Follicle-stimulating hormone enhances alveolar bone resorption via upregulation of cyclooxygenase-2

Chunxia Zhu1*, Yaoting Ji1*, Shengbo Liu2, Zhuan Bian1

1The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory of Oral Biomedicine Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan 430079, Hubei, China; 2Department of Endodontics, School and Hospital of Stomatology, Wuhan University, Wuhan 430079, Hubei, China. *Equal contributors.

Received April 5, 2016; Accepted June 29, 2016; Epub September 15, 2016; Published September 30, 2016

Abstract: This study aimed to investigate whether follicle-stimulating hormone (FSH)-induced alveolar bone resorption was mediated by a cyclooxygenase 2 (COX-2) enzyme related mechanism. Experimental periodontitis was induced in bilateral ovariectomized (OVX) rats, some of which were injected with triptorelin, an FSH inhibitor. After mandibles were collected, we performed micro-computed tomography to evaluate alveolar bone loss and immunohistochemical staining to assess COX-2 expression. As well, human periodontal ligament cells (PDLCs) were treated with FSH (30 ng/ml), and the COX-2 mRNA and protein expression levels were measured by quantitative real-time polymerase chain reaction (qPCR) and Western blotting, respectively; prostaglandin E2 (PGE2) levels were measured by enzyme-linked immunosorbent assay (ELISA). The results indicated that FSH significantly increased alveolar bone resorption and the expression of COX-2 in the bilateral OVX + Ligatured rats compared with the other treatment groups. FSH also increased the mRNA and protein expression of COX-2 and PGE2 (P < 0.01) in human PDLCs. Further, the analysis of signaling pathways revealed the activation of COX-2-mediated pathways including Erk, p38, and Akt. These data suggest that FSH aggravates alveolar bone loss via a COX-2-upregulation mechanism and that the Erk, p38, and Akt pathways are involved in this pathological process.

Keywords: Follicle-stimulating hormone, periodontitis, cyclooxygenase 2, ovariectomy, alveolar bone loss

Introduction

In the past, the singular cause of postmenopausal osteoporosis has been attributed to declining estrogen levels. Likewise, a prior report indicated that follicle-stimulating hormone receptor (FSHR) knockout mice did not exhibit bone loss, and that the activation of the MEK/Erk, NF-κB, and Akt pathways by precursors possessing Gi2α-coupled FSHRs resulted in enhanced osteoclast formation and function, suggesting that high circulating follicle-stimulating hormone (FSH) causes hypogonadal bone loss independent of estrogen [1]. In addition, recent research indicated that the experimental induction of periodontitis in bilateral ovariectomized (OVX) rats resulted in more severe alveolar bone resorption than in non-OVX rats with periodontitis. Furthermore, leuprolerin (LE), an FSH inhibitor, had a protective effect on alveolar bone loss in OVX rats, indicating that after menopause, systemic FSH could contribute to the progression of periodontitis [2].

Cyclooxygenase (COX), which is also called prostaglandin endoperoxide synthase, is an enzyme that catalyzes the stepwise conversion of arachidonic acid into two short-lived intermediates, prostaglandin G2 (PGG2) and prostaglandin H2 (PGH2), which are then metabolized into various prostaglandins (PGs) by the activity of specific synthases [3, 4]. There are two primary COX enzymes including COX-1, which is constitutively expressed in most cells and regulates normal physiological responses, and COX-2, which is not expressed under normal conditions but is induced by interleukin-1, tumor necrosis factor-α, and lipopolysaccharide (LPS) under pathological conditions [5]. Therefore, COX-2 appears to be the primary COX enzyme that controls prostaglandin E2 (PGE2) synthesis in response to inflammation. COX-2 and COX-2-mediated PGE2 play a key role in periodontitis,
Mechanism of FSH promoting alveolar bone resorption

including stimulating inflammatory factors and increasing osteoclast differentiation [6, 7]. It was likewise indicated that FSH could regulate the expression of COX-2 and PGE$_2$ production in ovarian cancer [8]. However, whether a link exists between FSH and COX-2 expression in the periodontal system remains unknown.

Prior research has demonstrated that the activated Erk1/2 [9, 10], p38 [10], and Akt [11] pathways participate in the signaling cascades that mediate the upregulation of COX-2 expression and PGE$_2$ production following exposure to pro-inflammatory factors in human periodontal ligament cells (PDLCS). Further, the Akt signaling pathway and its downstream protein, glycogen synthase kinase 3β (GSK3β), reportedly regulate COX-2 expression [12, 13]. The objectives of the current study were to investigate the hypothesis that COX-2 mediates FSH-induced alveolar bone loss and to elucidate the potential mechanism involved in this pathological process.

Materials and methods

Experimental groups

Eight-week-old female Sprague-Dawley rats, weighing between 200 and 220 g, were obtained from The Provincial Center for Disease Control (Wuhan, China). The rats were housed under standard conditions and had ad libitum access to food and water. All experimental protocols involving the rats were approved by the Wuhan University Institutional Animal Care and Use Committee. The rats were randomly assigned to four groups, including the bilateral OVX + Ligatured (L) group (Group 1; n = 8), the bilateral OVX + L + 1.6 mg/kg triptorelin group (Group 2; n = 7), the sham surgery (SHAM) + L group (Group 3; n = 6), and the SHAM + L + 1.6 mg/kg triptorelin group (Group 4; n = 6).

Treatments

The rats were anesthetized following a subcutaneous injection of a 4% chloral hydrate mixture (0.35 ml/100 g). Bilateral ovariectomies were performed in Groups 1 and 2, and rats in Groups 3 and 4 were subjected to SHAM ovariectomies, which entailed the resection of an equivalent size area of fatty tissue near the ovary. Triptorelin, a gonadotropin-releasing hormone agonist (Ipsen Pharma, Senna, France) was subcutaneously injected immediately after surgery. Once injected, triptorelin remained at a biologically effective concentration in the blood and inhibited the rise in circulating FSH levels for 4 weeks [14, 15].

To induce experimental periodontitis prior to OVX, surgeries were performed to place a sterile thread ligature around the cervix of the first mandibular molars as described in previous studies [2, 16]. All rats were euthanized by the administration of an overdose of anesthetic (4 weeks after surgery), and the mandibles were extracted and fixed in 4% paraformaldehyde. Histological analysis was performed on the left mandibles and micro-computed tomography (CT) scanning was performed on the right mandibles.

Micro-computed tomography

A mCT 50 micro-CT scanning system (Scanco Medical AG, Bassersdorf, Switzerland) was used to evaluate bone loss surrounding the right first mandibular molars. The micro-CT parameters were as follows: slice thickness, 20 μm; pixel size, 1024 × 1024; voltage, 70 kV; and electrical current, 114 μA.

According to a previous study [17], a fixed radius and length of the cylindrical region beginning at the furcation area and between the medial and distal root of the first mandibular molar was selected for the calculation of the bone volume fraction (BV/TV) for all of the samples (n = 6).

The mandibles were reconstructed using the Mimics® 17.0 software (Materialise Software, Leuven, Belgium). After reconstruction, the distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured along the buccal and lingual long axis of the root surfaces of the first molars [18]. Six sites were established per tooth, and the mean of the six values was calculated as the bone loss for each rat (n = 6). A trained and calibrated observer blinded to the groups carried out the measurements.

Immunohistochemistry

Specimens were embedded in paraffin and 4-μm thick serial sections were generated. A Streptavidin-Peroxidase (SP) kit (Zhongshan, Beijing, China) was then used to perform immunohistochemistry. A monoclonal antibody
against COX-2 was used as the primary antibody (Cayman Chemical, MA, USA). The expression of COX-2 in the slices was visualized using the avidin-biotin-peroxidase complex reaction. The slices were then counterstained with Mayer’s Hematoxylin Solution.

All of the slices were evaluated by light microscopy. Two trained and calibrated observers blinded to the groups performed the counting. In the event of a discrepancy, a consistent result in one field was achieved following a discussion between the two observers. We selected one slide from each sample (n = 5 samples for each group) and randomly examined two fields in the furcation region of each slide at a 400 × magnification. The mean value of these two fields represented the data for each sample.

Cell culture and characterization

Primary human PDLCs were obtained from the Wuhan University School of Stomatology. The human PDLCs were obtained from the premolar teeth for orthodontic reasons, and informed consent was obtained from each patient. The PDL tissues, which were attached to the mid-third of the root, were removed, cut into approximately 1 mm² pieces, and placed in T25 culture bottles under sterile conditions. The tissues were cultured in Dulbecco's Modified Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂ in compressed air. After confluency, the PDLCs were trypsinized and used in subsequent experiments at passages 4 or 5.

Immunofluorescence staining for type I collagen (COLI), fibronectin, vimentin, and keratin was performed to characterize the cell lineage of PDLCs, as previously described [19, 20]. PDLCs were seeded on slides, fixed with 4% (w/v) paraformaldehyde, blocked with 3% bovine serum albumin, and incubated with primary antibodies against vimentin, keratin, COLI, and fibronectin overnight at 4°C. The slices were then incubated with a fluorescent secondary antibody for 1 h at room temperature. Finally, 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Phosphate buffered saline (PBS) was used as a negative control. To characterize FSHR in PDLCs, a non-fluorescent secondary antibody was used and 3,3'-Diaminobenzidine (DAB) was used instead of DAPI to stain the nuclei.

PDLC treatments

The PDLCs were seeded in six-well plates at a density of 1 × 10⁶ cells per well and were allowed to attach for 12 h. The cells were then silenced by treatment with phenol red-free DMEM (Jinuo, Hangzhou, China) overnight. The PDLCs were subsequently treated with human recombinant FSH (30 ng/ml, Sigma, St. Louis, MO, USA) for 0, 2, 4, 6, 8, 12, and 24 h. To eliminate the effects of the different types of hormones, phenol red-free DMEM with 5% charcoal-stripped FBS (Serana, Aidenbach, Germany) was used.

To investigate the signaling pathways involved in FSH-induced COX-2 expression, the activated forms of Erk, p38, and Akt were examined in PDLCs at 0, 10, 15, 30, and 60 min after treatment with FSH. The PDLCs were then preincubated with PD98059 (5 µmol/L, Cell Signaling Technology, Inc., Beverly, MA, USA), a specific inhibitor of the Erk pathway, and SB203580 (Enzo, Biochem, Inc., New York, USA), a specific inhibitor of the p38 and Akt pathways, for 2 h before treating them for 6 h with FSH. SB203580 is a specific inhibitor of both the p38 and Akt pathways when used in different concentrations. Accordingly, 0.3 µmol/L was used for the p38 pathway, and 3 µmol/L was used for the Akt pathway. LPS (20 µg/ml, Sigma, St. Louis, MO, USA) was used as a positive control and PBS was used as a negative control.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from PDLCs using an RNAiso Plus kit (TaKaRa, Tokyo, Japan). A total of 1 µg of isolated RNA was used for cDNA synthesis using reverse transcription reagents (Roche, Switzerland), according to the manufacturer’s instructions. The COX-2 and β-Actin primer sequences were as follows: COX-2 Forward 5'-TTTCTACCAAGGGCAGGAT-3' and COX-2 Reverse 5'-TATCACAGGCTTCCATTGAC-3', and β-Actin Forward 5'-GCACCGTCAAGGCTGAGAAC-3' and β-Actin Reverse 5'-ATGGTGGTGAGAGCAGG-3' CCAGT. A 20-μL reaction mixture containing 10 μL SYBR Green Master, 2 μL primer mix, 2 μL cDNA, and 4 μL double-distilled water was prepared for each sample for the qPCR analyses. PCR conditions consisted of a 10 min preincubation step at 95°C followed by 45 cycles of denaturation (95°C, 10
Mechanism of FSH promoting alveolar bone resorption

s), annealing (60°C, 30 s), and extension (72°C, 1 min). All qPCR experiments were run in triplicate. A negative control containing water instead of sample cDNA was used in each plate. The data were analyzed according to the 2-ΔΔCT method, and the values were normalized to the housekeeping gene, β-Actin. All data are expressed as mean ± SD of three independent experiments.

Western blotting

After treatment, the PDLCs were washed in PBS and then lysed in radioimmunoprecipitation assay (RIPA) lysis buffer. After 10 min on ice, the lysate was transferred to a 1.5 ml Eppendorf microcentrifuge tube and centrifuged at 14,000 × g at 4°C for 10 min. The resulting protein (15 µg) was boiled with SDS-PAGE sample buffer, separated on 10% SDS-PAGE separation gels, and transferred to a PVDF membrane. The membranes were blocked with 5% nonfat milk in PBS containing 0.05% Tween-20 at room temperature for 2 h and then incubated with primary antibody overnight at 4°C. Proteins were detected by an enhanced chemiluminescence system followed by exposure to X-ray film. All of the Western blots were independently prepared in triplicate.

PGE_2 enzyme-linked immunosorbent assay (ELISA)

Concentrations of PGE_2 in the culture supernatants were determined using an ELISA kit according to the manufacturer’s recommendations (Cayman Chemical, MA, USA). Synthesized PGE_2 concentrations were normalized against protein concentrations measured in the corresponding whole-cell extracts. All data are expressed as mean ± SD of three independent experiments.

Statistical analysis

The data in this study are expressed as the mean ± SD. All of the qPCR, Western blting, and ELISA analyses were independently conducted in triplicate. The results of all analyses were compared by one-way ANOVA with Tukey’s post hoc tests using the SPSS17.0 software package (Chicago, IL, USA). P < 0.05 was considered statistically significant.

Results

Characterization of PDLCs

In accord with previous studies, primary PDLCs were fibroblast-like, highly transparent, and proliferative. The cells were likewise positive for...
Mechanism of FSH promoting alveolar bone resorption

the expression of COLI, fibronectin, and vimentin, but were negative for keratin expression, which indicated a mesenchymal origin. No specific staining was visualized in the negative control (Figure 1).

Alveolar bone loss

The three-dimensional images from the four groups are shown in Figure 2A. BV/TV was quantitatively analyzed in all groups, and the OVX + L group exhibited a significantly lower BV/TV compared with the other groups (P < 0.05; Figure 2B).

The mean CEJ-ABC distances of the four groups were 0.9 ± 0.01 mm (OVX + L), 0.71 ± 0.07 mm (OVX + L + triptorelin), 0.769 ± 0.08 mm (L + triptorelin), and 0.76 ± 0.08 mm (L) (Figure 2C). As well, significant differences in the mean CEJ-ABC distances were detected between the OVX + L + triptorelin and the L-only groups (P < 0.05), and between the OVX + L + triptorelin and the OVX + L groups (P < 0.05).

Figure 2. Effect of FSH on alveolar bone loss. After scanning, the reconstruction of mandibles and the distance from the CEJ to the ABC were processed using Mimics® 17.0 software and the bone fraction was analyzed using a micro-CT scanning system. A. The reconstructed three-dimensional micro-CT images of the four groups show buccal and lingual views of the first mandibular molars. The distance from the CEJ to the ABC was measured as a reference for bone loss and is shown by the white lines. The black arrow shows the area where the alveolar bone was resorbed. B. The bone fraction (BV/TV) in the interradicular regions of the first mandibular molars of each group. *= P < 0.05 versus the SHAM + L group and † = P < 0.05 versus the L group. No statistically significant differences were detected between the OVX + L + triptorelin and the SHAM groups or between the SHAM groups. C. The mean CEJ-ABC distances surrounding the first mandible molars in each group. ‡ = P < 0.05 versus the OVX + L + triptorelin group and § = P < 0.05 versus the L group. No statistically significant differences were detected between the OVX + L + triptorelin and the SHAM groups or between the SHAM groups. Data are expressed as mean ± SD.
**Mechanism of FSH promoting alveolar bone resorption**

**FSH-induced COX-2 expression in OVX rats and in non-OVX rats with experimental periodontitis**

COX-2 expression was detected by immunohistochemical staining in the periodontium, particularly in the periodontal ligament under the root furcation. As shown in Figure 3A, COX-2-positive cells were stained brown or yellow. The number of COX-2-positive cells in OVX + L rats was significantly higher than in the L-only rats (Figure 3B, P < 0.01). Furthermore, the administration of triptorelin significantly decreased the expression of COX-2 in the OVX + L rats (P < 0.05). No significant differences were found between the SHAM groups.

**Effect of FSH on COX-2 expression in PDLCs**

PDLCs exhibited positive expression for FSHR with immunocytochemical staining (Figure 4A); however, no FSHR expression was detected in the control (Figure 4B). The effect of FSH on

---

*Figure 3.* Follicle-stimulating hormone (FSH) increases cyclooxygenase-2 (COX-2) expression. Serial sections (4-µm) were generated and an SP kit was used to perform immunohistochemistry. A. Immunohistochemical staining for COX-2 in the interradicular regions of the first mandibular molars (original magnification: 400 ×, Bar: 50 µm). B. Quantitative analysis of COX-2-positive cells. Data are expressed as mean ± SD (/ hpf = per high-power field). The sample size was 5 for each group. **P < 0.01 versus the SHAM + L group and † P < 0.05 versus the OVX + L + triptorelin group.*
Mechanism of FSH promoting alveolar bone resorption

COX-2 expression was examined in PDLCs, which express a functional FSHR (Figure 4C).

PDLCs were treated with human recombinant FSH for 0, 2, 4, 6, 8, 12, and 24 h to investigate the role of COX-2 in alveolar bone loss. The results indicated that the expression of COX-2 occurred in a time-dependent manner in PDLCs treated with FSH (Figure 4D). COX-2 mRNA expression was also upregulated in a time-dependent manner (Figure 4E).

FSH-induced COX-2 upregulation via the p38, Erk, and Akt pathways in PDLCs

We evaluated whether the Erk, p38, and Akt pathways were activated in conjunction with FSH-induced COX-2 expression in PDLCs. First, Erk, p38, and Akt phosphoprotein and total protein levels were examined at 0, 10, 15, 30, and 60 min after treatment with 30 ng/ml FSH. The results indicated that FSH stimulated the activation of p-Erk, p-p38, and p-Akt (Figure 5A),...
Mechanism of FSH promoting alveolar bone resorption

whereas the expression of Akt and p38 expression remained unchanged, although Erk expression increased over time and p-Erk expression was increased based on Grayscale analysis (data not shown).

The effect of FSH on COX-2 expression in PDLCs after blocking the Erk, p38, and Akt pathways was also investigated. PDLCs were pretreated with PD98059, a selective Erk inhibitor, and SB203580, a selective Akt and p38 inhibitor,
Mechanism of FSH promoting alveolar bone resorption

for 2 h prior to treatment with FSH. The selective inhibitors of Erk, p38, and Akt markedly inhibited FSH-induced COX-2 expression (Figure 5B, 5C), indicating that activated Erk, p38, and Akt pathways participate in the signaling cascades that mediate the upregulation of COX-2 expression in PDLCs.

**FSH-mediated upregulation of PGE$_2$ production**

The level of PGE$_2$ released from PDLCs was measured by ELISA to determine whether the increase in COX-2 expression was associated with increased PGE$_2$ synthesis. The results showed that PGE$_2$ was significantly increased in PDLCs treated with FSH (Figure 5D, P < 0.01), indicating that FSH participates in PGE$_2$ production. PGE$_2$ levels were then measured in PDLCs treated with FSH after the Erk, p38, and Akt pathways were blocked, which revealed that blocking these pathways markedly repressed the expression of PGE$_2$ (Figure 5E, P < 0.01). The trend for PGE$_2$ production was the same as that for COX-2 expression, which suggests that COX-2 is related to PGE$_2$ production in PDLCs treated with FSH.

**Discussion**

Periodontitis is a chronic and destructive disease of the periodontium caused by inflammation of the periodontal tissue and alveolar bone absorption [5]. The progression of periodontal inflammation is regulated by systemic and oral environmental factors, including the changes in physiological hormone levels during postmenopause [21, 22]. In the past, postmenopausal osteoporosis has been attributed solely to declining estrogen levels. As well, estrogen replacement therapy has been effective in protecting from systemic bone loss and decreased alveolar bone loss [23-25]. However, a previous study indicated that high circulating FSH levels, not estrogen, caused the hypogonadal bone loss [2]. One of the complications of diseases characterized by high FSH levels, such as in Turner’s syndrome, is osteoporosis [26]. Further, clinical studies have shown a strong association between serum FSH levels and markers of bone resorption [27-29], which indicates that the rise in FSH appears to contribute to higher bone resorption.

In our animal model, we showed that FSH increased alveolar bone loss independent of estrogen. We observed that COX-2 expression was upregulated in OVX + L rats when compared with the L-only groups, and we demonstrated that the administration of triptorelin significantly inhibited COX-2 expression and alveolar bone loss. Hence, our results were in accord with the findings of the previous study indicating that FSH could increase alveolar bone loss and that the specific inhibitors of FSH had a protective effect on bone loss.

In the present study, we demonstrated for the first time that FSH induced the production of COX-2 and PGE$_2$ in a time-dependent manner in PDLCs. COX-2 serves as the main precursor of prostaglandin synthesis and is responsible for PGE$_2$ production in periodontitis. In addition, numerous studies have indicated that COX-2 and PGE$_2$ play a key role in the pathogenesis of periodontal disease [5, 30-32]. The content of PGE$_2$ in the gingival fluid is one of the criteria used to measure the severity of inflammation in periodontitis [33]. Further, in vitro studies have demonstrated that COX-2 and PGE$_2$ stimulate receptor activator of nuclear factor kappa-B ligand (RANKL) expression, downregulate osteoprotegerin (OPG) expression in various periodontal cell types, and that disruption of the balance of RANKL/OPG stimulates osteoclastogenesis [5, 9, 34]. Our results suggest that COX-2 and PGE$_2$ are the primary mediators of FSH-induced inflammatory response in PDLCs.

Due to the limitations of the animal model, we conducted in vitro experiments using PDLCs to explore the underlying mechanism in FSH-induced alveolar bone loss. It has been demonstrated that the Erk, p38, and Akt pathways are independently involved in COX-2/PGE$_2$ expression in stimulated PDLCs [31]. Hence, an anti-inflammatory drug could reduce enhancement of COX-2 by inflammatory cytokines by blocking the Akt and p38 pathways [11, 35]. Our study indicated that the Erk, p38, MAPK, and P13K/Akt pathways were activated by stimulation of FSH, and that the FSH-induced activation of COX-2/PGE$_2$ expression was significantly reduced by pretreatment with specific inhibitors of Erk, p38, and MAPK in PDLCs. However, we failed to detect the relationships between the cascades among these pathways. Hence, whether cross-talk existed in FSH-induced COX-2/PGE$_2$ production remains to be clarified.
Mechanism of FSH promoting alveolar bone resorption

PGE$_2$ production, suggesting that FSH increases alveolar bone loss through a COX-2-upregulated mechanism, and that the Erk, p38, and Akt pathways are involved in this pathological process. The results of our study provide additional evidence that FSH aggravates alveolar bone loss, as well as a more comprehensive understanding of the role of FSH in alveolar bone metabolism.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81120108010, 81470727 and 81102228 to Zhuan Bian and Shengbo Liu).

Disclosure of conflict of interest

None.

Address correspondence to: Zhuan Bian, The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory of Oral Biomedicine Ministry of Education, School and Hospital of Stomatology, Wuhan University, 237 Luoyu Road, Wuhan 430079, China. Tel: +86-27-87647443; Fax: +86-27-87647443; E-mail: bianzhuan@whu.edu.cn

References


Mechanism of FSH promoting alveolar bone resorption


