Regulation of ghrelin receptor by microbial and inflammatory signals in human osteoblasts

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Abstract : Recently, it has been suggested that the anti-inflammatory hormone ghrelin (GHRL) and its receptor GHS-R may play a pivotal role in periodontal health and diseases. However, their exact regulation and effects in periodontitis are not known. The aim of this in-vitro study was to investigate the effect of microbial and inflammatory insults on the GHS-R1a expression in human osteoblast-like cells. MG-63 cells were exposed to interleukin (IL)-1β and *Fusobacterium nucleatum* in the presence and absence of GHRL for up to 2 d. Subsequently, gene expressions of GHS-R1a, inflammatory mediators and matrix metalloproteinase were analyzed by real-time PCR. GHS-R protein synthesis and NF-κB p65 nuclear translocation were assessed by immunocytochemistry and immunofluorescence microscopy, respectively. IL-1β and *F. nucleatum* caused a significant upregulation of GHS-R1a expression and an increase in GHS-R1a protein. Pre-incubation with a MEK1/2 inhibitor diminished the IL-1β-induced GHS-R1a upregulation. IL-1β and *F. nucleatum* also enhanced the expressions of cyclooxygenase 2, CC-chemokine ligand 2, IL-6, IL-8, and matrix metalloproteinase 1, but these stimulatory effects were counteracted by GHRL. By contrast, the stimulatory actions of IL-1β and *F. nucleatum* on the GHS-R1a expression were further enhanced by GHRL. Our study provides original evidence that IL-1β and *F. nucleatum* regulate the GHS-R/GHRL system in osteoblast-like cells. Furthermore, we demonstrate for the first time that the proinflammatory and proteolytic actions of IL-1β and *F. nucleatum* on osteoblast-like cells are inhibited by GHRL. Our study suggests that microbial and inflammatory insults upregulate GHS-R1a, which may represent a protective negative feedback mechanism in human bone.

Keywords: Receptors, Ghrelin; Ghrelin; Osteoblasts; Periodontitis; *Fusobacterium nucleatum*.

Introduction

One of the most common diseases in humans worldwide is periodontitis. This chronic inflammatory disease is characterized by the destruction of the tooth-supporting tissues and can result in tooth loss and reduced quality of life.[1,2] Periodontitis is caused by pathogenic microorganisms of the subgingival biofilm, such as *Fusobacterium*

nucleatum, together with other risk factors, such as smoking, genetic predisposition and several systemic diseases.[1,2,3,4] The periodontopathogenic microorganisms induce an immunoinflammatory host response, whose aim is to battle the pathogens and, thereby, to stop the microbial attack. A plethora of proinflammatory mediators, such as interleukin (IL)-1β, IL-6, IL-8, cyclooxygenase (COX) 2, and chemokine CC motif ligand (CCL) 2 (CCL2), as well as proteolytic enzymes like matrix metalloproteinase (MMP) 1, are involved in these immunoinflammatory processes. Although the host response aims to protect the periodontal structures against the periodontopathogens, it may cause irreversible loss of the periodontal soft and hard tissues, if the immunoinflammatory processes are extended and/or excessive.[3]

Soft and hard tissues homeostasis, destruction, and healing are not only regulated by inflammation but also by hormones such as ghrelin (GHRL).[5,6] GHRL is a 28-amino acid peptide which induces the secretion of growth hormone at the hypothalamus-pituitary axis. GHRL exerts orexigenic and adipogenic effects and has been shown to regulate bone metabolism in an autocrine and paracrine manner.[7] There is fundamental evidence that GHRL stimulates proliferation and differentiation of osteoblasts and inhibits their apoptosis, suggesting that this hormone might also be a potential candidate to promote periodontal bone healing. Interestingly, periodontitis is associated with a great number of systemic diseases and conditions such as obesity, diabetes mellitus, cardiovascular diseases, and metabolic syndrome.[8,9] Therefore, GHRL might be a pivotal link in the relationships between periodontitis and systemic diseases.

The actions of GHRL are mediated by binding to the growth hormone secretagogue receptor (GHS-R) which is a G-protein-coupled receptor.[10] Two splice variants of GHS-R have been found: type 1a and 1b. Following binding of GHRL to GHS-R1a, a profound change in receptor conformation takes place, which then results in a diversity of physiological responses. In contrast to the biologically active GHS-R1a, GHS-R1b does not mediate any effects.[10]

It is only recently that the role of GHRL in periodontal health and diseases has become the focus of attention. Interestingly, GHRL levels in gingival crevicular fluid have been shown to be decreased in periodontally-diseased patients.[11] Moreover, GHRL has been found in saliva as well as cells and tissues of the tooth germ, such as the inner enamel epithelium, mesenchymal cells, ameloblasts, odontoblasts, and Hertwig's epithelial root sheath.[12,13,14,15]

Recently, we could demonstrate that inflammatory mediators and periodontopathogens are able to induce an initial GHS-R1a upregulation in periodontal fibroblasts.[16,17] Moreover, we found an increased GHS-R1a expression at periodontally-diseased sites as compared to healthy sites during the early stage of periodontitis in a rat experimental periodontitis model.[17] Our investigations also proved the anti-inflammatory nature of GHRL. Our in-vitro and in-vivo studies suggested that the GHS-R1a upregulation in periodontal fibroblasts in response to an inflammatory or microbial stimulus may represent a negative feedback mechanism to attenuate the initial inflammatory process in periodontal diseases. Furthermore, the anti-inflammatory GHRL/GHS-R system may, therefore, serve as a promising target for the prevention and therapy of periodontitis.[16,17]

Since periodontitis has a detrimental impact, not only on the soft connective tissue but also on bone, a better understanding of the GHRL/GHS-R system in hard connective tissue is of utmost importance. Therefore, the aim of this in-vitro study was to investigate the effect of microbial and inflammatory insults on the GHS-R1a expression in human osteoblast-like cells.

Methodology

Cell culture and treatment

Human osteoblast-like MG-63 cells (ATCC, CRL-1427™) were grown in Dulbecco's minimal essential medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin and 100 μg/ml streptomycin (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37°C. Then, 50,000 cells/well were seeded on tissue culture plates (Corning, NY,

USA) and grown to 80% confluence. Twenty-four hours prior to each experiment, the FBS concentration was reduced to 1%. The medium was changed every other day.

In order to simulate an inflammatory condition in vitro, cells were treated with IL-1β (1 ng/ml; (PromoKine, Heidelberg, Germany), because this proinflammatory cytokine had been widely shown to be increased at sites of periodontal inflammation.[18,19] To mimic a microbial infection in vitro, cells were exposed to various concentrations of the periodontopathogen *F. nucleatum* ATCC 25586 (optical density (OD): 0.05, 0.10, 0.20). *F. nucleatum* is a gram-negative anaerobic microorganism associated with both gingivitis and periodontitis.[20,21] The bacteria were pre-cultivated on Schaedler agar plates (Oxoid, Basingstoke, UK) in an anaerobic atmosphere, then suspended in phosphate-buffered saline (PBS) (OD_{660} =1.0, equivalent to $1.2×10^9$ bacterial cells/ml) and subjected twice to ultra-sonication (160 W for 15 min). Moreover, in a subset of experiments, cells were also incubated with GHRL (20 nM; human n-octanoylated ghrelin, Pepta Nova, Sandhausen, Germany) in the presence and absence of *F. nucleatum* or IL-1ß.

In order to unravel intracellular signaling pathways involved in the GHS-R regulation, cells were pre-treated with a specific MEK1/2 inhibitor (U0126; 10 µM; Calbiochem, San Diego, CA, USA) or a specific NF-κB inhibitor (PDTC; 10 µM; Calbiochem) 60 min prior to the stimulation with IL-1ß.

Real-time PCR

Total RNA was extracted using a specific RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA concentration was measured by the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and 500 ng of total RNA was reversely transcribed using the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer's instruction. The gene expressions of GHS-R1a, COX2, CCL2, IL-6, IL-8, MMP1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control were subsequently analyzed by using the iCycler iQ™

Real-Time PCR Detection System (Bio-Rad), SYBR Green QPCR Master Mix (Bio-Rad) and QuantiTect Primers (Qiagen), as in our previous studies.[16,17] For data analysis, the comparative threshold cycle (CT) method was used.

Immunocytochemistry

MG63 cells were seeded on plastic coverslips (Thermo Fisher Scientific) of 13 mm diameter placed in 24-well plates in the presence and absence of *F. nucleatum* or IL-1ß for 24 h. Afterwards, cells were washed twice with PBS (Invitrogen) and fixed in 4 % paraformaldehyde (Sigma-Aldrich, Munich, Germany) at pH 7.4 and at room temperature (RT) for 10 min. Next, cells were permeabilized in 0.1 % Triton X-100 (Sigma-Aldrich) for 5 min, washed again and then blocked using serum block (Dako, Hamburg, Germany) for 20 min. After an overnight incubation with a rabbit polyclonal primary antibody to GHS-R (Abcam, Cambridge, UK, 1:500) in a humid chamber at 4°C, cells were washed with PBS and, subsequently, incubated with a goat anti-rabbit IgG HRP-conjugated secondary antibody (Dako) for 45 min. After washing, the cells were incubated with 3,3'-diaminobenzidine substrate (DAB) for 5-10 min at RT and then counterstained 5 s in Mayer's hematoxylin solution. Finally, cells were mounted with DePeX (SERVA Electrophoresis, Heidelberg, Germany) and the production of GHS-R was assessed with an Axioskop 2 microscope (20×, Carl Zeiss, Germany). The images were captured with an AxioCam MRc camera and analyzed with the AxioVision 4.7 software (Carl Zeiss). Untreated cells were used as a control.

Immunofluorescence microscopy

MG63 cells were grown on plastic coverslips (Thermo Fisher Scientific) of 13 mm diameter placed in 24-well plates in the presence and absence of *F. nucleatum* or IL-1ß for up to 60 min. Afterwards, the cells were washed with PBS, fixed and permeabilized as described above and blocked with nonfat dry milk (Bio-Rad) for 1 h. After washing, the cells were incubated with a rabbit anti-nuclear factor (NF)-κB p65 primary antibody (E498; Cell Signaling Technology, Danvers, MA, USA, 1:100) at RT for 90 min. After the

incubation step, cells were washed twice with PBS and incubated with a CY3-conjugated goat anti-rabbit IgG secondary antibody (Abcam; 1:1000) at RT for 45 min. The NF-κB p65 nuclear translocation was observed by using the ZOE™ Fluorescent Cell Imager (Bio-Rad) with a 20× objective and an integrated digital 5MP CMOS camera. Untreated cells were used as a control.

Statistical analysis

Data analysis was performed using the IBM SPSS Statistics 22 software (IBM SPSS, Chicago, USA). For quantitative analysis, mean values and standard errors of the mean (SEM) were calculated. In order to test for significant ($p < 0.05$) differences between groups, parametric (t-test and ANOVA followed by the post-hoc Dunnett's and Tukey's tests) and non-parametric tests (Wilcoxon and Mann-Whitney-U tests) were applied. All experiments were performed in triplicate and repeated at least twice.

Results

Regulation of GHS-R1a in MG63 cells by F. nucleatum or IL-1β

As depicted in Figure 1A, *F. nucleatum* caused a significant upregulation of GHS-R1a by more than 50 % at 1 d and 2 d, as analyzed by real-time PCR. Interestingly, the stimulatory effect of *F. nucleatum* on GHS-R1a was significantly increased at the OD concentration of 0.1 (Figure 1B). When osteoblasts were exposed to IL-1β, the constitutive GHS-R1a expression was more than doubled at 1 d and 2 d (Figure 1C). Further analysis by immunocytochemistry revealed that the GHS-R1a upregulation by *F. nucleatum* or IL-1β at transcriptional level was paralleled at protein level. The immunoreactivity against GHS-R1a was strongly enhanced in osteoblasts treated with *F. nucleatum* or IL-1β, as compared with untreated cells (Figure 1D).

Intracellular signaling involved in the GHS-R1a regulation by F. nucleatum or IL-1β in MG63 cells

As shown in Figure 2A, exposure of MG63 cells to *F. nucleatum* or IL-1β resulted in a pronounced NF-κB (p65) nuclear translocation between 30 min and 60 min,

as determined by immunofluorescence microscopy. However, pre-incubation of cells with a specific inhibitor of NF-κB signaling had no significant effect on the GHS-R1a expression induced by *F. nucleatum* or IL-1β (data not shown). Nevertheless, when cells were pre-incubated with a specific inhibitor against MEK1/2, the IL-1β-induced GHS-R1a upregulation was significantly diminished, as revealed by real-time PCR (Figure 2B).

### Antiinflammatory and antiproteolytic effects of GHRL on MG63 cells under microbial condition

Both *F. nucleatum* and IL-1β increased significantly the expressions of COX2, CCL2, IL-6, IL-8, and MMP1 at 1 d (Figure 3A-E and Figure 4A-E). However, when the cells were simultaneously incubated with GHRL, the stimulatory actions of *F. nucleatum* or IL-1β on MG63 cells were significantly reduced (Figure 3A-E and Figure 4A-E).

GHS-R1a expression in MG63 cells in response to GHRL under microbial and inflammatory conditions

As demonstrated in Figures 5A and B, the GHS-R1a expression was significantly upregulated by GHRL at 1 d. The GHRL-stimulated GHS-R1 expression was even more increased when the cells were exposed to a combination of GHRL with *F. nucleatum* or IL-1β (Figures 5A and B).

Discussion

Our study provides original evidence that the periodontopathogen *F. nucleatum* and the proinflammatory cytokine IL-1β, which have been shown to be increased at inflamed periodontal sites, regulate the GHS-R1/GHRL system in osteoblast-like cells. Furthermore, we demonstrate for the first time that the proinflammatory and proteolytic actions of *F. nucleatum* and IL-1β on the osteoblast-like cells can be counteracted by GHRL. Our study suggests that microbial and inflammatory insults upregulate GHS-R1a, which may represent a protective negative feedback mechanism in human bone.
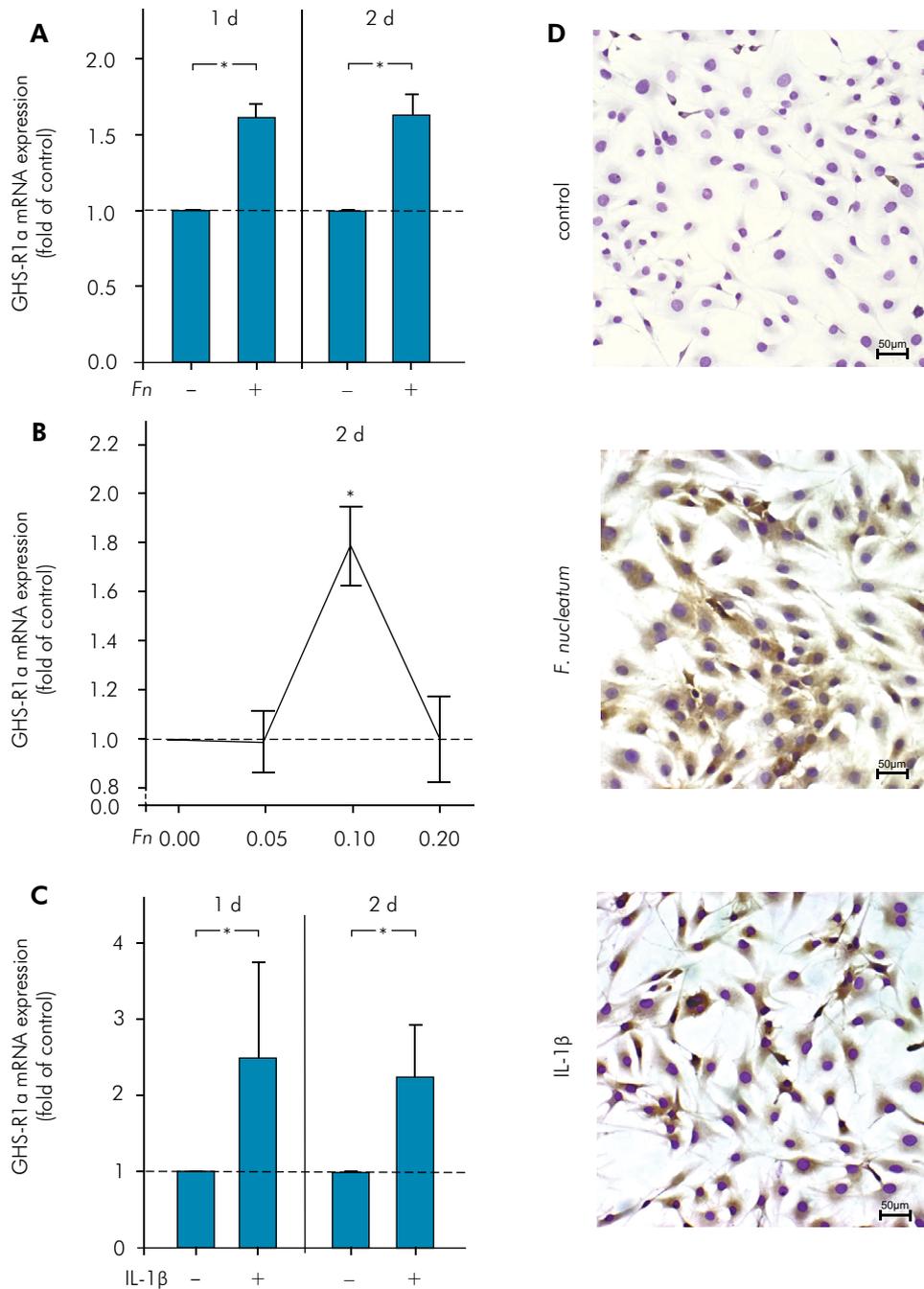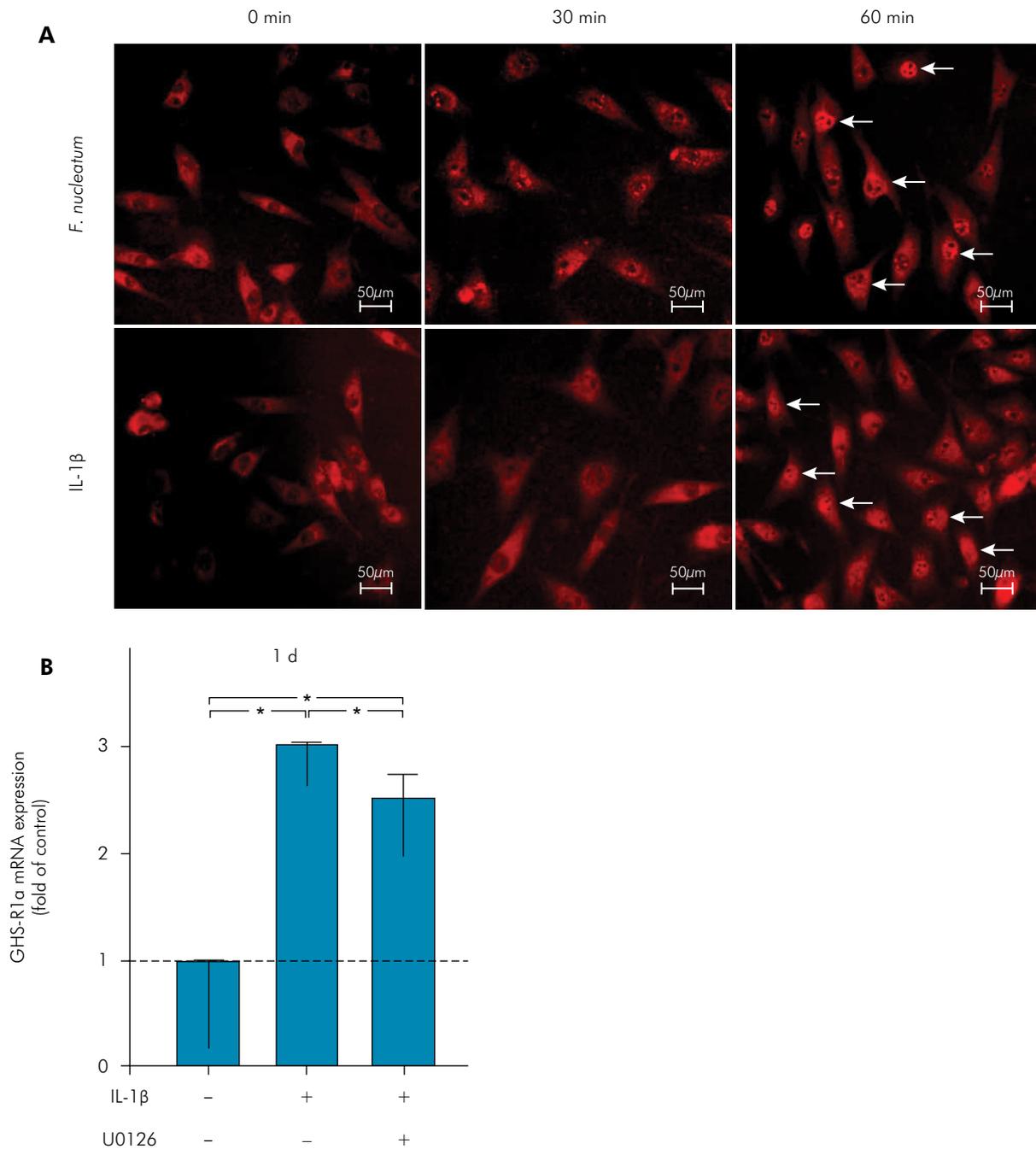
*significant (p < 0.05) difference between groups.

Figure 1. (A) GHS-R1a expression in the presence and absence of *F. nucleatum* (*Fn*; OD: 0.1) at 1 d and 2 d. Mean ± SEM (*n*=9). (B) GHS-R1a expression in response to various concentrations of *F. nucleatum* (*Fn*; OD: 0.05, 0.10, 0.20) at 1 d. Untreated cells served as control. Mean ± SEM (*n*=9). (C) GHS-R1a expression in the presence and absence of IL-1β (1 ng/ml) at 1 d and 2 d. Mean ± SEM (*n*=9). (D) GHS-R1a immunoreactivity in the presence and absence of *F. nucleatum* (OD: 0.1) or IL-1β (1 ng/ml) at 1 d. Representative images from one out of three experiments are shown.

GHRL is a potent growth hormone secretagogue, which is mainly produced by endocrine cells of the gastric oxyntic mucosa and linked to many physiological and pathophysiological aspects, such as bone metabolism, in addition to growth.[22,23] GHRL promotes proliferation and differentiation, as evidenced by matrix mineralization, alkaline phosphatase activity, osteoblast-specific gene
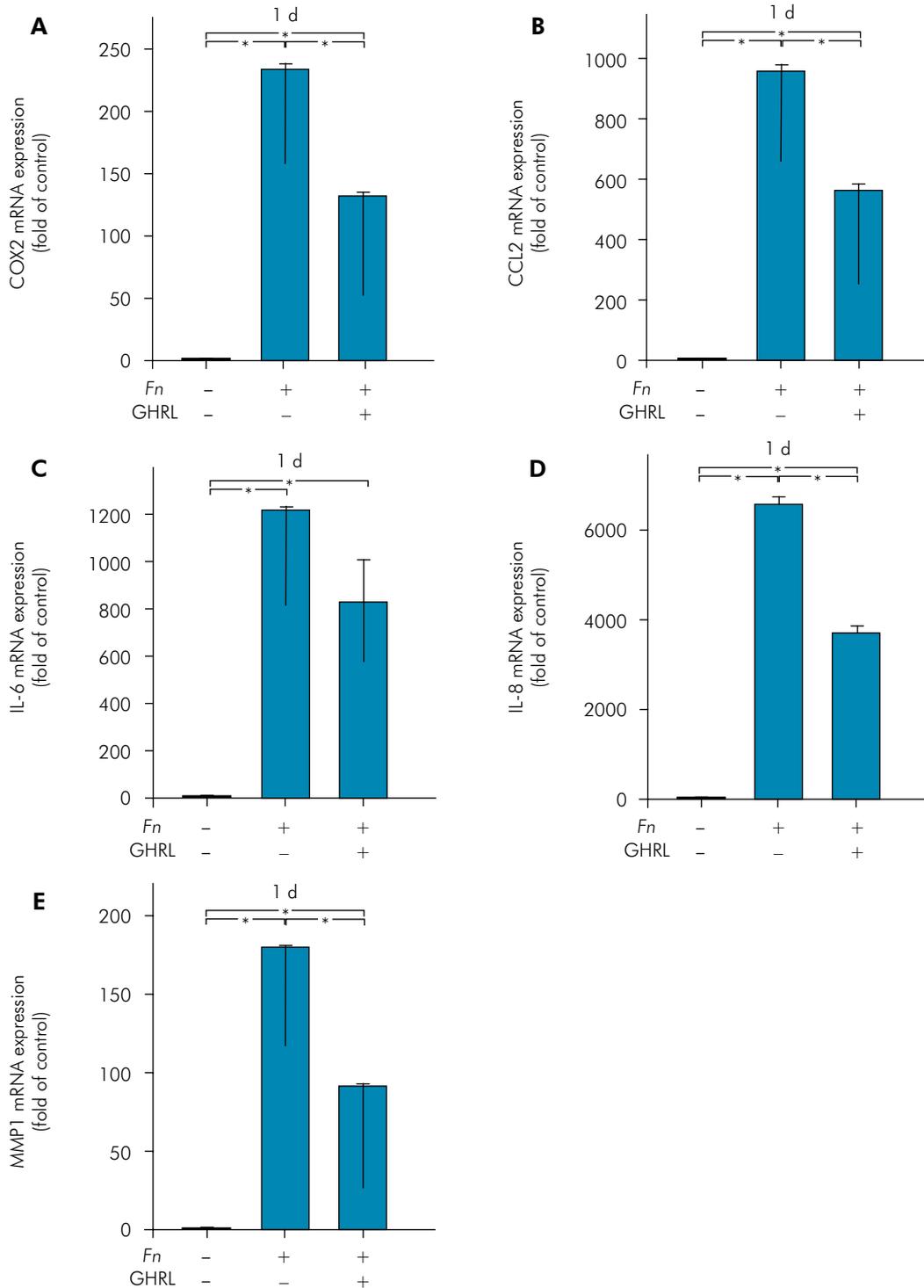
*significant (p < 0.05) difference between groups.

Figure 2. (A) NF-κB (p65) nuclear translocation in response to *F. nucleatum* (*Fn*; OD: 0.1) or IL-1β (1 ng/ml) over time. Representative images from one out of three experiments are shown. Indicator arrows show some cells presenting nuclear translocation of p65 at 60 min. (B) GHS-R1α expression in response to IL1β (1 ng/ml) in the presence and absence of a MEK1/2 inhibitor (U0126; 10 μM) at 1 d. Mean ± SEM (*n*=3).

expression, calcium accumulation in the matrix, inhibition of osteoblastic cells apoptosis, and exerts anabolic effect on osseous tissue.[7,24-26] GHRL has a positive relationship with the trabecular bone density.[27] However, the actions of GHRL on osteoclastogenesis are contradictory and it has even been postulated that the physiological effect of ghrelin on osteoclasts may be limited.[28] In-vivo
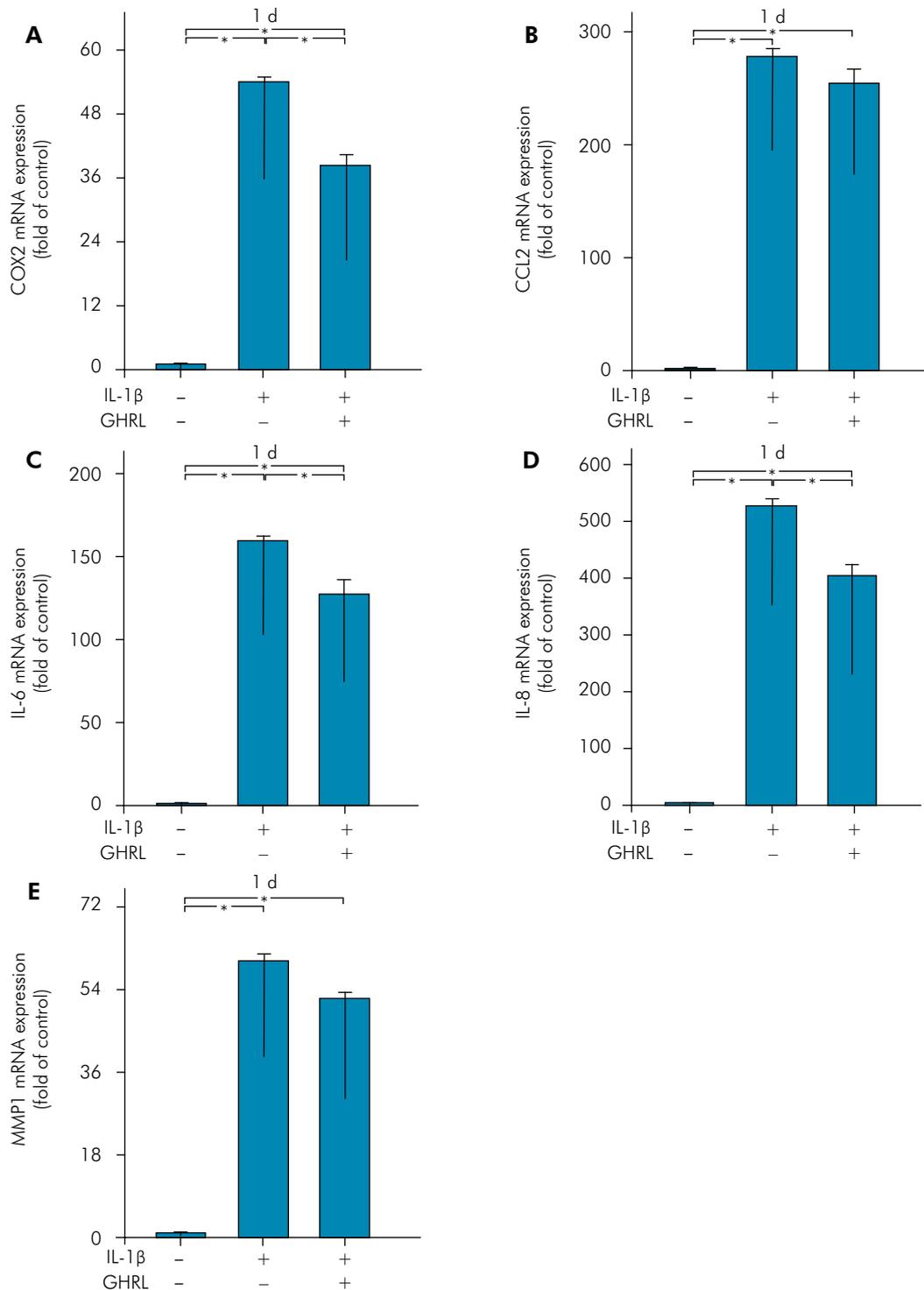
*significant (p < 0.05) difference between groups.

Figure 3. Expression of COX2 (A), CCL2 (B), IL-6 (C), IL-8 (D) and MMP1 (E) in response to *F. nucleatum* (*Fn*; OD: 0.1) in the presence and absence of GHRL (20 nM) at 1 d. Unstimulated cells served as control. Mean ± SEM (*n* = 3).

studies have demonstrated that intraperitoneal and central administration of GHRL enhance bone mineral density and mass.[27] Notably, GHS-R-null mice have a low bone mass phenotype with poor bone formation. Restoring the expression of GHS-R specifically in osteoblasts, and not

Figure 4. Expression of COX2 (A), CCL2 (B), IL-6 (C), IL-8 (D) and MMP1(E) in response to IL-1β (1 ng/ml) in the presence and absence of GHRL (20 nM) at 1 d. Untreated cells served as control. Mean ± SEM (*n*=3).

in osteoclasts or the central nervous system, ameliorated bone abnormalities in GHS-R-null mice, which indicates that the osteoblastic

expression of GHS-R is necessary and sufficient for the bone anabolic actions of GHRL.[28] GHRL promotes osteogenesis of intramembranous bone
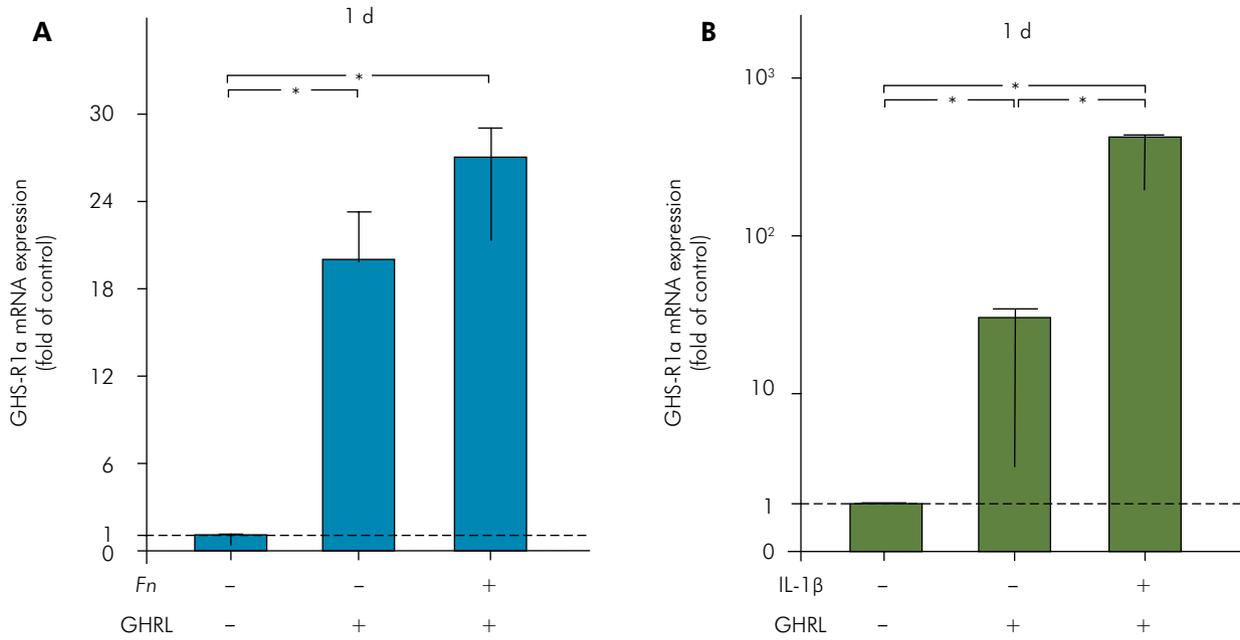
*significant (p < 0.05) difference between groups.

Figure 5. (A) GHS-R1a expression in response to GHRL (20 nM) in the presence and absence of *F. nucleatum* (*Fn*; OD: 0.1) at 1 d. Untreated cells served as control. Mean ± SEM (*n*=6). (B) GHS-R1a expression in response to GHRL (20 nM) in the presence and absence of IL-1β (1 ng/ml) at 1 d. Untreated cells served as control. Mean ± SEM (*n*=6).

and improves the repair of calvarial bone defect in rats.[29] Furthermore, clinical studies showed a positive association between circulating GHRL levels and trabecular bone mineral density in women and elderly men.[27] Moreover, gastrectomy results rapidly in osteopenia, independent of nutritional defects such as insufficient calcium absorption, which underlines the critical role of the stomach in bone remodeling.[30,31] Taken together, there is convincing evidence that GHRL exerts stimulatory effects on bone metabolism. GHRL has also been shown to be present in human osteoblasts and bone biopsies, suggesting an autocrine/paracrine role.[34] Pacheco-Pantoja et al. have demonstrated GHRL and GHS-R1a in osteoblast-like MG63 cells.[35] Taken together, the aforementioned studies show that GHRL can promote bone formation and, thereby, prevent bone loss.[7] Interestingly, *F. nucleatum* and IL-1β caused an upregulation of the functional receptor for GHRL in the present study, which suggests that the host may use this mechanism to maintain the bone volume and structure despite the presence of pathogens or

inflammatory mediators. Our previous studies on the GHRL/GHS-R1 system in fibroblasts support this assumption.[16,17] In addition to its beneficial actions on osteogenesis, GHRL has also the ability to inhibit inflammation.[9,24] In the present study, GHRL counteracted the proinflammatory and proteolytic effects of *F. nucleatum* and IL-1β on the osteoblast-like cells, which concurs with our previous findings in periodontal fibroblasts.[16,17] Both *F. nucleatum* and IL-1β induced an increase in the expressions of COX2, CCL2, IL-6, IL-8, and MMP1, which stimulate inflammation and thereby bone resorption. Therefore, the upregulation of the functional receptor for GHRL may not only protect bone loss through stimulation of osteogenesis but also through inhibition of bone resorption by reducing the proinflammatory effects of *F. nucleatum* and IL-1β. Cells response and tissues homeostasis can be affected by changes in ghrelin levels due to different physiological and/or pathological circumstances. Notably, GHRL stimulated the expression of its own receptor, suggesting a positive feedback to further amplify the bone-protective and

anti-inflammatory actions. However, the protective mechanisms may get lost at later stages of soft and hard tissue inflammation, as our previous in-vitro and in-vivo studies[16,17] have demonstrated that the continuous exposure to the inflammatory stimuli leads to a decrease in the expression of GHRL.

We also sought to unravel the intracellular signaling pathways involved in the stimulatory actions of *F. nucleatum* and IL-1β on GHS-R1a in osteoblast-like cells. Although both stimuli triggered an NF-κB nuclear translocation, pre-incubation of the cells with a specific inhibitor against NF-κB, did not abrogate the stimulatory effects of *F. nucleatum* and IL-1β, indicating that NF-κB might not be involved in the GHS-R1a upregulation. However, the GHS-R1a expressions induced by *F. nucleatum* or IL-1β was partially reduced in the presence of a specific inhibitor against MEK1/MEK2, two important components of the MAP kinase signaling pathway. Future studies should clarify what other signaling pathways are involved in the GHS-R1a regulation.

As in our previous studies, *F. nucleatum* was used to mimic microbial conditions in vitro.[17,36,37] This gram-negative, anaerobic microorganism acts as a bridge bacterium between early- and late-colonizing bacteria during plaque development, can invade periodontal cells, and is associated with both gingivitis and periodontitis.[20,21] However, since periodontitis is a polymicrobial disease, further experiments should also focus on the effects of other periodontopathogens or their combinations. Moreover, a sonicated lysate of *F. nucleatum* was applied in our experiments. Lipopolysaccharide, which binds to the toll-like receptor 2 and 4, as our previous studies have shown, might have been the major compound of this lysate.[17,36] Nevertheless, other virulence factors of *F. nucleatum* may also have participated in the GHS-R1a upregulation observed in our study. In our experiments, IL-1β was used to mimic inflammatory conditions in vitro, because this proinflammatory cytokine had been shown to be increased at inflamed periodontal sites.[18,19] Nevertheless, inflammation comprises a plethora of molecules, and further studies should also investigate their impact on the GHRL/GHS-R1a system. The applied concentrations of *F. nucleatum*, IL-1β and

GHRL were in the physiological range and the same as in our previous studies.[16,17,36,37]

In the present study, osteoblast-like MG63 cells were used instead of primary bone cells to avoid biological variations and to obtain reproducible results.[38,39] However, further studies should also involve primary alveolar osteoblasts to validate our findings. Moreover, the use of coculture models and ghrelin-knockout mice will further add to our understanding of the GHRL/GHS-R1a system under more complex and realistic conditions. Furthermore, GHRL might also mediate its effects on bone cells by binding to other receptors than GHS-R1a, which is another exciting topic of future research.

The present study expands the knowledge from our previous studies on the GHRL/GHS-R1 system in periodontal fibroblasts and soft tissue to bone cells. Since the periodontium also comprises hard tissues, such as bone, the findings of the present study are of utmost importance and contribute to a better understanding of the GHRL/GHS-R1 system in the entire periodontium. The GHRL/GHS-R1a system may serve as a potential target in periodontal disease and healing.

Conclusion

Our study provides original evidence that the periodontopathogen *F. nucleatum* and the proinflammatory cytokine IL-1β, which have been shown to be increased at inflamed periodontal sites, regulate the GHS-R1/GHRL system in osteoblast-like cells. Furthermore, we demonstrate for the first time that the proinflammatory and proteolytic actions of *F. nucleatum* and IL-1β on the osteoblast-like cells can be counteracted by GHRL. Our study suggests that microbial and inflammatory insults upregulate GHS-R1a, which may represent a protective negative feedback mechanism in human bone.

Acknowledgements

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