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Serotonin acts as a novel regulator of interleukin-6 secretion in osteocytes through the activation of the 5-HT_{2B} receptor and the ERK1/2 signalling pathway

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ABSTRACT

Interleukin-6 (IL-6) is a potent stimulator of osteoclastic bone resorption. Osteocyte secretion of IL-6 plays an important role in bone metabolism. Serotonin (5-HT) has recently been reported to regulate bone metabolism. The aim of this study was to evaluate the effect of serotonin on osteocyte expression of IL-6. The requirement for the 5-HT receptor(s) and the role of the extracellular signal-regulated kinase 1/2 (ERK1/2) in serotonin-induced IL-6 synthesis were examined. In this study, real-time PCR and ELISA were used to analyse IL-6 gene and protein expression in serotonin-stimulated MLO-Y4 cells. ERK1/2 pathway activation was determined by Western blot. We found that serotonin significantly activated the ERK1/2 pathway and induced IL-6 mRNA expression and protein synthesis in cultured MLO-Y4 cells. However, these effects were abolished by pre-treatment of MLO-Y4 cells with a 5-HT_{2B} receptor antagonist, RS127445 or the ERK1/2 inhibitor, PD98059. Our results indicate that serotonin stimulates osteocyte secretion of IL-6 and that this effect is associated with activation of 5-HT_{2B} receptor and the ERK1/2 pathway. These findings provide support for a role of serotonin in bone metabolism by indicating serotonin regulates bone remodelling by mediating an inflammatory cytokine.

1. Introduction

Serotonin or 5-hydroxytryptamine (5-HT), a well-known neurotransmitter in the central nervous system (CNS), is implicated in many psychiatric and neurological pathophysiologies. However, 95% of serotonin is found outside of the brain [1]. In the periphery, serotonin plays a multifunctional role serving as a paracrine factor to stimulate peristalsis and as an endocrine factor that mediates homeostasis, liver regeneration and cardiovascular activity [2–6].

Recent studies have revealed that, the skeleton is another target for serotonin activity. Yadav et al. report that the inhibition of peripheral serotonin production both prevented and treated osteoporosis [7]. Additionally, clinical observations indicate that selective serotonin re-uptake inhibitors (SSRIs), a widely used class of antidepressants, are associated with reduced bone mineral density [8–11]. However, the mechanism by which serotonin regulates bone metabolism remains controversial. Some researchers report that serotonin inhibits osteoblast proliferation [12,13] and promotes osteoclastogenesis [14,15], whereas other studies point to an opposite effect [16] or no effect [17,18].

IL-6 is a highly pleiotropic factor influencing many biological events in several organs [19]. It has been demonstrated that IL-6 is an important target of serotonin in the cardiovascular system. By triggering IL-6 secretion in smooth muscle cells, serotonin promotes the initiation or the progression of coronary atherosclerosis by activating the IL-6 mediated inflammatory processes in the vascular system [20]. The serotonin receptor antagonist, MCI, rescues IL-6 induced pulmonary hypertension [21]. Additionally, the connection between serotonin and IL-6 has been documented in the CNS, the adrenal gland and the liver, as increased IL-6 levels were found in the serotonin-stimulated zona glomerulosa cells [22], microglial cells [23] and DOI (5-HT_2 receptor agonist)-stimulated liver [24]. However, the connection between serotonin and IL-6 in bone requires further study. IL-6 plays a pivotal role in osteoarticular pathologies. The depletion of IL-6 significantly protects mice from bone destruction in both arthritis and osteoporosis models [25,26]. High IL-6 levels are

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detected in bone disorders, including trauma, rheumatoid arthritis, osteoporosis, and periodontitis [27–30]. Additionally, local serotonin levels rise in the bone diseases listed earlier [31–34]. Thus, we propose that in addition to its direct effects on osteoblast and osteoclasts, serotonin also regulates bone turnover by mediating the activity of IL-6.

Osteocytes are the most abundant and long-lived cells in bone. Past studies have shown that osteocytes are not only the mechanical sensor in bone but also the orchestrators of bone remodelling. However, the role of osteocytes in serotonin-regulated bone metabolism is unknown. It is known that the 5-HT receptors (5-HT_{1A}, 5-HT_{2A} and 5-HT_{2B} receptors), the 5-HT transporter (5-HTT), and the enzyme for serotonin synthesis (TPH-1) are expressed in osteocytes [35,36]. Moreover, higher levels of tryptophan, the precursor of serotonin, are detected in osteocytes and in their environment in an alcohol-induced osteoporosis model [37]. These results suggest a role for osteocytes in the function of serotonin in bone. In fact, the osteocyte network is an important source of soluble factors that regulate bone turnover [38]. Previously, we have demonstrated that mechanical stimulation could trigger IL-6 secretion in osteocytes [39]. As osteocytes compose 90–95% of bone cells [38], the osteocyte network may be an important source of IL-6 in bone, and fluctuations in levels of osteocyte-secreted IL-6 may contribute to bone remodelling. The activity of serotonin influences IL-6 secretion in many cell types. We hypothesized that serotonin may mediate IL-6 secretion in osteocytes and, in turn, may regulate bone metabolism. To test our hypothesis, we chose osteocytic MLO-Y4 cells to study the regulation of IL-6 secretion by serotonin in osteocytes and to identify the mediating receptor and signal transduction pathway.

2. Materials and methods

2.1. Cell culture

The cell line MLO-Y4 was generously gifted by Dr. Lynda F. Bonewald (Department of Oral Biology, University of Missouri at Kansas City, Kansas City, Missouri, USA). Cells were cultured in collagen-coated (rat tail collagen type I, 0.15 mg/ml in 0.02 N acetic acid; BD Biosciences, USA) flasks in α-modified essential medium (Hyclone, USA) supplemented with serotonin-depleted [40] 5% foetal bovine serum (Hyclone, USA) and 5% calf serum (Hyclone, USA).

2.2. RT-PCR

Total RNA (1 μg) was isolated using TRIzol Reagent (Invitrogen, USA) and reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (DRR047A; Takara, Japan). The RT-PCR test was performed by using SYBR® Premix RX Taq™ II (DRR820A; Takara, Japan) in the ABI PRISM 7300 Fast Real-Time PCR System. The primer sets (mouse) used were as follows: $\text{IL-6}_{forward}$: 5′-GACAAAGCCAGAGTCCTTCAGAG-3′; $\text{IL-6}_{reverse}$: 5′-TCCTTAGCCACTCCTTCT GTGAC-3′; $\text{GAPDH}_{forward}$: 5′-GACATCAAGAAGGTGGTGAAGC-3′; $\text{GAPDH}_{reverse}$: 5′-GAAGGTGGAAGAGTGGGAGTT-3′. The fold change was calculated as follows: $2^{-\Delta\Delta Ct}$, in which, $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$, $\Delta Ct = Ct_{target\ gene} - Ct_{GAPDH}$.

2.3. ELISA

The concentration of IL-6 was determined with an ELISA kit (Ray Biotech Inc, USA) according to the manufacturer's instructions. After incubation, supernatants of MLO-Y4 cell culture were collected and centrifuged at 1000*g* for 10 min to remove the pellet and cellular debris. Samples were stored at −80 °C. The optical absorbance of each well was measured in an ELISA reader (HTS7000+, USA) at 450 nm. Each sample was tested in duplicate.

2.4. Protein isolation and Western blot analysis

Total protein of MLO-Y4 cells was collected with a total protein extraction kit (KeyGen Biotech, China) and stored at −80 °C. Each sample was quantitatively assayed using the BCA method. Equal amounts of protein (20 μg) from each sample were diluted with SDS sample buffer (0.125 M Tris–HCl, pH = 6.8; 10% glycerol; 2% b-mercaptoethanol; 2% SDS; 0.1% bromophenol blue) and boiled for 5 min. Samples were resolved by SDS–PAGE and electro-transferred to PVDF membrane. Nonspecific protein binding was blocked by incubating the membrane with 5% (w/v) dried skim milk-TBST (10 mm Tris–HCl, pH 8.0; 150 mm NaCl; 0.05% Tween 20) for 1 h at room temperature. The membranes were washed in TBST and then incubated with anti-ERK1/2 or anti-phospho-ERK1/2 antibodies (Cell Signalling Technology, Medford, MA, USA) at 4 °C overnight. The membranes were washed with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membrane was washed in TBST to remove excess secondary antibody. The protein bands were visualized using the ECL detection system (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. Results were reproduced in three independent experiments with different samples.

2.5. Statistical analysis

The data were analysed by ANOVA of 16.0 SPSS (USA) for multiple comparisons between pairs, and a $p < 0.05$ was considered significant. All data are presented as the mean ±SD of triplicates.

3. Results

3.1. Effects of 5-HT on IL-6mRNA production

To test the effects of 5-HT on IL-6 expression, MLO-Y4 cells were incubated with 5-HT of various concentrations (10 nmol/L–10 μmol/L) for 4 h. As shown in Fig. 1A, 5-HT significantly increased IL-6 expression at 100 nM, 500 nM, 1 μM and 10 μM. However, 10 nM 5-HT failed to stimulate IL-6 expression. Among these concentrations, 1 μM 5-HT induced the highest increase in IL-6 expression (greater than 15-fold, $p < 0.05$ Fig. 1A).

We then evaluated IL-6 expression at different time points after 5-HT stimulation. MLO-Y4 cells were incubated with 1 μM 5-HT for various durations (1, 2, 4, 8 and 24 h). As shown in Fig. 1B, IL-6 mRNA expression increased significantly after 2 h of treatment, reached a peak at 4 h and then gradually decreased from peak levels. Nonetheless, 5-HT-stimulated cells expressed higher levels of IL-6 mRNA than the control level after 24 h ($p < 0.05$).

3.2. Effects of 5-HT on IL-6 production

The expression level of the IL-6 protein was measured by ELISA. As shown in Fig. 2A, 5-HT (1 μM) increased IL-6 level in a time-dependent manner. The expression level of IL-6 was significantly elevated from 4 h. After a 24-h incubation, the levels of IL-6 in the presence of 5-HT were approximately threefold higher than the control ($p < 0.05$).

3.3. 5-HT induces IL-6 increase in osteocytes via 5-HT_{2B} receptor

5-HT_{1A}, 5-HT_{2A} and 5-HT_{2B} receptors have been identified in osteocytes, and 5-HT_2 receptors have been shown to be involved

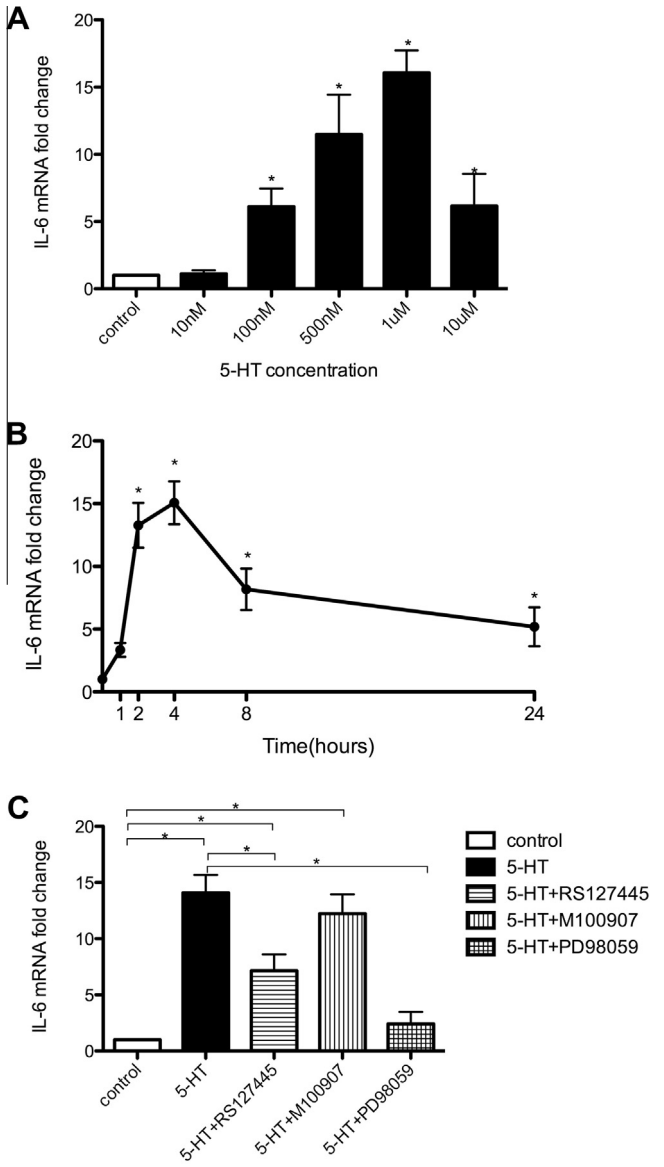

Fig. 1. Effects of 5-HT on IL-6 mRNA expression $^{*}p < 0.05$. (A) Effects of different concentrations of 5-HT: MLO-Y4 cells were incubated with various concentrations of 5-HT (10 nM, 100 nM, 500 nM, 1 μM and 10 μM) for 4 h. (B) Effects of incubation time: cells were incubated with 1 μM 5-HT for 1, 2, 4, 8 and 24 h, respectively. (C) Effects of serotonin receptor antagonists and ERK1/2 inhibitor: cells were pre-treated with 5-HT_{2B} receptor antagonist (RS127445, 20 nM, 20 min), 5-HT_{2A} receptor antagonist (M100907, 20 nM, 20 min), ERK1/2 inhibitor (PD98059, 10 μM, 30 min) respectively followed by 1 μM 5-HT treatment. IL-6 mRNA levels were determined after 4 h of 5-HT stimulation.

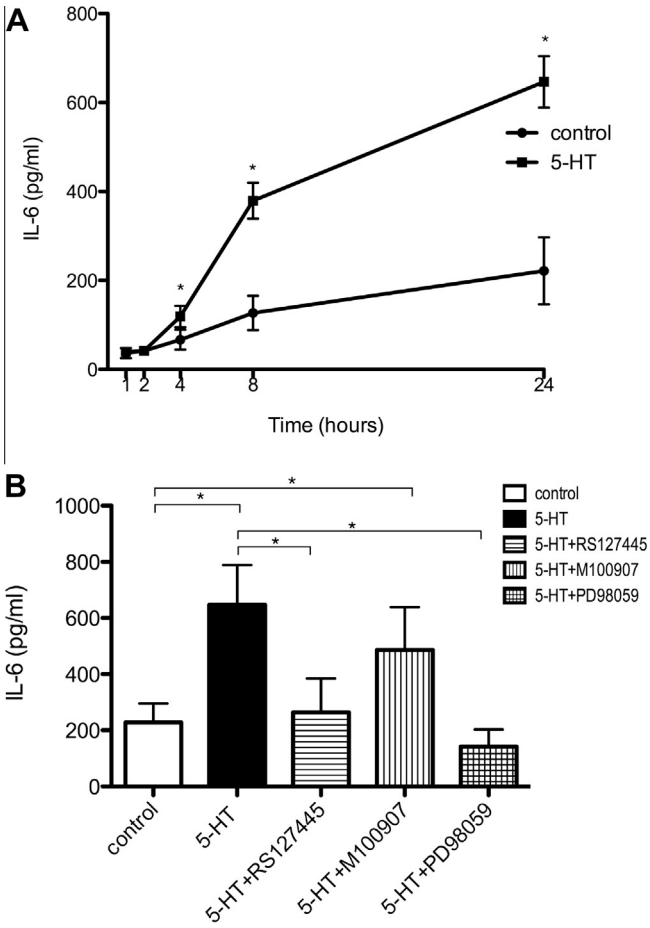

Fig. 2. IL-6 level determined by ELISA $^{*}p < 0.05$. (A) Effects of 5-HT incubation time: MLO-Y4 cells were incubated with or without 1 μM 5-HT for 1, 2, 4, 8 and 24 h, respectively. (B) Inhibitory effects of serotonin receptor antagonists and ERK1/2 inhibitor: cells were pre-treated with RS127445, M100907, PD98059 respectively followed by 5-HT treatment. IL-6 levels were determined after 24 h of 5-HT stimulation.

in IL-6 secretion [16,20]. Thus, the selective 5-HT_{2A} receptor antagonist M100907 and the selective 5-HT_{2B} receptor antagonist RS127445 were used to determine which 5-HT receptor subtype is involved in the 5-HT-induced IL-6 increase in MLO-Y4 cells. As shown in Fig. 1C, pre-treatment with RS127445 (20 nM; 20 min) but not M100907 (20 nM; 20 min) significantly inhibited 5-HT induced IL-6 mRNA expression. Additionally, we investigated the effect of RS127445 and M100907 on 5-HT-induced IL-6 secretion by ELISA. As shown in Fig. 2B, IL-6 increase is barely detectable in the presence of RS127445, whereas M100907 showed no inhibitory effect. MLO-Y4 cells were pre-incubated with the ERK inhibitor PD98059 (10 μM; 30 min) and followed by 5-HT treatment. As shown in Figs. 1C and 2B, PD98059 abolished 5-HT-induced IL-6 mRNA and protein expression. These data suggest that 5-HT may induce IL-6 secretion via the 5-HT_{2B} receptor and the ERK1/2 signal transduction pathway.

3.4. ERK1/2 is involved in 5-HT-induced IL-6 expression and secretion in osteocytes

We determined the levels of activation of ERK1/2 after 5-HT treatment by Western blot. Activation of ERK1/2 was analysed by determining the levels of p-ERK1/2 during 5-HT treatment of osteocytes. The levels of p-ERK1/2 increased between 5 min and 3 h after 5-HT treatment (Fig. 3A–D). The p-ERK/ERK ratio increased significantly and rapidly at the 5-min timepoint (greater than sevenfold to the control level), decreased until the 30-min timepoint (fourfold to the control) and then increased again from the 1-h timepoint and reached another peak at the 3-h timepoint.

To test the involvement of the 5-HT_{2B} receptor in ERK1/2 activation, MLO-Y4 cells were pre-treated with RS127445 (20 nM) for 20 min and subsequently received 5-HT stimulation. As shown in Fig. 3B–D, RS127445 significantly inhibited 5-HT-induced activation of the ERK1/2 signal transduction pathway.

5-HT-induced IL-6 secretion and ERK1/2 activation in MLO-Y4 cells (Figs. 1A and B, 2A and 3A). PD98059 abolished 5-HT-induced ERK1/2 activation (Fig. 3C and D) and blocked 5-HT induced IL-6 expression (Figs. 1C and 2B). The inhibition of the 5-HT_{2B} receptor inhibited ERK1/2 activation (Fig. 3B–D) and IL-6 upregulation

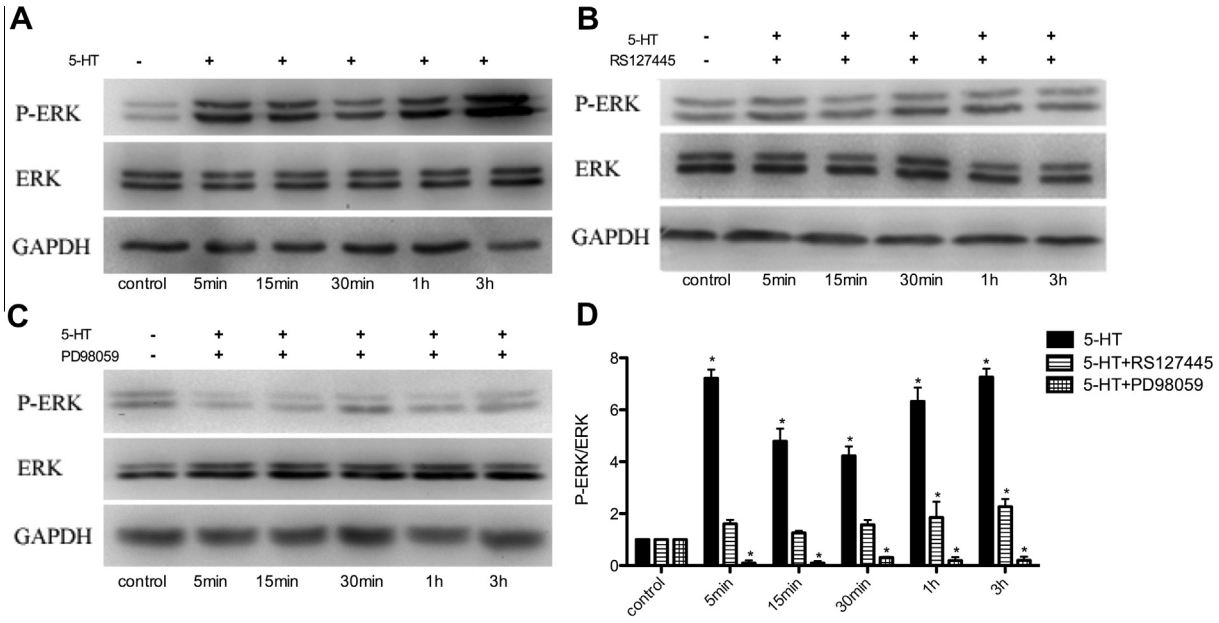

Fig. 3. Western blot analysis was performed using anti-p-ERK1/2 and anti-ERK1/2. (A) Effects of 5-HT on phosphorylation of ERK1/2: cells were incubated with 1 μM 5-HT for 5 min, 10 min, 30 min, 1 h or 3 h, respectively. (B) Effects of the 5-HT_{2B} receptor antagonist RS127445 on phosphorylation of ERK1/2: cells were pre-treated with RS127445 before 5-HT treatment. (C) Effects of the ERK1/2 pathway inhibitor on phosphorylation of ERK1/2: cells were pre-treated with PD98059 before 5-HT treatment. (D) Quantification of western blot: results are presented as the p-ERK/ERK fold change over control. $^{*}p < 0.05$.

(Figs. 1C and 2B). Collectively, these data demonstrate that 5-HT activates ERK1/2 via the 5-HT_{2B} receptor and that ERK1/2 is involved in 5-HT-induced IL-6 expression and secretion in osteocytes.

4. Discussion

In this study, we demonstrated that serotonin activates the ERK1/2 signal transduction pathway and induces the secretion of IL-6 in osteocyte-like MLO-Y4 cells. Moreover, we discovered that this effect is propagated through the activation of the 5-HT_{2B} receptor.

Our results suggest that serotonin acts on osteocytes and regulates bone turnover by mediating IL-6 level in bone, in addition to directly influence osteoblasts and osteoclasts. Collet et al. described decrease in the expression level of IL-6 in the 5-HT2b$^{-/-}$ osteoblast relative to wild-type osteoblasts [16]. However, the authors neither include data nor offer a mechanism in their studies. In this study, we present that serotonin significantly enhance the secretion of IL-6 in MLO-Y4 cells in a time- and dose-dependent manner. The increase in IL-6 level occurs as early as 4 h after serotonin stimulation. Peak levels of IL-6 increase were detected at 8 h. This pattern of IL-6 expression in MLO-Y4 cells is consistent with that observed in smooth muscle cells [20]. The effect of IL-6 in bone is complicated. Apart from its direct regulation of bone cells, IL-6 is a well-known pro-inflammatory cytokine and inducer of leukocyte recruitment. Interestingly, serotonin is a pro-inflammatory substrate. We hypothesize that the increase of IL-6 expression by serotonin may be an acute inflammation response in bone. We observed that the minimal effective serotonin concentration is 100 nM, whereas the most prominent effect occurred at a dosage of 1 μM 5-HT. The concentration of serotonin in serum is below 150 ng/ml. However, under inflammatory or traumatic conditions, the local serotonin level rises rapidly due to platelet activation [32]. Osteocytes are intertwined with vessels in such a way that the dendrites of osteocytes surround and encase the blood vessels in bone [41]. Furthermore, osteoclasts are able to secret serotonin with the existence of RANKL [14]. Thus, we propose that osteocytes may be exposed to sufficiently high levels of serotonin to trigger IL-6 secretion due to the release of a paracrine factor or hematoma, especially under pathological conditions.

We found that the 5-HT_{2B} receptor is required for serotonin triggered IL-6 upregulation. The 5-HT_{2B} receptors mediate the function of serotonin in the CNS and in the peripheral tissues. Recently, researchers have focused on the potential application of 5-HT_{2B} antagonists as a treatment for chronic heart disease, pulmonary hypertension and chronic liver disease [42–44]. Bone expresses higher levels of 5-HT_{2B} receptor mRNA compared with the brain, the heart, the lung and the liver. Among bone cells, osteocytes express the highest level of the 5-HT_{2B} receptor [36]. However, few researchers have focused on the function of the 5-HT_{2B} receptors in osteocytes. Here, we present that the 5-HT_{2B} receptor is required for the increase in IL-6 expression levels in serotonin-stimulated MLO-Y4 cells. However, the function of the 5-HT_{2B} receptor in bone is still not clear. The study by Collet et al. showed that the 5-HT_{2B} receptor is as important a factor in bone formation as it mediates osteoblast recruitment and proliferation [16]. In our study, we did not detect a fluctuation in proliferation rate in serotonin-stimulated MLO-Y4 cells. This may be due to the lack of proliferative ability in osteocytes. The observed increased IL-6 is a potential inducer of bone destruction. Thus, the 5-HT_{2B} receptor may play a role in the maintenance of bone mass through the regulation of the balance of bone formation and destruction.

Several signalling pathways are associated with the function of the 5-HT_{2B} receptor. For example, RAS, PKC and ERK/MAPK (mitogen-activated protein kinase) pathways are linked to the stimulation of cell proliferation via the 5-HT_{2B} receptor [45–47]. However, the pathway linking the 5-HT_{2B} receptor to the expression of IL-6 in bone cells has not been studied extensively. Previously, we reported that ERK1/2 is involved in the mechanically stimulated-induction of IL-6 secretion in MLO-Y4 cells [39]. In this study, we observe a rapid phosphorylation of ERK1/2 in serotonin-stimulated MLO-Y4 cells suggesting the activation of ERK1/2. The observation that PD98059, the inhibitor of ERK1/2, inhibited IL-6 production provides further evidence that ERK1/2 activation is

required for serotonin-induced IL-6 secretion in MLO-Y4 cells. Additionally, we found that the 5-HT_{2B} receptor antagonist suppressed the phosphorylation of ERK1/2 significantly while abolishing IL-6 upregulation. These data indicate that serotonin activates ERK1/2 through the 5-HT_{2B} receptor and support that ERK1/2 is involved in 5-HT-induced IL-6 synthesis. We observed that after stimulation, p-ERK/ERK ratio increased greatly and rapidly at 5 min, then decreased until a second increase at 1 h. This expression pattern suggests that a change occurred in a signal transduction pathway during this period. Interestingly, this time-dependent effect on ERK1/2 activation is similar to our previous findings in cyclic compressive force (CCF)-stimulated MLO-Y4 cells. In the latter case, CCF strongly activated ERK1/2 as early as 10 min followed by a decline, with another rise in expression level at 6 h [39]. We hypothesized that the 5-HT receptors may participate in CCF-induced IL-6 secretion. In fact, by RT-PCR analysis, we found that CCF affects the mRNA expression level of the 5-HT_{2A} and the 5-HT_{2B} receptor (data not shown). As osteocytes are considered the mechanosensors in bone, further investigation is required into the relationship between mechanical stimulation and 5-HT receptor function in osteocytes.

In conclusion, we successfully demonstrate that serotonin is able to regulate IL-6 secretion in osteocytes through the activation of the 5-HT_{2B} receptor and the ERK1/2 pathway. Our results strongly suggest a role for osteocytes in the function of serotonin in bone.

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