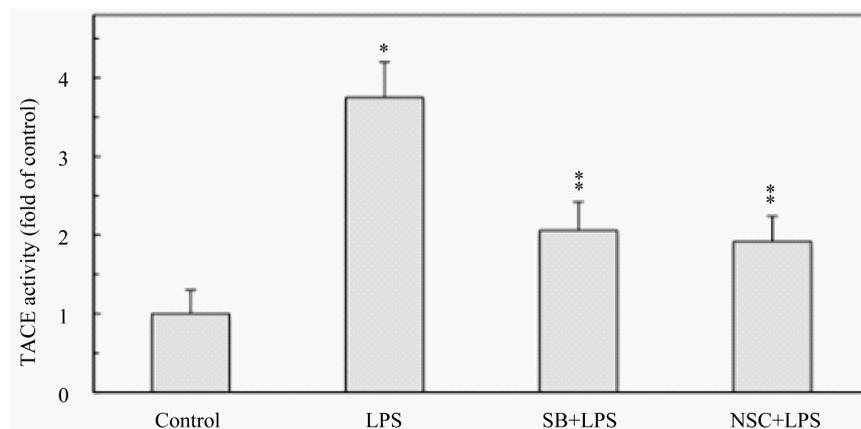


Figure 4. Effect of p38 and Rac1 inhibitors on *P. gingivalis* LPS-induced changes in sublingual salivary gland acinar cell expression of TACE activity. The cells, preincubated with 20 μ M p38 inhibitor, SB202190 (SB) or 50 μ M Rac1 inhibitor, NSC23766 (NSC), were treated with the LPS at 100 ng/ml and incubated for 10 min. 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.

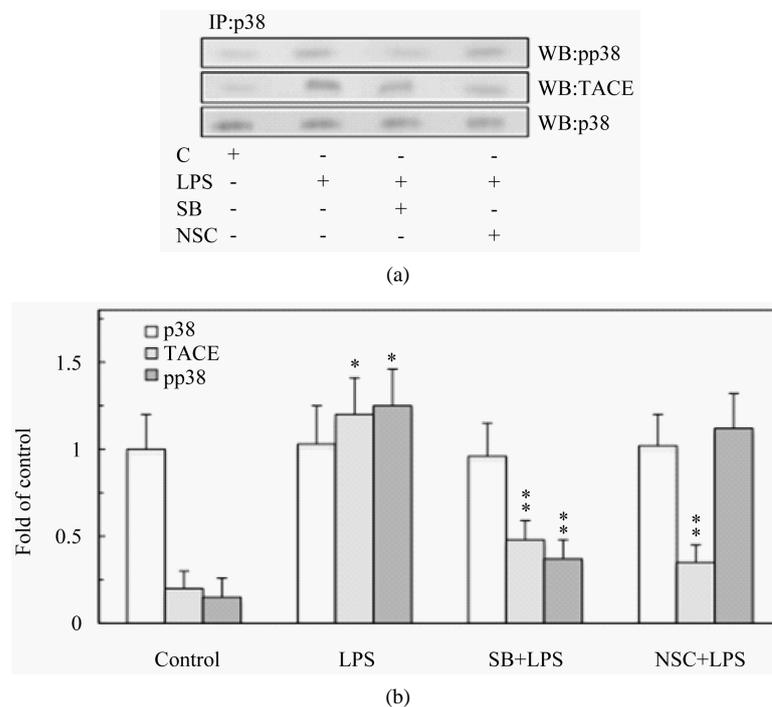


Figure 5. Impact of p38 and Rac1 inhibition on the changes induced by *P. gingivalis* LPS in sublingual salivary gland acinar cell p38 phosphorylation and its association with TACE. The cells, preincubated with 20 μ M SB202190 (SB), or 50 μ M NSC23766 (NSC), were treated with the LPS at 100 ng/ml and incubated for 1 h. Cell lysates were immunoprecipitated (IP) with anti-p38 antibody and immunoblotted (WB) with anti-p38 and anti-TACE antibody (a). The p38 immunoblots were also reblotted with anti-phospho-p38 antibody (pp38), and the relative densities of proteins (b) are expressed as fold of p38 control. 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.

membrane fraction (Figure 7). Further, we found that blocking p38 activation with SB202190, while affecting the membrane localization of p38 had no effect on the LPS-induced membrane recruitment of Rac1. On the other hand, blocking the Rac1 activation with NSC23766 led to a marked suppression in the membrane translocation of Rac1 as well as p38. Hence, we surmised that the recruitment of p38 for TACE activation by *P. gingivalis* LPS is dependent upon the membrane colocalization with Rac1.

4. Discussion

Oral mucosal responses to periodontopathic bacterium, *P. gingivalis* and its key endotoxin, LPS, are characterized by a massive rise in proinflammatory cytokine production, disturbances in cyclooxygenase (COX) and nitric oxide synthase (NOS) isozyme systems, and up-regulation in MAPK activation [2] [3] [6] [27]. Moreover, there are strong indications that LPS-induced proinflammatory signal propagation is also associated with EGFR transactivation [12]-[15]. Indeed, studies show that LPS-induced EGFR transactivation is an essential part of the inflammatory and repair process, and involves TACE-dependent release of inflammatory regulators, including EGFR ligand, TGF- α [12] [16]. As recent literature evidence has linked the course of TACE induction to LPS-elicited MAPK phosphorylation and Rac GTPase activation [12] [21], in the present study we investigated the nature of factors involved in *P. gingivalis* LPS-induced EGFR transactivation in salivary gland acinar cells.

Our data revealed that the exposure of the acinar cells to incubation with *P. gingivalis* LPS elicited a significant increase in ERK, JNK, and p38 MAPK phosphorylation, as well as produced a massive increase in the soluble TGF- α ligand release. However, while the LPS-induced ERK phosphorylation was prone to suppression by EGFR kinase inhibitor, AG1478, the extent of p38 and JNK phosphorylation remained essentially unaffected. Further, the LPS-induced increase in soluble TGF- α release was susceptible to inhibition by p38 inhibitor,

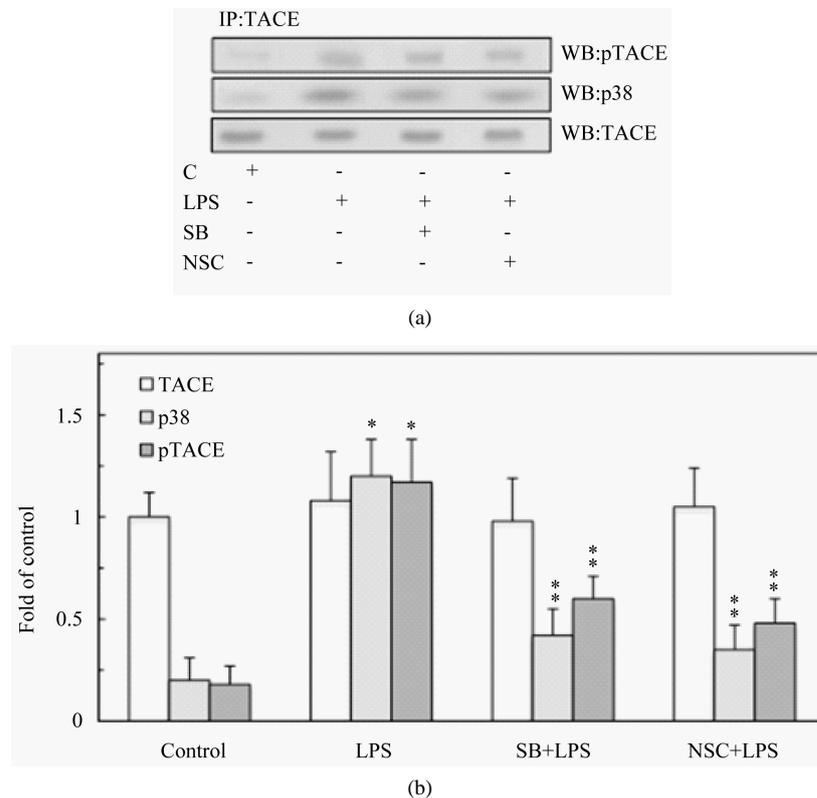


Figure 6. Effect of p38 and Rac1 inhibition on the changes induced by *P. gingivalis* LPS in sublingual salivary gland acinar cell TACE phosphorylation and its association with p38. The cells, preincubated with 20 μ M SB202190 (SB), or 50 μ M NSC23766 (NSC), were treated with the LPS at 100 ng/ml and incubated for 1 h. Cell lysates were immunoprecipitated (IP) with anti-TACE antibody and immunoblotted (WB) with anti-TACE and anti-p38 antibody (a). The TACE immunoblots were also reblotted with anti-phospho-TACE antibody (pTACE), and the relative densities of proteins (b) are expressed as fold of TACE control. 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.

SB202190, as well as the inhibitor of TACE, TAPI-1, whereas the inhibitors of ERK and JNK had no effect on the extent of TGF- α release. Moreover, examination of the acinar cell EGFR phosphorylation in the presence of neutralizing antibodies to the TGF- α and EGF revealed that *P. gingivalis* LPS-induced acinar cell EGFR transactivation occurs mainly in response to up-regulation in TGF- α ligand release. Indeed, the literature data indicate that TGF- α also plays the major role in LPS-induced EGFR phosphorylation in biliary epithelial cells [12] [16], and that metalloprotease, TACE, mediates ectodomain shedding of inflammatory cytokines and TGF- α family ligands [12] [16] [21] [28]. Moreover, our finding that *P. gingivalis* LPS-induced EGFR phosphorylation was susceptible to suppression by p38 inhibitor, SB202190 as well as the inhibitor of TACE, TAPI-1, attests to the involvement of p38 MAPK and TACE in the TGF- α ligand shedding.

Therefore, considering the evidence as to the involvement of Rac GTPase in p38 activation [23] [24] [29], we ascertained further the nature of the relationship between p38 MAPK and TACE in mediation of TGF- α release in response to *P. gingivalis* in the presence of p38 and Rac Inhibition. We observed a profound inhibitory effect on of the LPS-induced TACE activity not only in the presence of p38 inhibitor, SB202190, but also with the inhibitor of Rac1, NSC23766, thus pointing to the existence of crosstalk between Rac1 and p38 in TACE activation. This assertion is further supported by the results of co-immunoprecipitation analysis, in which the lysates of the acinar cells were immunoprecipitated with anti-p38 or TACE antibody, and subjected to probing with anti-phospho-p38 and anti-TACE, or anti-p38 and anti-phospho-TACE antibody. The results revealed that the association between the p38 and TACE was subject to interference by p38 inhibitor, SB202190, as well as the inhibitor of Rac1, NSC23766. Moreover, the inhibitor of p38, SB203190, not only blocked the p38 phosphorylation,

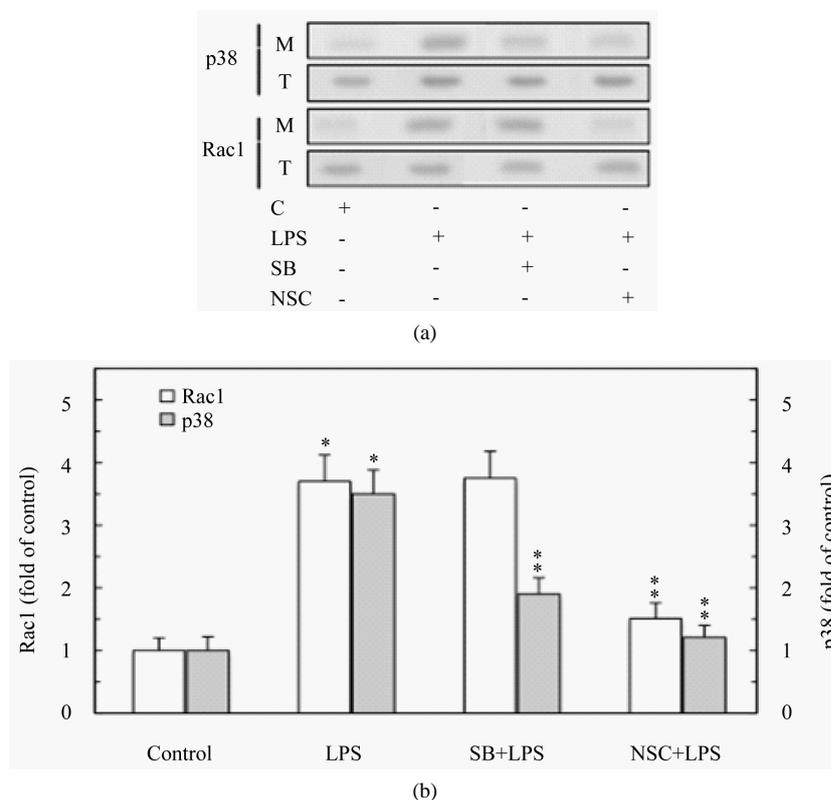


Figure 7. Impact of Rac and p38 inhibitors on the changes induced by *P. gingivalis* LPS in membrane recruitment of Rac1 and p38. The sublingual salivary gland acinar cells were treated with the LPS at 100 ng/ml in the presence of Rac1 inhibitor, NSC-23766 (NSC) at 50 μ M, or p38 inhibitor, SB202190 (SB) at 50 μ M, and incubated for 2 h. The lysates of whole cells (T) and the corresponding membrane (M) fractions were analyzed for p38 and Rac1 with specific antibodies (a). The relative densities of proteins are expressed as fold of control (b), and the total (T) p38 and Rac1 were used as loading control. 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.

but also interfered with the LPS-elicited TACE phosphorylation on Thr⁷³⁵, which is considered the target of p38-induced TACE activation [9] [21]. On the other hand, the inhibition of Rac1 with NSC23766, was found to interfere with the interaction between phosphorylated p38 and the TACE, as well as TACE phosphorylation. The fact that the activation of TACE by the LPS was susceptible to suppression by Rac1 inhibition led us to conclude that Rac1-GTP may be required for p38 localization with the membrane-anchored TACE for its activation. Indeed, studies show that in the absence of specific localization partners, the activated p38 preferentially accumulates in the cytosol [9].

Hence, taking into account that Rac proteins undergo regulatory control by GTP binding and membrane translocation for activation and hydrolysis to GDP for inactivation [30] [31], we evaluated the influence of *P. gingivalis* LPS on membrane recruitment of Rac1 and p38 translocation. Western blot analysis revealed that the effect of the LPS was reflected in the elevation in membrane recruitment of both p38 and Rac1, whereas inhibition of the p38 activation with SB202190 while affecting p38 translocation had no effect on the LPS-induced membrane translocation of Rac1. However, blocking the Rac1 activation with NSC23766 produced a marked suppression in the membrane translocation of Rac1 as well as p38. Therefore, we surmised that up-regulation by *P. gingivalis* LPS in Rac1 membrane translocation plays a major role in recruitment of the activated p38 to the cytosolic aspect of the membrane for TACE activation through phosphorylation of its cytoplasmic domain on Thr⁷³⁵. This assertion is supported by the findings demonstrating that membrane proximity of Rac1 is also essential for the recruitment and the enhancement in PLC γ 2 activation [25] [32].

Although the detailed functional paradigm of the role of Rac1 in p38 membrane recruitment and TACE acti-

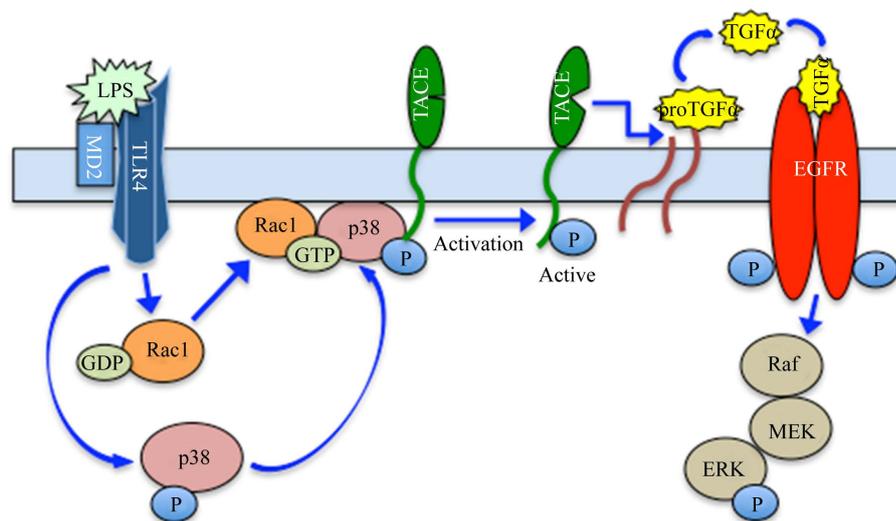


Figure 8. Schematic representation of the proposed signaling mechanism leading to *P. gingivalis* LPS-induced EGFR transactivation by TGF- α . Ligation by the LPS of salivary gland acinar cell TLR4 triggers up-regulation in p38 MAPK phosphorylation, as well as prompts the activation and translocation of Rac1-GTP to the vicinity of membrane-anchored latent TACE. This, in turn, leads to the recruitment and localization of p38 to the complex, and TACE activation through phosphorylation. The subsequent cleavage by the TACE of pro-TGF- α results in a soluble TGF- α ligand that binds to and activates EGFR, thus causing the increased signaling to ERK/MAPK.

vation requires further defining, our findings are consistent with the with the studies suggesting that Rac GTPases function as sophisticated modulators of a remarkably complex and diverse range of cellular processes, including regulation of p38 expression, PLC γ 2 membrane localization, and metalloproteinase activation [9] [25] [28] [30]-[34].

5. Conclusion

The data presented in this report reveal that stimulation of salivary gland acinar cells with *P. gingivalis* LPS leads to p38 MAPK-dependent increase in TGF- α ligand shedding and EGFR transactivation. We further show that the LPS-induced TGF- α ectodomain shedding and TGF α transactivation involve the activation of membrane-anchored TACE through phosphorylation by p38 that requires Rac1 participation (**Figure 8**). Moreover, we demonstrate that the LPS-elicited Rac1 membrane translocation serves as an essential platform for the localization of p38 with the TACE, its activation through phosphorylation on Thr⁷³⁵, and subsequent increase in TGF- α ectodomain shedding for EGFR transactivation.

References

- [1] Nonnenmacher, C., Mutters, R. and deJacoby, L.F. (2001) Microbiological Characteristics of Subgingival Microbiota in Adult Periodontitis, Localized Juvenile Periodontitis and Rapidly Progressive Periodontitis Subjects. *Clinical Microbiology and Infection*, **7**, 213-217. <http://dx.doi.org/10.1046/j.1469-0691.2001.00210.x>
- [2] Wang, P.L. and Ohura, K. (2002) *Porphyromonas gingivalis* Lipopolysaccharide Signaling in Gingival Fibroblasts-CD14 and Toll-Like Receptors. *Critical Reviews in Oral Biology and Medicine*, **13**, 132-142. <http://dx.doi.org/10.1177/154411130201300204>
- [3] Dareau, R.P., Arbabi, S., Gracia, I., Bainbridge, B. and Maier, R.V. (2002) *Porphyromonas gingivalis* Lipopolysaccharide Is Both Agonist and Antagonist for p38 Mitogen-Activated Protein Kinase Activation. *Infection and Immunity*, **70**, 1867-1873. <http://dx.doi.org/10.1128/IAI.70.4.1867-1873.2002>
- [4] Slomiany, B.L. and Slomiany, A. (2007) Alteration by Indomethacin in Proinflammatory Consequences of Salivary Gland Cytosolic Phospholipase A₂ Activation by *Porphyromonas gingivalis*: Role of Leptin. *Journal of Applied Research*, **7**, 127-136.
- [5] Kawai, T. and Akira, S. (2010) The Role of Pattern-Recognition Receptors in Innate Immunity: Update on Toll-Like

- Receptors. *Nature Immunology*, **11**, 373-384. <http://dx.doi.org/10.1038/ni.1863>
- [6] Slomiany, B.L. and Slomiany, A. (2011) Ghrelin-Induced cSrc Activation through Constitutive Nitric Oxide Synthase-Dependent S-Nitrosylation in Modulation of Salivary Gland Acinar Cell Inflammatory Responses to *Porphyromonas gingivalis*. *American Journal of Molecular Biology*, **2**, 43-51. <http://dx.doi.org/10.4236/ajmb.2011.12006>
- [7] Carpenter, S. and O'Neill, L.A.J. (2009) Recent Insights into the Structure of Toll-Like Receptors and Post-Translational Modifications of Their Associated Signaling Proteins. *Biochemical Journal*, **422**, 1-10. <http://dx.doi.org/10.1042/BJ20090616>
- [8] Garrington, P.T. and Johnson, G.L. (1999) Organization and Regulation of Mitogen-Activated Protein Kinases Signaling Pathways. *Current Opinion in Cell Biology*, **11**, 211-218. [http://dx.doi.org/10.1016/S0955-0674\(99\)80028-3](http://dx.doi.org/10.1016/S0955-0674(99)80028-3)
- [9] Cuadrado, A. and Nebreda, A.R. (2010) Mechanism and Function of p38 MAPK Signaling. *Biochemical Journal*, **429**, 403-417. <http://dx.doi.org/10.1042/BJ20100323>
- [10] Grishin, A.V., Wang, J., Potoka, D.A., et al. (2006) Lipopolysaccharide Induces Cyclooxygenase-2 in Intestinal Epithelium via a Non-Canonical p38 MAPK Pathway. *Journal of Immunology*, **176**, 580-588. <http://dx.doi.org/10.4049/jimmunol.176.1.580>
- [11] Slomiany, B.L. and Slomiany, A. (2013) Involvement of p38 MAPK-Dependent Activator Protein (AP-1) Activation in Modulation of Gastric Mucosal Inflammatory Responses to *Helicobacter pylori* by Ghrelin. *Inflammopharmacology*, **21**, 67-78. <http://dx.doi.org/10.1007/s10787-012-0141-9>
- [12] Finzi, L., Shao, M.X.G., Paye, F., Housset, C. and Nadel, J.A. (2009) Lipopolysaccharide Initiates a Positive Feedback of Epidermal Growth Factor Receptor Signaling by Prostaglandin E2 in Human Biliary Carcinoma Cells. *The Journal of Immunology*, **182**, 2269-2276. <http://dx.doi.org/10.4049/jimmunol.0801768>
- [13] Hsu, D., Fukata, M., Hernandez, Y.G., Sotolongo, J.P., Goo, T., Maki, J., et al. (2010) Toll-Like Receptor 4 Differentially Regulates Epidermal Growth Factor-Related Growth Factors in Response to Intestinal Mucosal Injury. *Laboratory Investigation*, **90**, 1295-1305. <http://dx.doi.org/10.1038/labinvest.2010.100>
- [14] McElroy, S.J., Hobbs, S., Kallen, M., Tejera, N., Rosen, M.J., Grishin, A., et al. (2012) Transactivation of EGFR by LPS Induces COX-2 Expression in Enterocytes. *PLoS ONE*, **7**, e38373. <http://dx.doi.org/10.1371/journal.pone.0038373>
- [15] Slomiany, B.L. and Slomiany, A. (2013) Role of EGFR Transactivation in the Amplification of *Helicobacter pylori*-Elicited Induction in Gastric Mucosal Expression of COX-2 and iNOS. *OA Inflammation*, **1**, 1. <http://dx.doi.org/10.13172/2052-787X-1-1-412>
- [16] Trussoni, C.E., Tabibian, J.H., Splinter, P.L. and O'Hara, S.P. (2015) Lipopolysaccharide (LPS)-Induced Biliary Epithelial Cell NRas Activation Requires Epidermal Growth Factor Receptor (EGFR). *PLoS ONE*, **10**, e0125793. <http://dx.doi.org/10.1371/journal.pone.0125793>
- [17] Carpenter, G. (1999) Employment of the Epidermal Growth Factor Receptor in Growth Factor-Independent Signaling Pathways. *Journal of Cell Biology*, **146**, 697-702. <http://dx.doi.org/10.1083/jcb.146.4.697>
- [18] Ohsu, H., Dempsey, P. and Eguchi, S. (2006) ADAMs as Mediators of EGF Receptor Transactivation by G Protein-Coupled Receptors. *American Journal of Cell Physiology*, **291**, C1-C10. <http://dx.doi.org/10.1152/ajpcell.00620.2005>
- [19] Slomiany, B.L. and Slomiany, A. (2004) *Porphyromonas gingivalis* Lipopolysaccharide-Induced Up-Regulation in Endothelin-1 Interferes with Salivary Mucin Synthesis via Epidermal Growth Factor Receptor Transactivation. *IUBMB Life*, **56**, 601-607. <http://dx.doi.org/10.1080/15216540400020361>
- [20] Bergin, D.A., Greene, C.M., Sterchi, E.E., Kenna, C., Geraghty, P., Belaouaj, A., et al. (2008) Activation of the Epidermal Growth Factor Receptor (EGFR) by a Novel Metalloprotease Pathway. *Journal of Biological Chemistry*, **283**, 31736-31744. <http://dx.doi.org/10.1074/jbc.M803732200>
- [21] Xu, P. and Derynck, R. (2010) Direct Activation of TACE-Mediated Ectodomain Shedding by p38 MAP Kinase Regulates EGF Receptor-Dependent Cell Proliferation. *Molecular Cell*, **37**, 551-566. <http://dx.doi.org/10.1016/j.molcel.2010.01.034>
- [22] Slomiany, B.L. and Slomiany, A. (2000) Aspirin Ingestion Impairs Oral Mucosal Ulcer Healing by Inducing Membrane-Bound Tumor Necrosis Factor- α Release. *IUBMB Life*, **50**, 391-395.
- [23] Kong, L. and Ge, B.X. (2008) MyD88-Independent Activation of a Novel Actin-Cdc42/Rac Pathway Is Required for Toll-Like Receptor-Stimulated Phagocytosis. *Cell Research*, **18**, 745-755. <http://dx.doi.org/10.1038/cr.2008.65>
- [24] Yao, H.Y., Chen, L.H., Wang, J.Y., Wang, J.R., Chen, J.Q., Xie, Q.M., et al. (2011) Inhibition of Rac Activity Alleviates Lipopolysaccharide-Induced Acute Pulmonary Injury in Mice. *Biochimica et Biophysica Acta*, **1810**, 666-674. <http://dx.doi.org/10.1016/j.bbagen.2011.03.020>
- [25] Slomiany, B.L. and Slomiany, A. (2015) *Porphyromonas gingivalis*-Induced GEF Dock180 Activation by Src/PKC δ -Dependent Phosphorylation Mediates PLC γ 2 Amplification in Salivary Gland Acinar Cells: Effect of Ghrelin. *Journal of Biosciences and Medicines*, **3**, 66-77. <http://dx.doi.org/10.4236/jbm.2015.37008>

- [26] Slomiany, A. and Slomiany, B.L. (2012) Phosphatidylglycerol-Containing ER-Transport Vesicles Built and Restore Outer Mitochondrial Membrane and Deliver Nuclear DNA Translation Products to Generate Cardiolipin in the Inner Mitochondrial Membrane. *Advances in Biological Chemistry*, **2**, 132-145. <http://dx.doi.org/10.4236/abc.2012.22016>
- [27] Slomiany, B.L. and Slomiany, A. (2010) Suppression by Ghrelin of *Porphyromonas gingivalis*-Induced Constitutive Nitric Oxide Synthase S-Nitrosylation and Apoptosis in Salivary Gland Acinar Cells. *Journal of Signal Transduction*, **2010**, Article ID: 643642. <http://dx.doi.org/10.1155/2010/643642>
- [28] Kenny, P.A. and Bisseli, M.J. (2007) Targeting TACE-Dependent EGFR Ligand Shedding in Breast Cancer. *Journal of Clinical Investigations*, **117**, 337-345. <http://dx.doi.org/10.1172/JCI29518>
- [29] Li, C., Hu, Y., Sturm, G., Wick, G. and Xu, Q. (2000) Ras/Rac-Dependent Activation of p38 Mitogen-Activated Protein Kinase in Smooth Muscle Cells Stimulated by Cyclic Strain Stress. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **20**, e1-e9. <http://dx.doi.org/10.1161/01.ATV.20.3.e1>
- [30] Wennerberg, K., Rossman, K.L. and Der, C.J. (2005) The Ras Superfamily at Glance. *Journal of Cell Science*, **118**, 843-846. <http://dx.doi.org/10.1242/jcs.01660>
- [31] Parri, M. and Chiarugi, P. (2010) Rac and Rho GTPases in Cancer Cell Motility Control. *Cell Communication and Signaling*, **8**, 23-37. <http://dx.doi.org/10.1186/1478-811X-8-23>
- [32] Walliser, C., Retlich, M., Harris, R., Everett, K.L., Josephs, M.B., Vatter, P., *et al.* (2008) Rac Regulates Its Effector Phospholipase C γ 2 through Interaction with a Split Pleckstrin Homology Domain. *Journal of Biological Chemistry*, **283**, 30351-30362. <http://dx.doi.org/10.1074/jbc.M803316200>
- [33] Slomiany, B.L. and Slomiany, A. (2015) Mechanism of Rac1-Induced Amplification in Gastric Mucosal Phospholipase C γ 2 Activation in Response to *Helicobacter pylori*: Modulatory Effect of Ghrelin. *Inflammopharmacology*, **23**, 101-109. <http://dx.doi.org/10.1007/s10787-015-0231-6>
- [34] Murai, T., Miyauchi, T., Yanagida, T. and Sako, Y. (2006) Epidermal Growth Factor-Regulated Activation of Rac GTPase Enhances CD44 Cleavage by Metalloprotease Disintegrin ADAM10. *Biochemical Journal*, **395**, 65-71. <http://dx.doi.org/10.1042/BJ20050582>