

Role of α -Tubulin Acetylation and Protein Kinase D2 Ser/Tyr Phosphorylation in Modulation by Ghrelin of *Porphyromonas gingivalis*-Induced Enhancement in Matrix Metalloproteinase-9 (MMP-9) Secretion by Salivary Gland Cells

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Abstract

Matrix metalloproteinase-9 (MMP-9) is a glycosylated endopeptidase, and hence its processing between the endoplasmic reticulum (ER), Golgi and trans-Golgi (TGN) network remains under a strict control of factors that affect the microtubule (MT) stabilization, and the recruitment and activation of coat and cargo proteins, including ADP-ribosylation factors (Arfs) and protein kinase D (PKD). Here, we report on the factors implicated in the regulation of MMP-9 secretion by salivary gland acinar cells in response to *P. gingivalis* LPS, and the effect of hormone, ghrelin. We show that the LPS-elicited induction in MMP-9 secretion is associated with the increase in α -tubulin acetylation and the enhancement in MT stabilization, while the modulatory effect of ghrelin is reflected in a decrease in α -tubulin acetylation. Further, the effect of the LPS occurs in concert with up-regulation in Arf-guanine nucleotide exchange factor (GEF)-mediated Arf1 activation and the TGN recruitment of PKD2, while ghrelin exerts the modulatory effect on Arf-GEF activation. Moreover, we reveal that the LPS-induced up-regulation in MMP-9 secretion is reflected in a marked increase in PKC δ -mediated PKD2 phosphorylation on Ser, while the modulatory effect of ghrelin is manifested by the SFK-PTKs-dependent phosphorylation of PKD2 on Tyr. The findings demonstrate that MT stabilization along with Arf-GEF-mediated Arf1/PKD2 activation play a major role in *P. gingivalis* LPS-induced up-regulation in salivary gland acinar cell MMP-9 secretion, and point the modulatory mode of action by ghrelin.

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Keywords

***Porphyromonas gingivalis*, Oral Mucosa, Ghrelin, MMP-9, α -Tubulin Acetylation, Arf1, PKD2 Ser/Tyr Phosphorylation**

1. Introduction

Porphyromonas gingivalis 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 [1]-[3]. The oral mucosal reaction to *P. gingivalis* triggers a pattern of inflammatory responses characterized by the disturbances in nitric oxide synthase and cyclooxygenase systems, up-regulation in EGFR and MAPK activation, and the induction in secretion of the glycosylated metalloproteinase-9 (MMP-9) [4]-[8]. Like the secretion of other salivary proteins and glycoproteins, the processing of MMP-9 along the secretory pathway relies heavily on the co- and posttranslational modifications acquired during the protein transit between the endoplasmic reticulum (ER), Golgi, and trans-Golgi network (TGN) [9] [10]. Moreover, the trafficking of MMP-9 cargo occurs in concert with the changes in cytoskeleton microtubule (MT) stabilization, and remains under a strict control of factors that affect the membrane recruitment and activation of various coat and cargo proteins, including ADP-ribosylation factors (Arfs) and protein kinase D (PKD), [11]-[15].

Indeed, studies show that cytoskeleton plays an important role not only in cell division and motility, but also is involved in the intracellular transport and positioning of organelles and vesicles, and its largest component, MTs, have been directly implicated in the regulation of MMP-9 secretion in several different cell systems [11] [15]-[18]. These diverse aspects of MT specialization arise from the accumulation of a variety of posttranslational modifications on the tubulin subunits, one of which is α -tubulin acetylation, which is known to affect the binding of MT-associated proteins and cause the enhanced MT stabilization associated with the increased in MMP-9 secretion [11] [15].

Equally important in controlling the secretory cargo trafficking and sorting through Golgi-TNG is the role of small GTP-binding proteins of the ADP-ribosylation factor (Arf) family [14] [19]. The Arfs are active when bound to GTP and inactive when bound to GDP, and the activation status is controlled by the guanine nucleotide exchange factors (GEFs) [19] [20]. All six members of the Arf family are characterized by presence the N-terminal myristoylate tail, which allows their association with membranes, and based on their amino acid sequence homology are grouped into three classes. Of these, class I and class II Arfs are associated with the Golgi apparatus and implicated in the regulation of cell secretory function, while class III Arf, Arf6, functions in the endocytic system [13] [21]. While inactive Arfs are cytosolic, the stimulus activated GTP-bound class I Arfs (Arf1, Arf2, and Arf3) rapidly translocate to the Golgi membrane compartments and assume the principal role in the recruitment of various cytosolic coat and cargo adaptor proteins, exchange factors, and lipid modifying enzymes, including phospholipase D and phosphatidylinositol 4-kinase, that are essential for regulation of ER-to-Golgi traffic [12] [14].

Of particular significance to the secretory cargo processing is the recently established involvement of Arf1 in the recruitment from the cytoplasm to the TGN of PKD [21]-[23]. The PKD family includes three closely related serine/threonine kinases (PKD1, PKD2, and PKD3), that play a crucial role in the regulation of Golgi structure and function by phosphorylation of the TGN-localized substrates required for subsequent shedding of cargo-containing vesicles [12] [23]. Upon cell stimulation, the members of PKD family are first recruited from the cytosol to the diacylglycerol-rich membrane environment, including the Golgi complex, via interaction with Arf-GTP and then undergo activation by protein kinase C (PKC)-mediated transphosphorylation of their activation loop on Ser⁷⁴⁴, followed by autophosphorylation on Ser⁷⁴⁸ [12] [24]. There are also indications that in addition to PKC-mediated activation loop phosphorylation on Ser, the PKD activation may also involve Src-mediated phosphorylation on Tyr [12] [25]. Thus, PKD along with PKC form a key convergence and integration node for signals triggered by Toll-like receptor (TLR) activation as well as those arising through G protein-coupled receptor (GPCR) stimulation.

Indeed, PKD/PKC axis is not only the signaling target of LPS-induced TLR4 activation that defines the extent of inflammatory response, but also plays a major role in modulation of inflammation by a peptide hormone,

ghrelin [26]-[28]. This endogenous ligand for the growth hormone secretagogue receptor type 1a (GHS-R1a), initially isolated from the stomach, and later identified in saliva and the acinar cells of salivary glands, has emerged recently as a principal modulator of the local inflammatory responses to bacterial infection [29]-[32].

As there are indications that *P. gingivalis* LPS along with the elevated levels of MMP-9, routinely detected in the circulation of periodontal disease patients, may be responsible for a decrease in cardiac function and cardiovascular disease [3] [33], while the anti-inflammatory effect of ghrelin observed during progressive heart failure has been linked to the suppression in MMP-9 expression [34], in this study we investigated the factors implicated in the regulation of MMP-9 secretion by salivary gland acinar cells in response to *P. gingivalis* LPS and ghrelin.

2. Materials and Methods

2.1. Salivary Gland Cell Incubation

The acinar cells of rat sublingual salivary gland were suspended in five volumes of ice-cold Dulbecco's modified (Gibco) Eagle's minimal essential medium (DMEM), supplemented with fungizone (50 µg/ml), penicillin (50 U/ml), streptomycin (50 µg/ml), and 10% fetal calf serum, and gently dispersed by trituration with a syringe and settled by centrifugation [35]. 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 [35]. *P. gingivalis* used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 33277 [27]. In the experiments evaluating the effect of ghrelin (rat) and wide spectrum PKC inhibitor, GF109203X (Sigma), Src family protein tyrosine kinase (SFK-PTK) selective inhibitor, PP2, ER-to-Golgi transport inhibitor, Brefeldin A (BFA), microtubule stabilizing agent, tubacin (Tb) and microtubule destabilization agent, nocodazole (Noc), (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. 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 [8] [15]. The spent acinar cell media, collected by centrifugation, were mixed with Laemmli buffer, lacking 2-mercaptoethanol, and subjected to electrophoresis using 8% SDS-PGE 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 [15], 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. Arf1-GTP Activity Assay

The measurement of Arf1 activation was carried out with Arf1 Pull-Down and Detection Kit (EMD Millipore). The salivary gland acinar cells from the control and experimental treatments were lysed in the lysis/binding/wash buffer (25 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 5 mM MgCl₂, 1% Nonidet P-40, and 5% glycerol), 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 the beads were incubated with a 100 µg of GST-GGA3-PBD proteins PAK1 PBD-agarose for 1 h at 4°C. The beads were washed three times in the lysis buffer, resuspended in Laemmli reducing sample buffer, resolved on 12% SDS-PAGE, and immunoblotted for GTP-bound Arf1 using anti-Arf1 antibody.

2.4. Golgi Membranes

An intact Golgi apparatus fraction of the salivary gland acinar cells was prepared essentially according to the previously described procedure [36]. The acinar 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, 5 mM EDTA, 25 mM KCl, 1 mM PMSF, 10 mM aprotinin, 10 mM leupeptin, and 10 mM chymostatin. The homogenate was filtered through a layer of gauze and centrifuged at 10,000 rpm for 20 min. The supernatant was collected, layered onto 1.25 M sucrose, buffered with 10 mM Tris-HCl, pH 7.4, and centrifuged at 25,000 rpm for 90 min in SW28 rotor. The crude membranes sedimented above the interface with the sucrose layer were collected by aspiration, adjusted to 1.2 M sucrose, and overlaid with 1.1, 1.0, and 0.5 M buffered (10 mM Tris-HCl, pH 7.4) sucrose. Following centrifugation for 2.5 h at 25,000 rpm in the SW28 rotor, the Golgi-enriched membranes were collected from the 0.5/1.0 sucrose interface.

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 [5]. 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 cell lysates as well as those of Golgi membrane preparations were then used either for immunoblots analysis, or proteins of interest were subjected to immunoprecipitation by incubation 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 PKD2, phospho-PKD2 (Ser⁸⁷⁶), Arf1, and phosphotyrosine (4G10) (EMD Millipore), PKD1, α -tubulin and acetylated α -tubulin (Sigma).

2.6. Data Analysis

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

Although salivary glands are recognized as the principal contributor of proteins, glycoproteins and mucins found in saliva, their acinar cells are also known to respond rapidly to microbial challenge by the increased in nitric oxide and proinflammatory cytokine production, and the enhancement in secretion of the glycosylated metalloproteinase, MMP-9 [3] [37] [38]. As the elaboration of glycosylated proteins relies on their processing along the ER-to-Golgi secretory pathway, in this study we investigated the nature of factors implicated in the regulation of salivary gland acinar cell MMP-9 secretion in response to *P. gingivalis* stimulation, as well as the influence of peptide hormone, ghrelin, on these processes. Employing zymography and Western blot analyses of the extracellular levels of MMP-9 released into the incubation medium by the acinar cells exposed to incubation with LPS of periodontopathic bacterium, *P. gingivalis*, we showed that the effect of the LPS was manifested in a marked up-regulation in MMP-9 secretion, while preincubation with ghrelin elicited a significant reduction in the LPS effect (Figure 1). Furthermore, we found that the stimulatory effect of the LPS on MMP-9 secretion was susceptible to suppression by a well-known inhibitor of protein glycosylation, Brefeldin A, which also produced an additive result on the inhibitory effect of ghrelin. These data, thus suggest that the LPS as well as ghrelin exert their effect on MMP-9 elaboration at the level of ER-to-Golgi processing.

Next, following on the established evidence as to the involvement of cytoskeleton in the secretory vesicle trafficking [16], we have evaluated the impact of the LPS and ghrelin on MMP-9 secretion in the presence of pharmacological agents affecting microtubule (MT) stability. The results of Western blot analysis revealed that pretreatment of the acinar cells with MT destabilizing agent, nocodazole, led not only to a marked inhibition in MMP-9 secretion induced by the LPS, but also produced further reduction in the secretion of MMP-9 elicited by ghrelin, while the effect of MT stabilizing agent, tubacin, was reflected in the enhancement in the LPS-induced

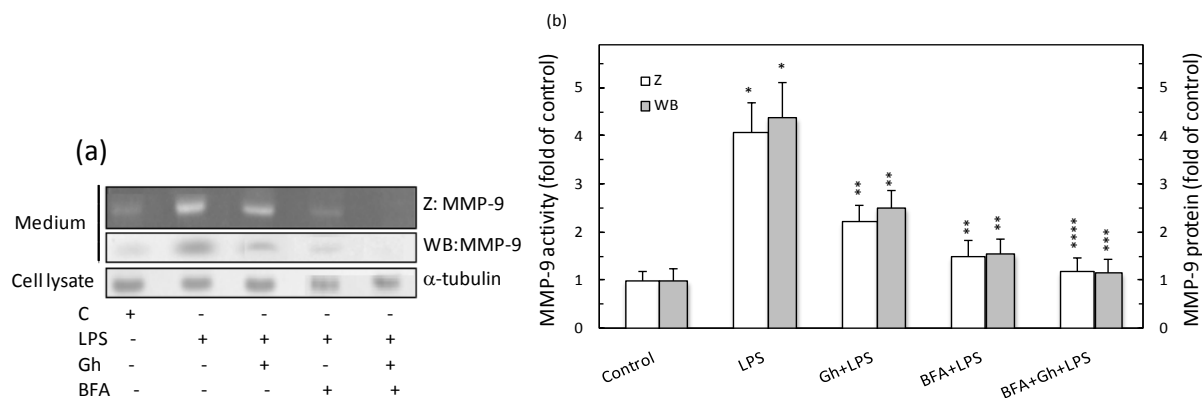


Figure 1. Effect of Brefeldin A (BFA) on the changes induced by *P. gingivalis* LPS and ghrelin (Gh) in MMP-9 secretion by sublingual salivary gland acinar cells. The cells, preincubated with BFA at 5 μ g/ml, were treated with 0.5 μ g/ml Gh, and incubated for 16 h in the presence of 100 ng/ml LPS. Following centrifugation, the cell culture supernatants were collected and subjected to gelatin zymography (Z) or immunoblotting (WB) for MMP-9, while the total cell lysate was analyzed for α -tubulin, used as a lane loading control (a). The relative level of MMP-9 protein and its gelatinolytic activity are expressed as fold of control (b). 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. *** P < 0.05 compared with that of Gh + LPS.

MMP-9 secretion (**Figure 2**). Tubacin, furthermore, exerted countering effect on the suppression in MMP-9 secretion observed in the presence of ghrelin. Moreover, we have also assessed the relationship between the degree of MT stabilization and MMP-9 secretion by monitoring the level of acetylated α -tubulin, a marker of MT stability. As shown in **Figure 2**, a significant increase in the acetylated α -tubulin level was attained following the acinar cell stimulation with the LPS and tubacin, while the effect of ghrelin was manifested in a marked reduction in the LPS-induced α -tubulin acetylation. Together, these results strongly infer that the enhancement in MT stabilization plays an important role in the salivary gland acinar cell secretion of MMP-9 in response to *P. gingivalis* LPS challenge, and that the effect of ghrelin is associated with MT destabilization.

As the regulation of ER-to-Golgi and TGN trafficking relays on the sequential activation and inactivation of a small GTPase of the ADP-ribosylation factor family, Arf1 [14], in further assessment of the mechanism of MMP-9 secretion, we have examined the influence of *P. gingivalis* LPS and ghrelin on the acinar cell Arf1 activation. The results of Arf1 GTPase activation assay revealed that effect of the LPS was manifested in the elevation in Arf1-GTP, which paralleled that of the rise in MMP-9 secretion, while the effect of ghrelin was reflected in the reduction in the LPS-induced Arf1-GTP formation as well as MMP-9 secretion (**Figure 3**). Further, we found that preincubation of the acinar cells with Brefeldin A led to a significant suppression in the LPS-induced MMP-9 secretion as well as Arf1-GTP formation, thus implicating *P. gingivalis* LPS as well as ghrelin in modulation of MMP-9 secretion and Arf1-GTP activation at the level of the ER-to-Golgi trafficking. Moreover, as Arf1 activation occurs through the exchange of GDP for GTP that is catalyzed by guanine nucleotide exchange factors (GEFs), we have followed the leads as to role of Src/PKC in Arf-GEF activation. The results of Arf-GEF activity assays revealed that the effect of the LPS was manifested by a significant increase in the acinar cell GEF activation, whereas preincubation with ghrelin elicited reduction in the LPS effect (**Figure 4**). Moreover, the activation of Arf-GEF by the LPS was susceptible to suppression not only by Brefeldin A, but also to a wide spectrum PKC inhibitor, GF109203X, as well as the inhibitor of SFK-PTKs, PP2, thus affirming the involvement of SFK-PTKs and PKC, identified earlier as PKC δ [39], in the processes of BFA-susceptible salivary gland Arf-GEF activation.

Taking into account the role of Arf in the secretory product trafficking through Golgi by affecting the membrane recruitment and activation of various coat and cargo proteins, including PKDs required for phosphorylation of Golgi-localized substrates [21], we next assessed the acinar cell Arf1 requirement with respect to PKD recruitment. For this, the cell lysates were immunoprecipitated with anti-Arf1 antibody and subjected to analysis using anti-Arf1, anti-PKD1 and anti-PKD2 antibody (**Figure 5**). We found that while Arf1 was present in the complex with PKD2 following the cell incubation with the LPS as well as ghrelin, neither the LPS nor ghrelin promoted the association of Arf1 with the PKD1, thus suggesting the involvement of PKD2 in the processes of MMP-9 cargo trafficking in the Golgi. It is also pertinent to note that the LPS-induced association of Arf1 with

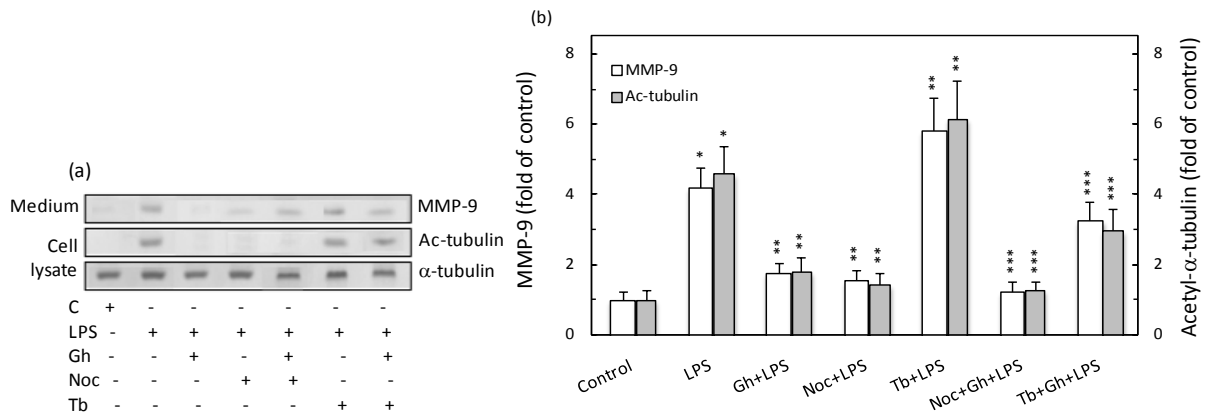


Figure 2. Effect of *P. gingivalis* LPS and ghrelin (Gh) on MMP-9 secretion and α -tubulin acetylation in the salivary gland acinar cells. The cells, preincubated with 10 μ M of microtubule destabilizing agent, nocodazole (Noc) or 5 μ M of microtubule stabilizing agent, tubacin (Tb), were treated with 0.5 μ g/ml Gh, and incubated for 16 h in the presence of 100 ng/ml LPS. The cell culture supernatants were collected and subjected to Immunoblotting for MMP-9, while the total cell lysate was analyzed for acetylated α -tubulin and α -tubulin, used as a lane loading control (a). The relative levels of MMP-9 protein and acetyl- α -tubulin are expressed as fold of control (b). 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. *** P < 0.05 compared with that of Gh + LPS.

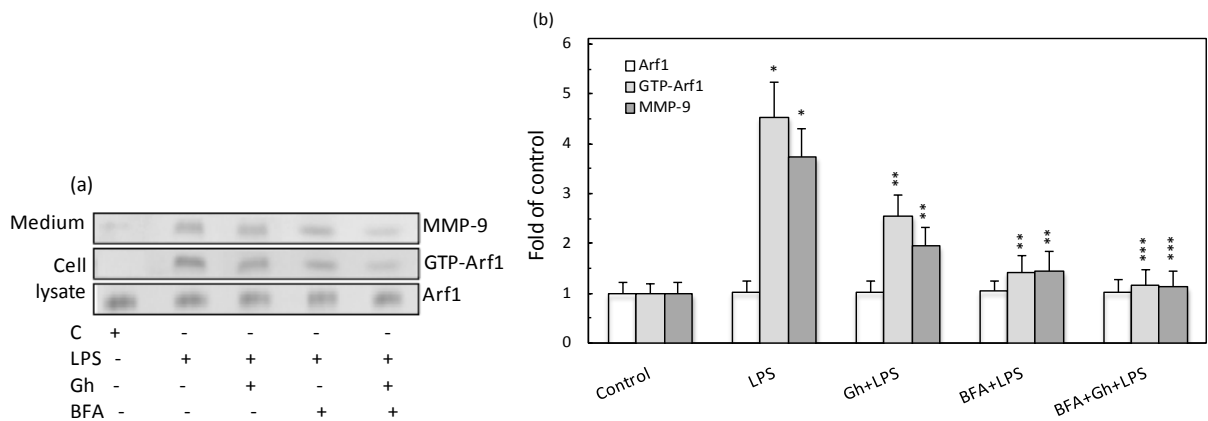


Figure 3. Influence of ER-to-Golgi transport inhibitor, Brefeldin A (BFA), on the changes induced by *P. gingivalis* LPS and ghrelin (Gh) in Arf1 activation in the salivary gland acinar cells. The cells, preincubated with 5 μ g/ml BFA, were treated with 0.5 μ g/ml Gh, and incubated with the LPS at 100 ng/ml. After 2 h of incubation the cell lysates were analyzed for Arf1 and Arf1-GTP, whereas the conditioned media following 16 h of incubation were subjected to immunoblotting for MMP-9 (a). The relative levels of protein are expressed as fold of control (b). 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 control. *** P < 0.05 compared with that of LPS. **** P < 0.05 compared with that of Gh + LPS.

the PKD2 was subject to interference by BFA.

To gain further insights into the role of PKD2 in mediation of the processes associated with MMP-9 secretion, we examined the impact of *P. gingivalis* LPS and ghrelin on the recruitment and phosphorylation of PKD2 in the Golgi. The analyses revealed that the effect of the LPS was manifested by a marked elevation in the Golgi membrane content of PKD2, and its phosphorylation on Ser and some on Tyr, while the effect of ghrelin was associated with a significant decrease in PKD2 phosphorylation on Ser and the increase in phosphorylation on Tyr (Figure 6). Therefore, in further assessment of factors affecting the PKD2 phosphorylation, we investigated the requirements and selectivity of PKD2 interaction with PKC δ and Src by co-immunoprecipitation. The results, presented in Figure 7, revealed that PKC δ was present in association with PKD2 following the acinar cell stimulation with the LPS, while preincubation with ghrelin promoted the association of PKD2 with Src. Moreover, the interaction between PKD2 and PKC δ was dependent on PKD2 phosphorylation on Ser as well as the activity of PKC δ , as the wide spectrum PKC inhibitor, GF109203X, interfered with the co-immunoprecipitation. The

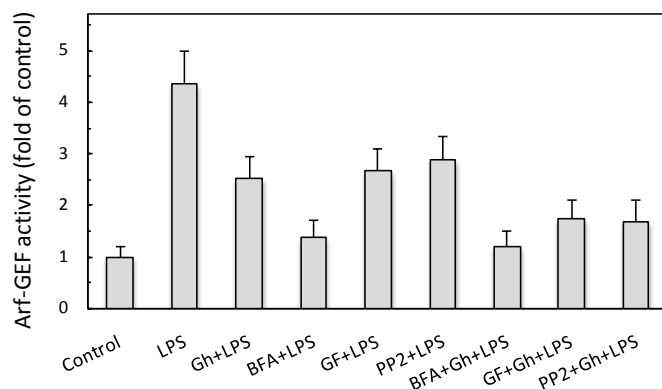


Figure 4. Effect of ghrelin (Gh) on *P. gingivalis* LPS-induced changes in sublingual salivary gland acinar cell expression of Arf-GEF activity (GTP-Arf1). The cells, preincubated with 5 µg/ml of Brefeldin A (BFA), 30 µM of SFK-PTKs inhibitor, PP2 or 5 µM of wide spectrum PKC inhibitor, GF109203X (GF), were treated with 0.5 µg/ml Gh, and incubated for 2 h in the presence of 100 ng/ml LPS. The data represent the mean ± SD of four experiments. **P* < 0.05 compared with that of control. ***P* < 0.05 compared with that of control. ****P* < 0.05 compared with that of Gh + LPS.

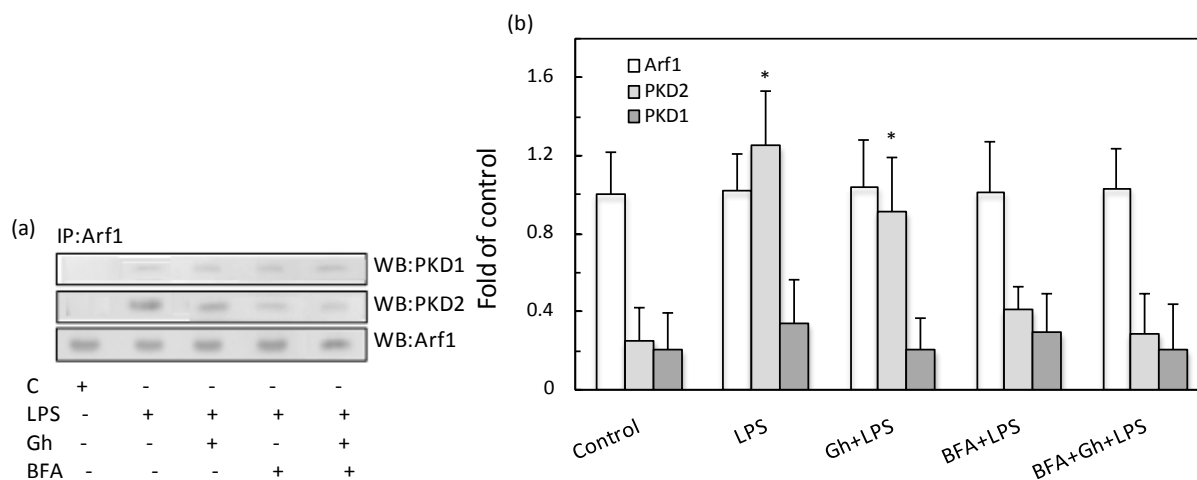


Figure 5. Impact of Brefeldin A (BFA) on the association of Arf1 with PKD1 and PKD2 induced in the acinar cells by *P. gingivalis* LPS and ghrelin (Gh). The cells, preincubated with 5 µg/ml BFA, were treated with 0.5 µg/ml Gh, and incubated for 2 h in the presence of 100 ng/ml LPS. Cell lysates were immunoprecipitated (IP) with anti-Arf1 antibody and immunoblotted (WB) with anti-PKD1, and anti-PKD2 antibody (a). The relative densities of proteins are expressed as fold of Arf1 control (b). The data represent the mean ± SD of four experiments. **P* < 0.05 compared with that of control.

association of PKD2 with Src, on the other hand was dependent on PKD2 phosphorylation on Tyr, and displayed susceptibility to SFK-PTKs inhibitor, PP2. Together, these data suggest that PKCδ-mediated phosphorylation of PKD2 on Ser plays a major role in the LPS-induced up-regulation in MMP-9 secretion, while the SFK-PTKs-mediated phosphorylation of PKD2 on Tyr reflects the modulatory influence on the acinar cell MMP-9 secretion by ghrelin.

4. Discussion

Oral mucosal reaction to periodontopathogen, *P. gingivalis* and its key endotoxin, LPS, triggers a pattern of inflammatory responses that include the rise in proinflammatory cytokine production, disturbances in nitric oxide synthase and cyclooxygenase systems, changes in EGFR and MAPK activation, and the induction in MMP-9 secretion [4]-[8]. Indeed, studies show that the elevated levels of MMP-9 are found in gingival tissue of patients with periodontitis, a chronic inflammatory disease that leads to periodontal lesions and progressive destruction of teeth-supporting tissue, including bone loss [1]-[3] [6]. There are also strong indications that *P. gingivalis*

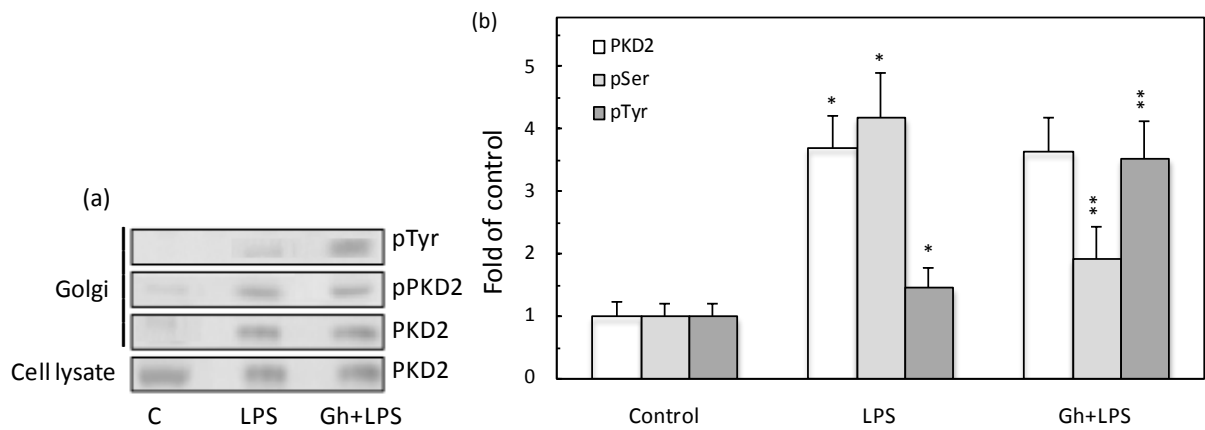


Figure 6. Effect *P. gingivalis* LPS and ghrelin (Gh) on PKD2 phosphorylation in the salivary gland acinar cells. The cells were preincubated with 0 or 5 $\mu\text{g/ml}$ Gh and incubated for 2h in the presence of 100 ng/ml LPS. The lysates of whole cells and the corresponding Golgi membrane fractions were analyzed for PKD2, phospho-PKD2 (Ser), and phosphotyrosine (pTyr) with specific antibodies (a). The relative densities of proteins are expressed as fold of control (b). The data represent the means \pm SD of four experiments. * $P < 0.05$ compared with that of control. ** $P < 0.05$ compared with that of LPS.

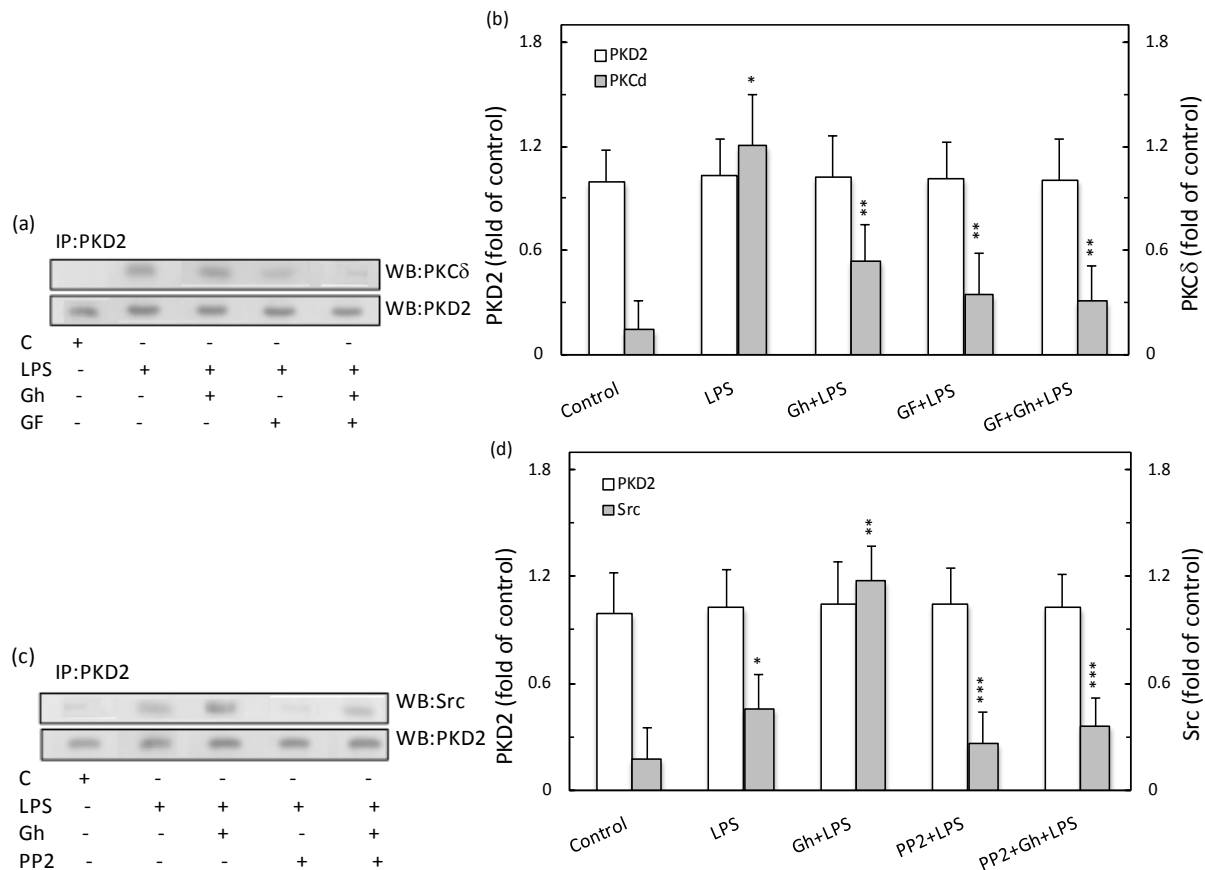


Figure 7. Effect of *P. gingivalis* LPS and ghrelin (Gh) on the association of PKD2 with PKC δ in the salivary gland acinar cells. The cells, preincubated with 5 μM GF109203X (GF) or 30 μM PP2, were treated with 0.5 $\mu\text{g/ml}$ Gh, and incubated for 2 h in the presence of 100 ng/ml LPS. (a) cell lysates were immunoprecipitated (IP) anti-PKD2 antibody and immunoblotted (WB) with anti-PKD2 and anti-PKC δ antibody, and the relative densities of proteins (b) are expressed as fold of PKD2 control. (c) cell lysates were immunoprecipitated with anti-PKD2 antibody, immunoblotted with anti-PKD2 and anti-Src antibody, and the relative densities of proteins (d) are expressed as fold of PKD2 control. * $P < 0.05$ compared with that of control. ** $P < 0.05$ compared with that of LPS. *** $P < 0.05$ compared with that of Gh + LPS.

LPS along with the elevated content of MMP-9, routinely detected in the circulation of patients with periodontitis, may be responsible for a decrease in cardiac function and cardiovascular disease [3] [33]. Moreover, the literature data on the course of inflammatory responses associated with progressive heart failure as well as the spinal cord injury suggest that up-regulation in MMP-9 secretion is subject to suppression by a peptide hormone, ghrelin [34] [40]. Hence, in the present study we investigated the nature of factors implicated in the modulatory influence of ghrelin on *P. gingivalis* LPS-induced up-regulation in salivary gland acinar cell MMP-9 secretion.

Employing zymography and Western blot analyses; our results revealed that incubation of sublingual salivary gland acinar cells with the LPS elicited a marked enhancement in MMP-9 secretion, while the effect of ghrelin was reflected in a significant reduction in the LPS effect. Furthermore, we found that the stimulatory effect of the LPS on MMP-9 secretion was susceptible to suppression by a well-known ER-to-Golgi secretory cargo inhibitor, Brefeldin A [41], which also produced an additive result on the inhibitory effect of ghrelin. Taking into consideration that MMP-9 is a highly glycosylated protein comprising mainly of O-glycans, and the fact that N-glycosylation is a co-translational event taking place in the ER, while the O-glycosylation occurs later in the Golgi and TGN [9] [10], the above findings attest to the modulatory influence of ghrelin on the rate of MMP-9 elaboration at the level of ER-to-Golgi processing.

Moreover, relying on the literature evidence as to the role of cytoskeleton MT stabilization in the secretory vesicle trafficking [11] [15] [16], we assessed the impact of the LPS and ghrelin on the acinar cell secretion of MMP-9 in the presence of pharmacological agents affecting MT stability. We observed that the LPS elicited induction in MMP-9 secretion was susceptible to suppression by MT destabilizing agent, nocodazole, which also produced further amplification in the countering effect of ghrelin, while preincubation with MT stabilizing agent, tubacin, led to the enhancement of the LPS-induced MMP-9 secretion and the inhibition in the countering effect of ghrelin. Our findings are thus in keeping with the data indicating that microtubule stabilization is not only of paramount importance to cell shape, motility and division, but also plays a major role in MMP-9 secretion [15]-[18]. Furthermore, as the extent of MT stabilization has been directly linked to the degree of its tubulin subunit acetylation on Lys⁴⁰ [11] [15], we have monitored the impact of *P. gingivalis* LPS and ghrelin on the level of acetylated α -tubulin in the acinar cells. A significant increase in the acetylated α -tubulin level was observed following the acinar cell stimulation with the LPS and MT stabilizing agent, tubacin, while the effect of ghrelin and MT destabilizing agent, nocodazole, was reflected in a marked reduction in the LPS-induced α -tubulin acetylation. Accordingly, we concluded that *P. gingivalis* LPS as well as ghrelin exert their influence on the acinar cell secretion of MMP-9 by affecting MT stabilization through α -tubulin acetylation. In this connection, it is pertinent to note that MT α -tubulin acetylation is reversible [11] [42], and hence presents a tempting target for therapeutic intervention in the secretory processes of MMP-9.

In further assessment of the role of ghrelin in modulation of pathways utilized by *P. gingivalis* LPS to enhance the acinar cell secretion of MMP-9, we have turned our attention to factors that affect trafficking and sorting of secretory cargo through the Golgi-TGN, namely Arf and PKD [12]-[14]. Following the literature leads as to the involvement of Arf1 in controlling the Golgi-TGN cargo trafficking through GDP to GTP-dependent activation [14], we have identified Arf1 in the acinar cells and demonstrated that the elevation in Arf1-GTP formation upon the LPS-stimulation paralleled that of the rise in MMP-9 secretion, while the effect of ghrelin was reflected in the reduction of the LPS-induced Arf1-GTP formation as well as MMP-9 secretion. Furthermore, as the activation status of Arfs is controlled by GEFs [19] [20], we have assessed the requirements for the acinar cell Arf-GEF activity and found that the effect of the LPS was manifested by a significant increase in the Arf-GEF activation, whereas preincubation with ghrelin led to the reduction in the LPS effect. Moreover, the activation of Arf-GEF by the LPS was susceptible to suppression not only by Brefeldin A, but also to the inhibitors of PKC δ and SFK-PTKs. The sensitivity to Brefeldin A indicates that the acinar cell Arf1-GEF belongs to the class of high (>100 kDa) molecular weight GEFs [14], while the susceptibility to the inhibition by PP2 and GF109203X suggests that the process of the acinar cell Arf1-GEF activation, like that of Rac1-GEF, Dock180 [35], occurs with the involvement of Src/PKC δ -dependent phosphorylation.

As Arf1 plays a key role in the Golgi recruitment and activation of a pool of PKDs essential for phosphorylation of TGN-localized substrates, including phosphatidylinositol-4-kinase required for export of cargo material from the Golgi to the plasma membrane [12] [14] [19] [43], we first employed co-immunoprecipitation analysis to identify the acinar PKD isoform recruited by Arf1 from the cytoplasm to the TGN upon the LPS stimulation as PKD2, thus confirming the involvement of Arf1/PKD2 in the processing of MMP-9 secretory cargo [22] [23]. Then, to gain further insights onto the role of PKD2 in mediation of the processes associated with the acinar cell

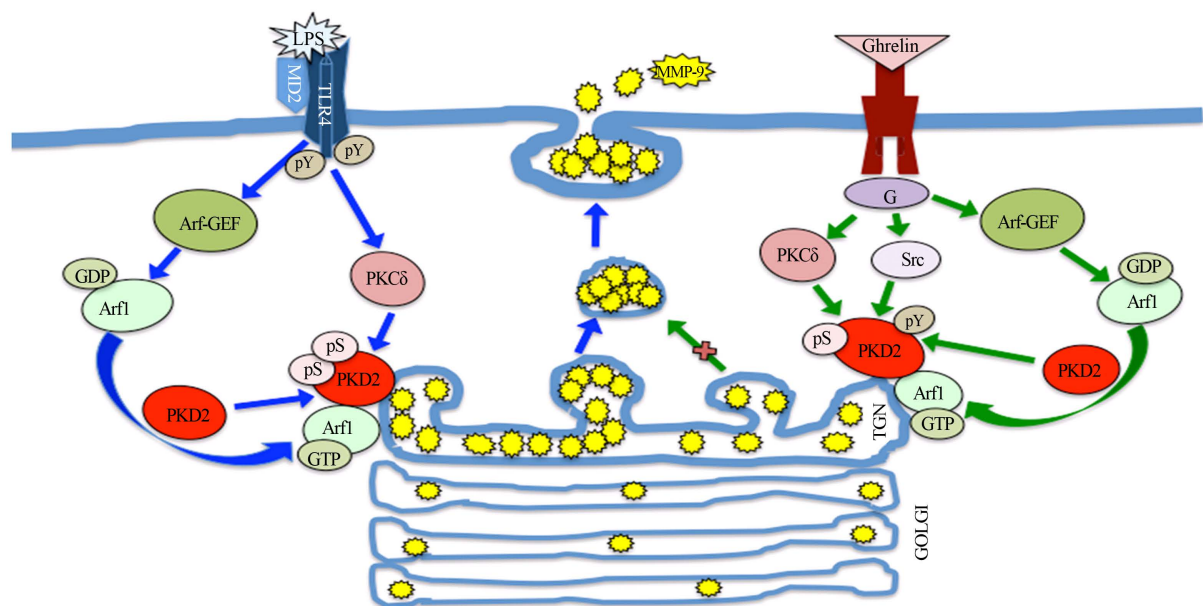


Figure 8. Schematic diagram of the regulatory role of Arf1/PKD2 in mediation of salivary gland acinar cell MMP-9 secretion in response to *P. gingivalis* LPS stimulation, and the modulatory influence of ghrelin. Engagement of TLR4 by the LPS triggers Arf-GEF-mediated Arf activation and its translocation to the Golgi. This promotes the localization of PKD2 with Arf1-GTP at the TGN, and its activation by phosphorylation on Ser by the PKC δ . The activated PKD2, by acting on the TGN-localized substrates, affects the vesicle fission events and thus enhances the secretion of MMP-9. Ligation by ghrelin of GHS-R1a, on the other hand, leads to Arf-GEF-mediated Arf1 activation and PKD2-to-TGN localization, followed by the SFK-PTKs-mediated phosphorylation of PKD2 on Tyr that maintains the PKD2 activity at its normal regulatory level. G heterotrimeric G protein, pS phosphoserine, pY phosphotyrosine.

MMP-9 secretion, we examined the effect of the LPS and ghrelin on the recruitment and phosphorylation of PKD2 in the Golgi. Taking into account evidence as to the PKDs activation through phosphorylation on Ser as well as Tyr residues [12] [25], we demonstrated that the effect of the LPS was manifested by a marked elevation in the Golgi membrane content of PKD2, and its phosphorylation on Ser and some Tyr, while preincubation with ghrelin evoked to a significant increase in PKD2 phosphorylation on Tyr and was accompanied by a marked decrease in the phosphorylation on Ser. Moreover, employing co-immunoprecipitation analysis in conjunction with pharmacological inhibitors we have established that the LPS-induced up-regulation in the acinar cell MMP-9 secretion is associated with PKC δ -mediated PKD2 phosphorylation on Ser, while the modulatory influence of ghrelin is reflected in the SFK-PTKs-dependent phosphorylation of PKD2 on Tyr.

Thus it is becoming increasingly apparent that PKC along with PKD form a key convergence and integration node for signals triggered by TLR activation as well as those arising through GPCR stimulation. Indeed, PKD/PKC axis is not only the signaling target of LPS-induced TLR4 activation that defines the extent of inflammatory involvement, but also plays a major role in modulation of inflammation by ghrelin, an endogenous ligand of GPCR. The schematic diagram of the regulatory role of Arf1/PKD2 in mediation of salivary gland acinar cell MMP-9 secretion in response to *P. gingivalis* LPS and the modulatory influence of ghrelin is depicted in Figure 8.

5. Conclusion

The findings presented in this report show that *P. gingivalis* LPS-induced enhancement in salivary gland acinar cell MMP-9 secretion is associated with the increase in α -tubulin acetylation and MT stabilization, and occurs with the involvement of Arf-GEF-mediated Arf1 activation and recruitment to the Golgi of PKD2. We further demonstrate that the effect of the LPS on MMP-9 secretion is reflected in PKC δ -mediated PKD2 phosphorylation on Ser, while the modulatory influence of ghrelin is manifested in the SFK-PTKs-dependent phosphorylation of PKD2 on Tyr. Hence, Arf1/PKD2 junction presents a target for developing more effective treatments of periodontal disease.

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