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Original Article

Expression of toll-like receptor-1 by bradykinin in human gingival fibroblasts

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Abstract: Background: Periodontal disease is a chronic inflammatory condition characterized by a host immune response against periodontopathogenic bacterial infection. It results in a variety of inflammatory responses including vasoactive amine sensitization to histamine and bradykinin (BK). Gingival fibroblasts in the inflamed tissues were found to be dependent on bradykinin B2 receptor (B2R). Molecular studies revealed a link between vasoactive amines and Toll like receptors (TLRs) expression. In the present study, bradykinin induced Toll like receptor-1 expression in human gingival fibroblasts. Results: Using western blot and RT-PCR we found that bradykinin promoted TLR-1 expression through p38, PKC, AKT and NFkB dependence. Conclusion: This finding revealed a molecular GPPR signaling platform to potentiate inflammatory response in periodontium through TLR and subsequent signaling.

Keywords: Periodontitis, bradykinin, toll like receptors

Introduction

Periodontitis is characterized by the destruction of connective tissue, loss of periodontal attachment and resorption of alveolar bone [1-3]. Periodontitis comprehends a group of infectious diseases triggered by periodontopathogens [4, 5]. It is a multi-factorial disease sharing common inflammatory pathways [6]. Bacterial pathogens are the major etiological agents in periodontal disease. Immunological response to the microorganisms is thought to play an important role in the pathogenesis of this chronic inflammation, which results in tissue destruction and bone loss [8-10]. The presence of immunoglobulin's [10], complement and lymphocytes in the gingival fluid and tissue suggests that both the cell-mediated and humoral immune systems are involved in tissue destruction.

Knowledge of periodontal disease pathogenesis showed a wide variation in inflammatory response magnitude, which would explain a subgroup of periodontitis with rapid progression. Certain factors such as smoking, diabetes, stress or inflammatory biochemical mediators may also contribute to disease progression [11-13]. The following biochemical markers can be found among inflammatory mediators and products such as Histamine, BK, Prostaglandin E2, Tumor necrosis factor-alpha and Cytokines [14-17]. The immune system is activated in order to protect gingival tissue against local microbial attack and their damaging products from spreading or invasion, the defense mechanism might thus be sometimes harmful to the host [18, 19]. Innate immune system forms the first line of defense against microbial infections, as it exerts an immediate response. Innate immunity works through Toll-like receptors (TLR) which function as primary pathogen sensors. TLR activates multple signaling cascades leading to the induction of genes responsible for the release of inflammatory cytokines and are said to be responsible for many of the destructive host responses in inflammatory diseases [20-23].

BK, one of the major metabolites in the tissue kallikrein-kinin system, mediates multiple pro-inflammatory effects including vasodilation, increases vascular permeability, eicosanoid synthesis, and neuroprotection [24-26]. Several in

vivo and in vitro studies have revealed beneficial metabolic, cardiovascular and neuroprotective effects following stimulation of the endogenous Bradykinin B2 receptor. The glycosilated serine protease, tissue kallikrein-1 generates Kallidin (Lys-BK) from circulating low-molecular-kininogen. BK is generated following aminopeptidase cleavage of Lys-BK [27], Both peptides are agonists of the BK receptor B2R-induced stimulation of the B2R results in a cycle of desensitization-endocytosis, followed by essentially complete recycling of the receptor to the plasma membrane. Bradykinin (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) and its N-terminal extended from Lys-BK (Kallidin), they selectively activated B2 receptors, belonging to the seven transmembrane G-protein receptor family, expressed in human gingival fibroblasts [28, 29]. Once bound to B2 receptors, BK induces activation of phospholipase C and following signaling involving protein kinase C and phospholipase A2 phosphorylation, nitric oxide synthase, activation and the subsequent intracellular downstream signaling [30, 31]. In consequence, it would elicit release of inflammatory mediators which contribute to cell inflammatory responses and activation/sensitization of physiological responses. In a previous report we found that BK promoted TLR4 expression [32], for this reason, we hypothesized that BK-induced TLR-1 expression would increase expression and secretion.

Materials and methods

Materials

Dulbecco's Modified Eagle Medium, PI3K inhibitors Wortmanin and LY294002), JNK inhibitor (SP600125); MEK inhibitor (PD98059); p38 inhibitor (SB203580); PKC inhibitors (Claphostin C, bisindolylmaleimide II; Phospholipase C inhibitor (U73122); Bradykinin, Lys-(des-Arg, Leu^8)-Bradykinin trifluoroacetate (antagonist B1), HOE140, Curcumin were obtained from Sigma Aldrich (St. Louis MO, USA). Antibodies phospho-AKT/PKB; phospho-p38, γ-tubulin, TLR1, TLR2 and luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Human gingival fibroblasts cultures

Human gingival fibroblasts were isolated from gingival papillar explants obtained from three clinical and systemically healthy voluntary donors, whose rights were protected by the Ethical Committee of Universidad Nacional Autónoma de México, which granted approval to the study. Written consents were received. Gingival explants were placed at the bottom of culture dishes 75 cm^2 (Corning, Midland Michigan USA) with Dulbecco's modified Eagle medium (SIGMA-ALDRICH, St. Louis Mo, USA) supplemented with 10% fetal bovine serum (GIBCO-BRL/Life Technologies) and 10 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C and 5% CO_2 in a fully humidified incubator, for 10 days until outgrowth of fibroblasts from the explants was observed. Fibroblasts were detached and seeded at a density of 4×10^4 cells and cultured until cells were 80% confluent. Media was changed and cells were incubated in the absence (basal) or presence of BK at time and doses indicated in the figure legends [32].

Flow cytometric analysis

Human gingival fibroblasts (1 × 10^6 well) were grown in 6-well plates (Corning). Briefly, cells were treated with Bradykinin (1 μM) for 16 hours, following the aforementioned treatment, cells were obtained by trypsin; cell viability was assessed by trypan blue exclusion. Only samples with at least 98% cell viability were selected for the study. After cells were fixed with paraformaldehyde, they were collected and stained for TLR1, using specific antibodies (Santa Cruz Biotechonologies) and analyzed by flow cytometry using a FACSC anto II flow cytometer (BD Biosciences). Results were given as MFI (mean fluorescence intensity), which indirectly equals the level of receptors' density on the cell surface. All analyses were performed at a maximum 2 h after collecting the sample. All experiments were repeated at least three times.

Immunofluorescence staining

Human gingival fibroblasts cells were cultured directly on glass coverslips in 24-well plates for 24 hours to detect TLR-2 and TLR-1 localization by immunofluorescence assays using a fluorescence microscope. After stimulation with Bradykinin, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 minutes at room temperature and permeabilized with 100% MeOH for 10 minutes at 20°C. Polyclonal antibodies against anti-TLR1 or anti-

TLR-2 were applied for 1 hour followed by a 1 hour incubation period with FITC-conjugated goat anti-mouse IgG. After washing with phosphate-buffered saline, nuclei were stained with DAPI, and fluorescence was visualized using a fluorescence microscope. All experiments were repeated at least three times.

PGE2 production assay

Human gingival fibroblasts were cultured in 6-well culture plates in DMEM containing 10% fetal bovine serum. When cells were confluent, the medium was changed to serum-free DMEM for 18 h, and PGE2. Contents in the supernatant were assayed by commercially available enzyme immunoassay Kits, following manufacturer's instructions (ADI-900-001, 96 well; Enzo Life Sciences).

RNA interference

The siRNA targeting the PKC the isoforms (α, δ, ζ and MYD88) were obtained from Santa Cruz Biotechnology. HGF cells were seeded in 6-well plates for 24 hours, resulting in a 70% confluent cell monolayer. Cells were then transfected with the PKC (α, δ, ζ) and MYD88 siRNA, or a scramble siRNA duplex as a negative control, using siRNA Transfection medium and siRNA transfection reagent Santa Cruz Biotechnology. After 72 hours, depletion of PKC (α, δ, ζ) and MYD88r was confirmed by western blotting, cells were subsequently used for further experiments

Polymerase chain reaction with reverse transcription (RT-PCR)

Total cellular RNA was isolated from human gingival fibroblasts by using the method described by Chomczynski and Sacchi [33]. Total cell RNA (1 μg) was reverse-transcribed using the One Step RT-PCR kit (Invitrogen). PCR was performed using oligonucleotides 5'-CTGAGGGTCCTGATAATGTCCTAC-3' (coding sense) and 5'-GATCACCTTTAGCTCATTGTGGG-3' (anticoding sense) derived from TLR1 gene and 5'-CCACCCATGGCAAATTCCATGGCA-3' (coding sense) and 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (coding sense) derived from glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene. Conditions for PCR amplification included denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min; PCR was carried out for 35 cycles. RT-PCR gave rise to a single 510 bp TLR1 band and a single 309 bp GADPH band. Fragment identity was characterized by apparent fragment size on ethidium bromide-stained agarose gels. Five independent experiments were performed for each treatment. Data were analysed by using Labs Works 4.0 (Upland, CA, USA.) commercial software. All experiments were repeated at least three times. Each densitometric value was expressed as mean ± standard deviation (S.D.).

Western blot analysis

Total cell layer proteins were extracted with 100 μl homogenization buffer containing, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM Sodium pyrophosphate, 25 μg/ml aprotinin, 25 μg/ml leupeptin and 10 μM phenylmethylsulfonyl fluoride, on ice. Proteins were quantified by Bradford method. Briefly 30 μg of protein were separated using 10% SDS-PAGE to be then transferred to PVDF membrane. Membrane was blocked in 5% skim milk for 1 hr and then incubated with in mouse-anti-phospho AKT (1:1000) or mouse-anti-phosho-p38 (1:1000) or goat-anti-TLR1 (1:1000) or goat-anti-TLR2 (1:1000) primary antibodies buffer TBS at 4°C overnight. Blots were then washed three times in TBS-0.1% Tween-20 and incubated for 1 hour at room temperature in secondary antibodies goat-anti-rabbit (1:1000). Blots were washed and analyzed by DIGI Doc It system.

Statistical analysis

Data were expressed as means ± SE. To compare groups, we used the two-way ANOVA by least-square fit to determine the significance of the effects of and interactions between the two categorical parameters: Bradykinin and inhibitors. *P value lower than 0.05 was considered to be significant.

Results

Bradykinin promotes TLR-1 expression in transcription and translation levels in fibroblast gingival human

We assessed the effect of BK on TLR1 in transcription and translation levels in HGF. Our results showed that BK promoted a time dependent increment in TLR-1 expression, maximum expression of TLR-1 was found at 60

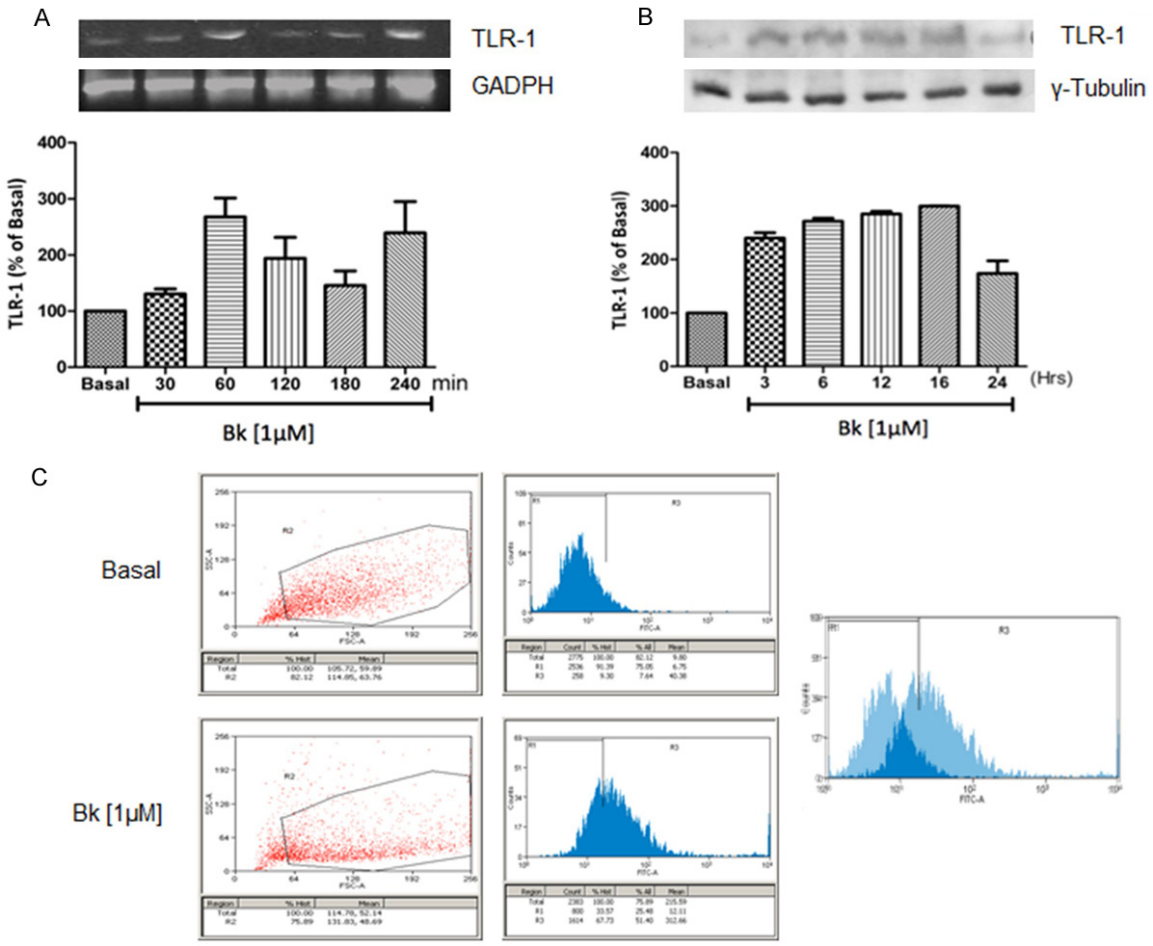

Figure 1. Bradykinin promotes TLR-1 expression in transcription and translation levels in fibroblast gingival human. Human gingival fibroblasts were cultivated in 6 well plates overnight. A. The monolayers were incubated with Bradykinin (1 μM) for different periods of time by RT-PCR assay as described in Material and methods section. B. The cells were incubated with Bradykinin (1 μM) for different periods of time. After incubation the total protein was extracted, and the Western-Blot was conducted as described in Materials and methods. C. The HGF were incubate with Bradykinin (1 μM) for 16 h. The results shown are from a representative experiment chosen from three separate repetitions. Each bar represents the mean ± SD calculated from three independent experiments. *P<0.05.

minutes, suggesting that BK regulates the expression of Toll-like receptor 1 at transcription level in HGF (**Figure 1A**). Western-blot results showed that Bk increased TLR expression, maximum expression was found at 3 hours (**Figure 1B**). In order to localize toll like receptor-1 in cytoplasmic membrane, flow cytometry analyses we conducted. Results revealed that BK promoted increase in the expression of these receptors and their localization in cytoplasmic membrane (**Figure 1C**).

Bradykinin promotes the interaction of TLR-1 and TLR-2 in HGF

Functional TLR receptors can form homo or heterodimers, particularly TLR-2 can group with TLR-1 or TLR-6. In order to assess the effect of BK in heterodimer formation, we conducted immunoprecipitation assays of TL-2 and blotting to TLR-1. Results showed that BK promoted dose-dependent increase in formation of TLR-2 TLR-1 heterodimer, maximum expression occurred at 1 μM (**Figure 2A**). Similar results were obtained by cytoimmunoflorescence (**Figure 2B**) where BK increase the co-localization to the TLR-2 and TLR-1 in HGF treated with BK (1 μM) for 16 hours.

Effect of the inhibitors in TLR-1 expression induced by bradykinin

As shown in **Figures 1** and **2**, BK increased TLR-1 expression in a dose and time dependent

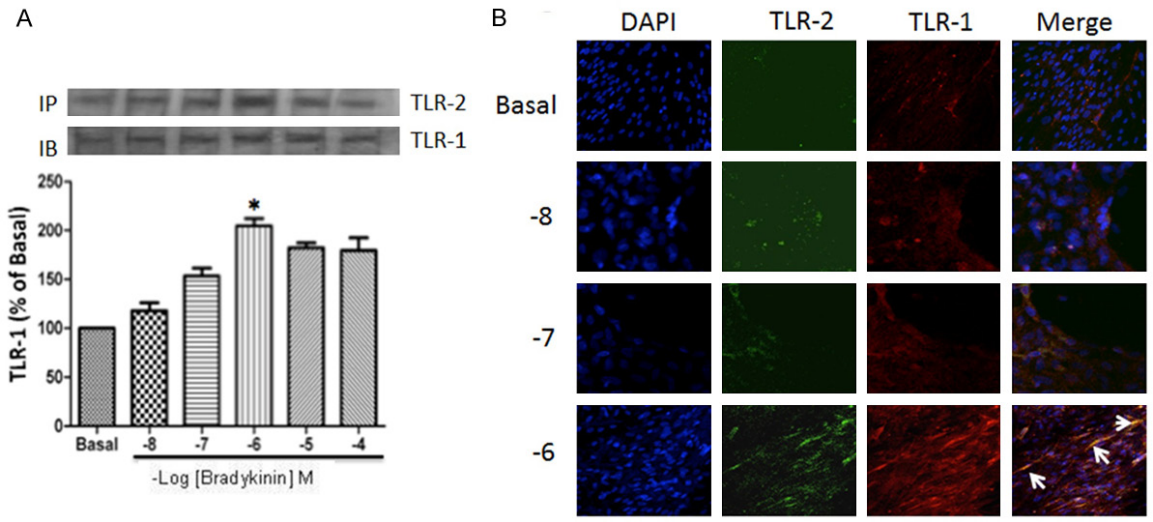

Figure 2. Bradykinin promotes the interaction of TLR-1 and TLR-2 in HGF. Human gingival fibroblasts were cultivated in 6 well plates overnight. A. The monolayers were incubated with different dose of Bradykinin (10 nm, 100 nm, 1 μM, 10 μM, 100 μM) for a period of 16 h, after incubation the total protein was immunoprecipitated with TLR-2 and blotted to TLR-1 and the Western blot was conducted as described in Materials and methods. B. Human gingival fibroblasts were cultivated in 24 well plates overnight in microscope slide covers, allowed to adhere the entire night and grow until confluence. The cells were incubated in DMEM+ 2% SBF (basal) and treated with different dose of Bradykinin (10 nM, 100 nM, 1 μM) for 16 h. After stimulation the cells were fixed with formaldehyde, permeabilized with triton 100, and then stained with anti-TLR2 and anti-TLR1 antibodies and later with anti-goat antibodies bound to fluorescein. The samples were visualized under a confocal microscope. The images are representative ones chosen from three separate repetitions. The results shown are from a representative experiment chosen from three separate repetitions. Each bar represents the mean ± SD calculated from three independent experiments. *P<0.05.

manner. We therefore decided to study the implicated receptor as well as intracellular signalling mechanism involved in TLR-1 expression induced by Bradykinin (1 μM). B1R antagonist Lys-[Leu^{8}] des-Arg^{9}-BK showed a non inhibitory effect on TLR-1 receptor expression, but TLR-1 expression was inhibited when the cells were incubated in the presence of HOE 140, as both were detected by Western-Blot (**Figure 3A**). These results suggested that TLR-1 overexpression mediated by BK occurred via binding to B2R type receptors.

To evaluate the intracellular signalling mechanism involved in TLR-1 expression, cells were preincubated with different inhibitors: for MEK (PD98059; 30 μM), p38 (SB 203580; 20 μM), JNK (SP600125; 10 μM) and NFκB (Curcumin; 30 μM), the aforementioned was assessed by Western Blot. In **Figure 3B**, we found that only p38 and NFkB blocked TLR-1 expression induced by Bk. We next assessed the effect of inhibitors on Phospholipase C (PLC) (U73122; 1 μM), PI3K/AKT (LY294002; 20 μM and Wortmanin 1 μM) and Protein kinase C (PKC) (BIM; 1 μM) in presence of BK (1 μM) in TLR-1 expression (**Figure 3C**). Results showed t that PLC, PKC and PI3K inhibitors blocked the effect of Bradykinin on TLR-1 expression. These results suggested that the signal pathways involved were p38, PI3K/AKT, PLC, PKC and NFkB.

Effect of the inhibitors in phosphorylation of p38 and AKT induced by bradykinin

As previously shown Bradykinin-induced TL-1 expression was regulated by p38 and AKT. Thus, we decided to evaluate BK-induced phosphorylation of p38 and AKT. Cells were incubated in the presence of different inhibitors for PI3K/AKT (LY294002; and Wortmanin 1 μM), PLC (U73122; 1μM) and PKC (Bisindolylmaleimide I (BIM I) (1 μM) in a presence of BK (1 μM). We found that blocking PLC reduced AKT phosphorylation (**Figure 4A**). We next analyzed the effect of same inhibitors on BK-induced p38 phosphorylation and we found that PLC and PKC reduced p38 phosphorylation. These results suggest that PLC and AKT regulate AKT and p38 phosphorylation.

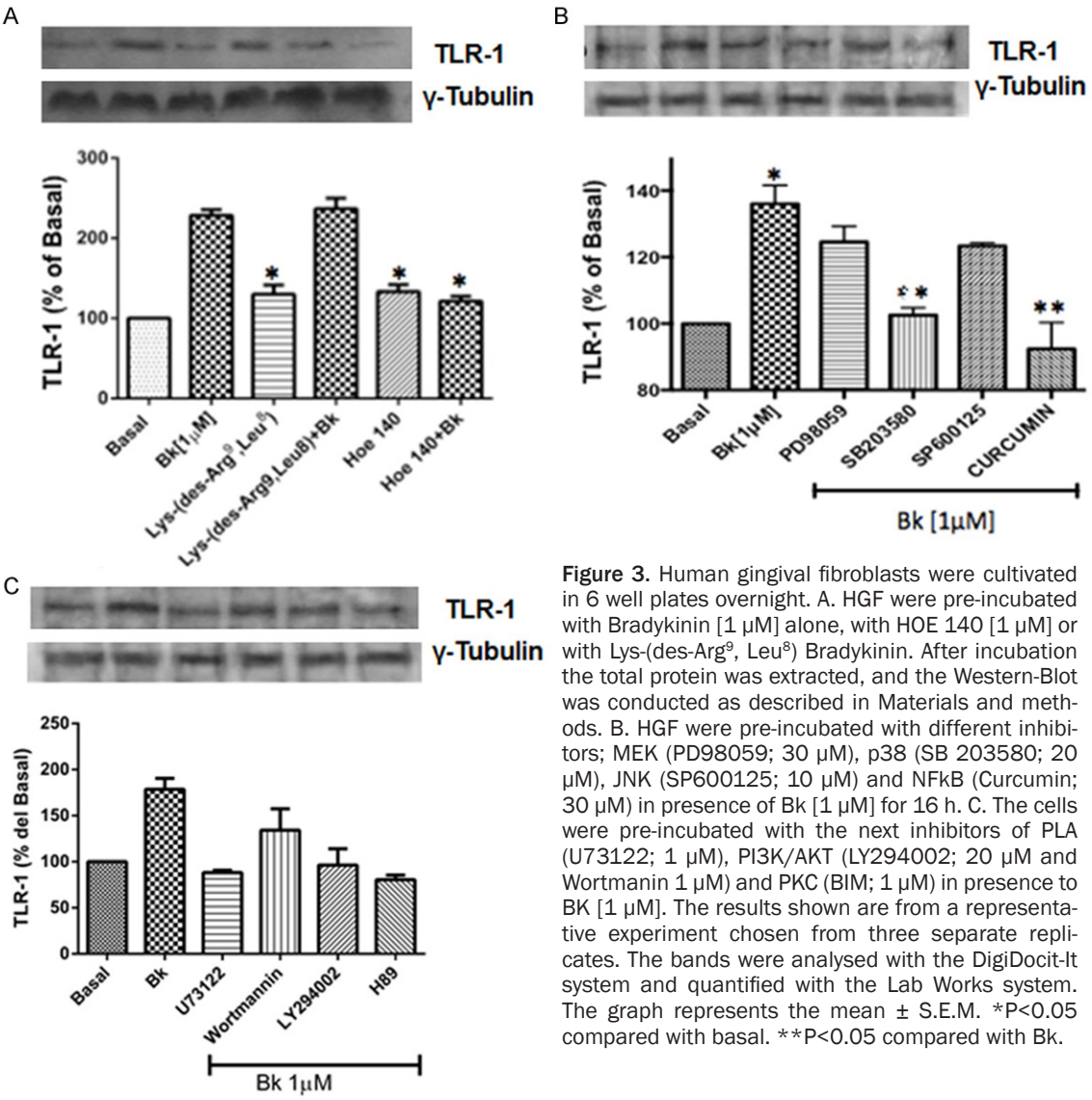

Figure 3. Human gingival fibroblasts were cultivated in 6 well plates overnight. A. HGF were pre-incubated with Bradykinin [1 μM] alone, with HOE 140 [1 μM] or with Lys-(des-Arg^9, Leu^8) Bradykinin. After incubation the total protein was extracted, and the Western-Blot was conducted as described in Materials and methods. B. HGF were pre-incubated with different inhibitors; MEK (PD98059; 30 μM), p38 (SB 203580; 20 μM), JNK (SP600125; 10 μM) and NFkB (Curcumin; 30 μM) in presence of Bk [1 μM] for 16 h. C. The cells were pre-incubated with the next inhibitors of PLA (U73122; 1 μM), PI3K/AKT (LY294002; 20 μM and Wortmanin 1 μM) and PKC (BIM; 1 μM) in presence to BK [1 μM]. The results shown are from a representative experiment chosen from three separate replicates. The bands were analysed with the DigiDocit-It system and quantified with the Lab Works system. The graph represents the mean ± S.E.M. *P<0.05 compared with basal. **P<0.05 compared with Bk.

Bradykinin regulates TLR-1 expression through the action of protein kinase C

To determine which PKC isoform(s) is responsible of BK-induced TLR-1 expression by silence α, δ, and ζ PKC isoforms (**Figure 5A**) scramble control and antisense oligodeoxinucleotides were used. Treatment of the cells with siRNA to PKC isoforms resulted in a significant down-regulation of these specific isoforms for PKC for PKC α (**Figure 5B**); PKC δ (**Figure 5C**) and PKCζ (**Figure 5D**). BK-induced TLR-1 expression was largely blocked by when PKC isoforms were knocked down, indicating that at least that PKC isoforms were responsible for TLR-1 upregulation by BK (**Figure 5F**). The control siRNA had no effect on BK-induced TLR-1 expression (**Figure 5E**). These results suggest that BK-induced TLR-1 expression requires the activation of PKC isoforms.

Discussion

Results of the present study demonstrated that Bradykinin-induced TLR-1 receptor expression, was transcriptionally and translationally regulated. As well as PKC α, δ and ζ are involved in the formation of heterodimers TLR-1/2.

In oral cavity's connective tissue there is a variety of microorganisms. When hygiene is poor dental bacterial plaque develops. It has the possibility to induce an inflammatory process integrated by the activation of different inflammatory mediators such as Bradykinin, recognized by B2 receptor; it promotes calcium mobi-

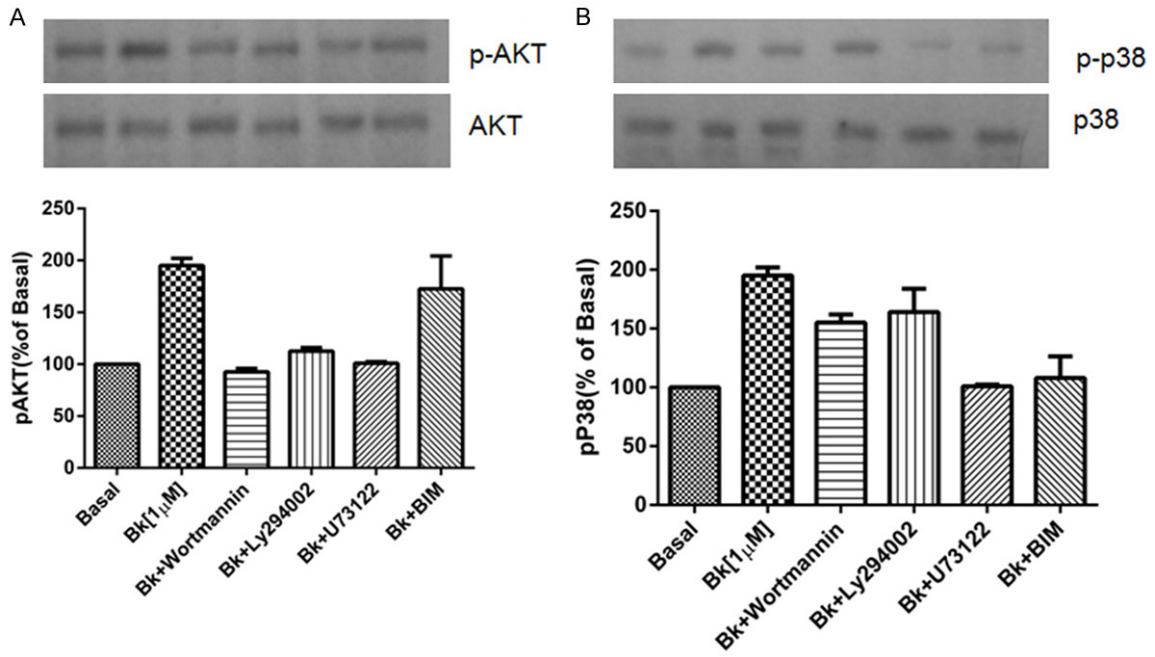

Figure 4. Effect of the inhibitors in phosphorylation of P38 and AKT induced by bradykinin. Human gingival fibroblasts were cultivated in 6 well plates overnight. HGF were pre-incubated with different inhibitors; PI3K/AKT (LY294002; 20 μM and Wortmanin 1 μM), PLA (U73122; 1μM) and PKC (BIM; 1 μM) in presence to BK [1 μM]. A. It was performed western blot for p-P38. B. It was performed western blot for p-AKT. The results shown are from a representative experiment chosen from three separate replicates. The bands were analysed with the Digit Doc-It system and quantified with the Lab Works system. The graph represents the mean ± S.E.M. *P<0.05 compared with basal. **P<0.05 compared with Bk.

lization[1] and activation of other proteins like PKC [30-32].

It is well known that the immune system utilizes TLRs to recognize pathogen-associated microbial patterns (PAMPs). In the oral cavity, different events could develop epithelial rupture, where gingival fibroblasts play an important role in mediating inflammatory response [34]. Human gingival fibroblast express the totality of Toll like receptors, because these cells have the need to recognize a large range of pathogens that are integrated of different structures. Therefore, HGF have evolved for a better reconnaissance, with the formation of TLR complexes. For instance, TLR-2 recognizes bacterial lipopeptides in a heterodimeric complex with TLR6 or TLR1, thereby discriminating between di or triacylated lipopeptides, respectively [35, 36].

The present results demonstrate for the first time that Bradykinin is a potent inflammatory factor recognized by B2R, which induces increment of expression and TLR1 protein synthesis. This effect is a very important mechanism in activation of pathogen recognition and formation of heterodimers with TLR-2. The regulatory action of Bradykinin on TLR expression occurs by binding it to the B2 receptor, which activates the signalling pathway. We found that the expression of TLR-1 by BK decreases when cells have a pre-treatment with HOE 140, indicating that receptor involved is B2. This receptor is a G protein couple receptor (GPCR) that recognized Bk, it activates the phospholipase C (PLC) via GTP-binding proteins. After the activation of PLC, the membrane phospholipid phosphatidyl-inositol4,5-bisphosphate hydrolyses, it generates two; second messengers: inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 promotes the mobilization of calcium from intracellular stores, and together with diacylglycerol, activates PKC in the cytosol. We found that the expression of TLR-1 by Bk is for the pathway of PKC α, δ, ζ and MYD88, which could lead to amplification of inflammatory responses and development of periodontal diseases. Other pathways involved with Bk activation are associated with signalling of MAPKs and AKT.

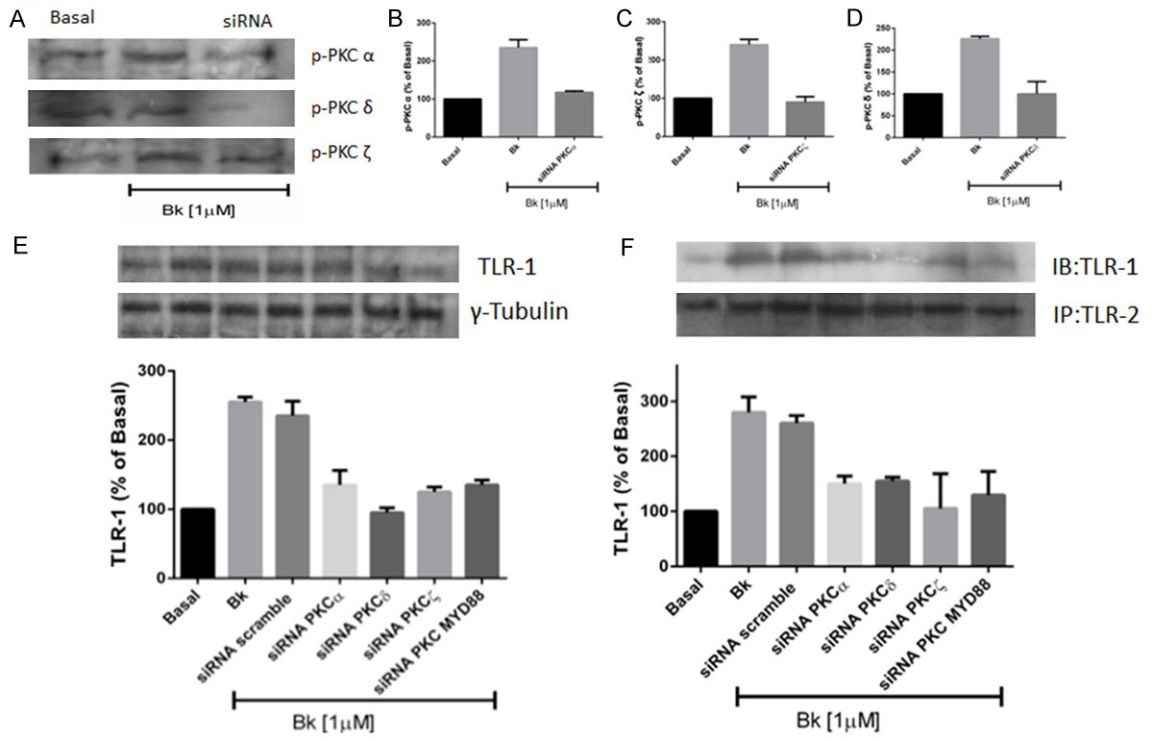

Figure 5. Bradykinin regulates TLR-1 expression through the action of protein kinase C and MYD88 signaling. The cells HGF was ground in plated in 6-well for 24 hours, resulting in a 70% confluent cell monolayer. The cells were then transfected with the PKC (α, δ, ζ) and MYD88 siRNA, or a scramble siRNA duplex as a negative control, using siRNA transfection medium and siRNA transfection reagent Santa Cruz Biotechnology after 72. After the cells were incubated in DMEM+ 2% SBF (basal) and treated with Bk [1 μM] for demonstrated the knockdown are correctly It was performed Western-Blot with antibodies target to p-PKC α, δ, ζ it is demonstrated the image in (A) and the representation graphic to (B-D), the cell that have a transfection for PKC (α, δ, ζ) and MYD88 It was performed western blot to determinate the expression of TLR-1 (E) the cell that have a transfection for PKC (α, δ, ζ) and MYD88. It was performed immunoprecipitation of TLR-2 and the blotting to TLR-1. (F) HGF cells were immunoprecipitated for TLR-2 and then blotting to TLR-1, cells were knocked down with different PKC isoforms (α, δ, ζ) and MYD88. The results shown are from a representative experiment chosen from three separate replicates. The bands were analysed with the DigiDocit-It system and quantified with the Lab Works system. The graph represents the mean ± S.E.M. *P<0.05 compared with Bk.

We found that the activation of AKT and P38 is very necessary for the expression of TLR-1 in HGF.

In conjunction with the TLR dimerization process, TLR receptors need to undergo conformational changes following ligand binding, which allow proper orientation of the ectodomains of TLR for receptor association. Indeed, ligand-induced conformational changes have been shown to allosterically activate. It has become evident that TLRs require additional proteins to be activated by their respective ligands. As an example, not only is CD14 associated with MyD88-dependent TLR4 receptors on the cell surface, but also it constitutively interacts with the MYD88-dependent TLR. It was found that CD14 was necessary for TLR-7 and TLR-9 dependent induction of proinflammatory cytokines in vitro and for TLR-9 dependent innate immune responses in mice. In addition, the absence of CD14 led to reduced nucleic acid uptake in macrophages. Using various types of vesicular stomatitis virus, the report showed that CD14 is dispensable for viral uptake but it is required for the triggering of TLR-dependent cytokine responses. We found that BK induced an increment in the expression of TLR-1 but when the cell have knockdown in MYD88 the expression decreases and the formation of heterodimers with TLR-2. These findings suggest that MYD88 has a dual role in the pathway in the expression of TLR-1 and the makes a complex of TLR.

Conclusions

In the present work, the data presented in this report represent a novel role of Bk signalling in the process in the expression of TLR-1 and the

formation a heterodimer with TLR-2 at the ectodomain in Human Gingival Fibroblast, as it was previously suggested. Secondly, PKC α, δ, ζ and MYD88 are intermediate requirements in regulating in the expression of TLR-1 and the heterodimer TLR-1/-2. Our results showed the Bradykinin mechanism in the feedback of the immune process in Human Gingival Fibroblast in order to promote pathogen recognition.

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Disclosure of conflict of interest

None.

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