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紫河車가 칼슘재흡수, cyclooxygenase의 발현, PGE2 생합성에 미치는 영향

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ABSTRACT

***Hominis Placenta* suppress Calcium release, cyclooxygenase expression and PGE_2 synthesis**

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Purpose : 자하거 (*Hominis Placenta*; HP) 는 건강한 사람의 태반을 烘製하여 건조한 것으로 한의학에서는 氣血을 大補하고 腎精을 補益시켜 久病으로 인한 身體虛弱이나 혹은 體質虛弱과 氣血不足 및 腎虛精虧 등 證을 治療하는데 單味 또는 複方에 配伍하여 쓰여왔다. 또한 자하거는 면역학적으로 골대사 활성이 있는 것으로 알려져 있어 본 연구에서는 자하거의 항골다공증 활성을 분자세포생물학적으로 검정하고자 하였다.

Methods : Osteoblast cells에서 자하거가 COX-2 mRNA의 발현과 PGE_2 생합성을 억제시키는지를 관찰하기 위해 먼저 TNF-α, IL-β 와 IL-6를 처리한 후 PGE_2의 생합성과 더불어 COX-2 mRNA의 발현을 확인하였다. 그 후 TGF-β, 紫河車와 이 둘의 조합인 자하거+TGF-β가 COX-2 mRNA 발현과 PGE_2 생합성을 저해시키는지 관찰하였다. 또한 자하거가 IL-1β로 유발된 흰쥐의 과칼슘혈증을 감소시키는지를 확인하였다.

Results : IL-6, IL-1β와 TNF-α를 동시에 처리하면 이것을 단독으로 처리한 것과 비교해 볼 때 PGE_2의 생합성과 더불어 COX-2 mRNA의 수치가 상승작용을 일으키며 증가하였다. TGF-β, 자하거와 이 둘의 조합인 자하거+TGF-β은 COX-2 mRNA 발현, PGE_2 생합성 및 골재흡수를 감소시켰다. 紫河車는 IL-1β, TNF-α와 IL-6 각각 또는 이들의 조합으로 인해 증가하는 COX-2 mRNA 발현과 PGE_2 생성을 감소시키는 반면 COX-1 mRNA 발현에는 유의성 있는 영향을 미치지 않았다. 한편 자하거는 농도의존적으로 IL-1β로 유발된 흰쥐의 과칼슘혈증을 감소시켰다. 이러한 결과는 흰쥐의 두개골 골아세포에서 PGE_2 생산에 대한 IL-β, TNF-α, IL-6의 상승작용이 COX-2의 유전자 발현 증가에 기인함을 보여주었다.

Conclusions : 이러한 결과들로부터 자하거가 골대사과정중 골재흡수를 억제하는데 효과적임을 밝히게 되었으며, 자하거의 골다공증의 억제기전이 골재흡수관련 단백질들의 전사조절에 있음을 최초로 해명하게 되었다.

Key words : IL-1β; Osteoblast; Osteoporosis; Osteolysis; IL-6; TNF-α; TGF-β; *Hominis Placenta* (HP); *Jahage*

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Ⅰ. Introduction

It is well known that Korean traditional medicine, *Jahage* (*Hominis Placenta; HP*) is effective for the treatment of Tuberculosis, arteriosclerosis, and gynecological diseases such as osteoporosis and bone resorption. According to the ancient Chinese and Korean medicinal and herbal literature[1]. The HP is also known as an effective biological response modifier for augmenting host homeostasis of body circulation[1]. The pharmacological action of HP has been limitedly studied in regard to gynecological diseases. This traditional medicine has been shown to express diverse activities such as immunomodulating, anti-infarction, anti-allergic and anti-inflammatory effects[2]. Thus, it still occupies an important place in traditional Korean medicine.

The cytokine interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), which are produced mainly by activated monocytes or macrophages, stilumate bone resorption as well as enhance PGE_2 production in several type of cells including calvarial osteoblasts[3-6]. The phospholipase A_2 catalyzes the liberation of arachidonic acid (AA) from membrane phospholipids whereas the cyclooxygenase (COX) mediates conversion of AA to prostaglandins[6]. The enzyme COX exists at least in two isoforms, the constitutive COX and the inducible COX-2[7,8,9].

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to enhanced bone fragility, and a consequent increase in fracture risk. The low bone mass results from an imbalance between bone formation and bone resorption, coupled processes that maintain skeletal integrity. Most conditions that lead to osteoporosis (estrogen deficiency in postmenopausal women, hyperparathyroidism, hyperthyroidism, and corticosteroid treatment) are associated with increased bone resorption, which is determined by the number and activity of bone-resorbing cells, or osteoclasts. The differentiated osteoclast is a large, multinucleated, highly motile cell of hematopoietic origin.

Since the effectiveness of HP for gynecological and inflammatory diseases has been widely demonstrated by clinical administration, anti-bone resorption activity was assessed by the effect on osteoblastic cells. The present paper reports the effect of extracts obtained from HP on cytokine-induced experimental bone resorption in mouse calvarial cells.

Ⅱ. Materials and Methods

Medicinal herbal formulation

The aqueous extracts of HP, which was massproduced as for clinical use, were kindly supplied by the Oriental Medical Hospital of Dongguk University College of Oriental Medicine (Kyungju, Korea).

Reagents

Radiochemicals were from Amersham International Co. (Seoul, Korea). All other chemicals and biochemicals were of analytical grade and were purchased from Sigma Chem. Co. (St. Louis, MO) or Boehringer Mannheim Biochemicals (Seoul, Korea).

IL-1β, TNF-α and IL-6 were purchased from R&D Systems (Funakoshi, Co., Ltd., Tokyo, Japan). Herbimycin A was from Sigma Chemical CO. (St. Louis, MO, USA). Radioimmunoassay kits for PGE_2 were from Ammersham. It was stored in aliquots of 100 mg dissolved in 91 mL of 100 mmol/L Tris buffer (pH 7.8) containing 2 mmol/L sodium azide, and diluted with phosphate-buffered saline (PBS) prior to use.

Calvarial osteoblast cultures

Cultures of osteoblast cells were established from calvaria obtained from healthy 10 mice with

2 months of age. Minced pieces of the tissue were explanted to 25 cm^2 Falcon tissue culture flasks containing 5 ml of Eagle's basal medium (BME). The osteoblasts were obtained by trypsinisation of the primary outgrowth of cells as previously described[10]. Osteoblasts were seeded and grown in BME supplemented with 5% fetal calf serum, L-glutamine, penicillin-streptomycin and HEPS for 24 h. The cell layers were then rinsed three times with serum-free BME medium and incubated in the absence or presence of IL-1 β, TNF-α, IL-6 and HP alone or in combinations at the concentrations indicated in the legends to figures and tables. At the end of the incubation period, indicated in the legends to figures and tables, the monolayer was frozen for isolation of total RNA. The cells used for the experiments proliferated in the logarithmic phase between the 6th and the 12th passage.

Quantification of mRNA for COX-1 and COX-2.

The cells were scraped in 2 ml of 10 mM EDTA (pH8.0), 0.5% SDS and RNA was isolated according to Sambrook et al. (1989)[11]. The levels of mRNA for COX-1 and COX-2 were determined by southern hybridization which was carried out by using RNA probes, synthesized in vitro using a RNA trasncription kit, and labeled with ^{35}S-rCTP[11].

Prostaglandin E_2 determination assays

The amount of PGE_2 was determined in the medium by using commercially avaiable radioimmunoassay kits with [^{125}I]-PGE_2 antiserum. The PGE_2 antiserum has 30% cross-reactivity with in PGE_2.

In vitro bone resorption assay

Fetal rat long bones were prepared and cultured as described by Feyen *et al.*[12]. In brief, timed pregnant Sprague-Dawley rats were injected with radiolabeled ^{45}Ca subcutaneously (s.c.) (100 μCi) on the 18th day of gestation. The following day, radii and ulnae were dissected and then precultured in 0.5 mL of BGJ medium supplemented with 1 mg/mL of bovine serum albumin (BSA) in 24 well tissue culture plates in a CO_2 incubator at 37℃ for 24 h. The bone explants were then cultured in the presence or absence of the agents to be tested for 2 days. The medium was removed and replaced with fresh medium supplemented with the test agents, and culture was continued for another 3 days before terminating the experiment. Aliquots of conditioned medium of day 2 and day 5 and the acid extract [trichloroacetic acid (TCA), 5% (w/v)] of the bone explants were counted for ^{45}Ca by liquid scintillation. Bone resorption was assessed as the percentage of total ^{45}Ca that was released into the medium.

IL-1β-induced hypercalcemia in mice

The method of Sabatini *et al.*[13] was used to induce hypercalcemia in mice. An Alzet minipump (type 1003D; Alza Corporation, Palo Alto, CA) was implanted on the upper side of the neck of each mouse while in anesthesia with halothane/air (4:96%). The minipump infused IL-1β dissolved in PBS at a rate of 2 mg/day for 72 h. The control group received a similar minipump infusion of PBS. The test compounds were administered subcutaneously twice daily at 6:30 and 2:30 The last administration (seventh injection) was given in the morning of day 4; 2 h thereafter the mice were killed and blood samples were collected. The total serum calcium concentration was determined colorimetrically at 570 nm with *o*-cresolphthalein complexone using a commercially available kit (MPR Calcium; Boehringer Mannheim, Germany) in a microtitre plate format. The serum amyloid protein concentration of the blood samples was determined by an ELISA method[14].

Statistics

Results of the above animal studies are given as mean ± standard error of the mean (SEM). Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) and by Tukey's multiple comparison test against the control group.

Ⅲ. Results

1. Synergistic Inhibitory effect of HP and TGF-β on TNF-α, IL-1β and IL-6 -induced COX mRNA levels and PGE_2 production.

The cytokine TGF-β (10 U/ml) significantly reduced COX-2 mRNA levels in cultures of osteoblasts (Fig. 1) when TGF-β were treated after TNF-α, IL-β and IL-6 treatment. When 100 ㎍/ml of HP was treated with TGF-β (10 U/ml) to the osteoblast cells to examine whether the HP inhibits synergistically the COX mRNA levels and PGE_2 production after TNF-α, IL-β and IL-6 treatment, COX-2 mRNA expression and PGE_2 production were greatly reduced by HP(100 ㎍/ml) synergistically with a significant difference ($P<0.001$). This result also indicated that the HP is acting in stage of COX-2 mRNA transcription and PGE_2 synthesis of the osteoblast cells (Fig. 1).

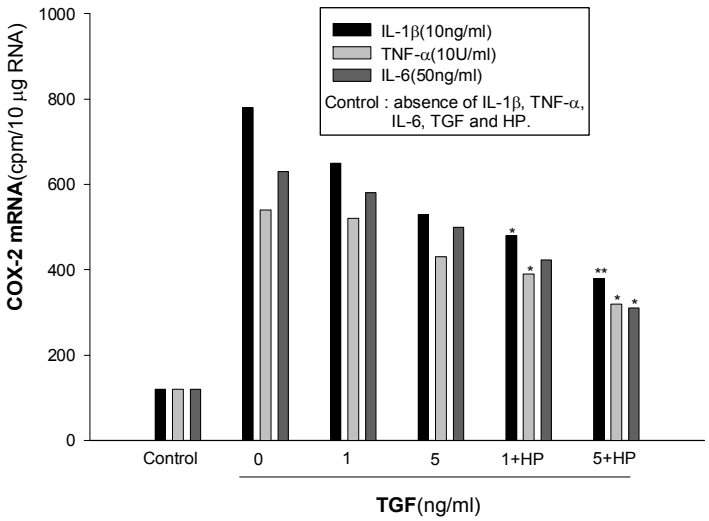


Fig. 1. Synergistic inhibitory activity of HP (100 ㎍/ml) on inhibitory effect of TGF-β on IL-1β induced COX mRNA levels.

Effect of TGF-β, at different concentrations, in the absence or presence of IL-1 β(10 ng/ml), in 6 h cultures, was assayed to see the expression of COX-2 mRNA level in mouse osteoblasts (N=6).

Mean±SD of triplicate experiments representing one of three separate experiments with similar results.

*, significantly different from untreated control cells ($P<0.01$),

**, significantly different ($P<0.001$).

2. Inhibitory activity of HP on effect of TNF-α, IL-1β and IL-6 alone or in combination on PGE_2 production.

The cytokine IL-1β and to a lesser extent IL-6 (≥10 ng/ml), stimulated PGE_2 production in osteoblasts (Fig. 2). Furthermore, the increase of COX-2 mRNA levels stimulated by the combination of IL-1β and IL-6 were accompanied by a synergistic increase of PGE_2 production which was dependent on the concentration of IL-6. HP treatment with 100ng/ml of IL-6 in the presence or absence of IL-1β (Fig. 2) and TNF-α (Fig. 2) showed the significant inhibition of PGE_2 production which was dependent on the concentration of IL-6.

3. Inhibitory effect of HP and TGF-β on TNF-α, IL-1β and IL-6 induced PGE_2 production.

The cytokine TGF-β (10 U/ml) significantly reduced PGE_2 production levels in cultures of osteoblasts (Fig. 3-A,B,C) when TGF-β were treated after TNF-α, IL-1β and IL-6 treatment. Inhibitory effect of TGF-β and a combination of TGF-β plus(+) HP on IL-1β-induced PGE_2 production were examined. Effect of TGF-β at different concentrations, in the absence or presence of IL-1β (10 ng/ml), in 6 h cultures, was assayed to see the PGE_2 production level in mouse osteoblasts (N=6). As shown in Fig. 3-A,B,C, TNF-α, IL-1β and IL-6 -induced PGE_2 production were significantly decreased (P<0.001) by 5 or 10ng/ml TGF-β, and more stronlgy by TGF-β+ HP.

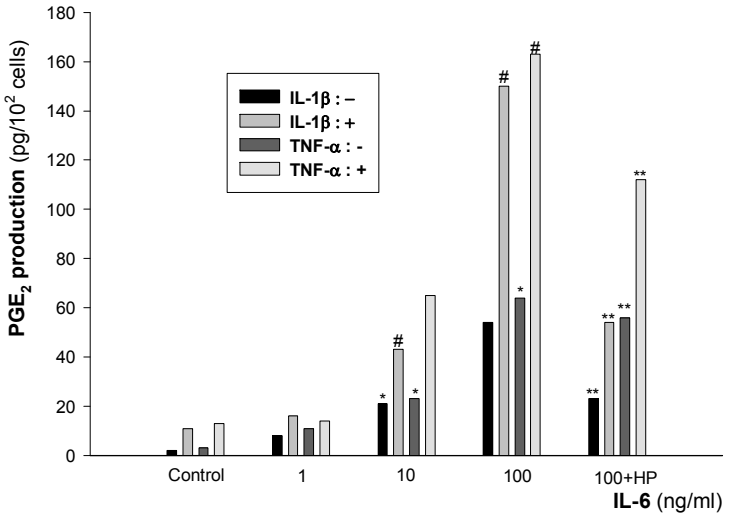


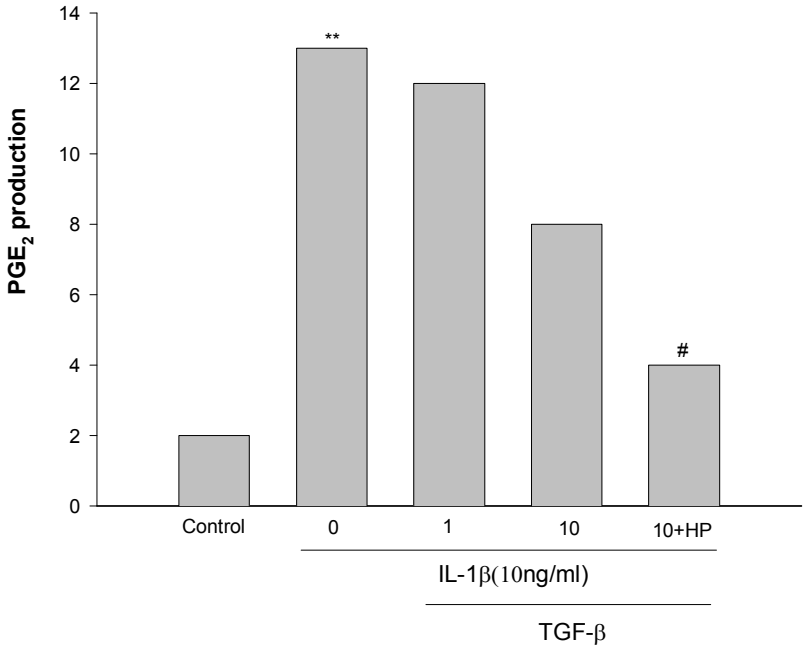
Fig. 2. Inhibitory activity of HP (10㎍/ml) of effect of IL-6, at different concentrations, in the absence or presence of IL-1β (10 ng/ml) or TNF-α, in 6 h cultures, on the PGE_2 production in mouse osteoblasts (N=6).

Mean±SD of triplicate experiments representing one of three separate experiments with similar results.

*, significantly different from untreated control cells (P<0.01),

#, significantly different from IL-1β or IL-6-treated cells (P<0.001).

**, significantly different from HP and 100ng/ml IL-6-treated cells (P<0.001).


14
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PGE2 production
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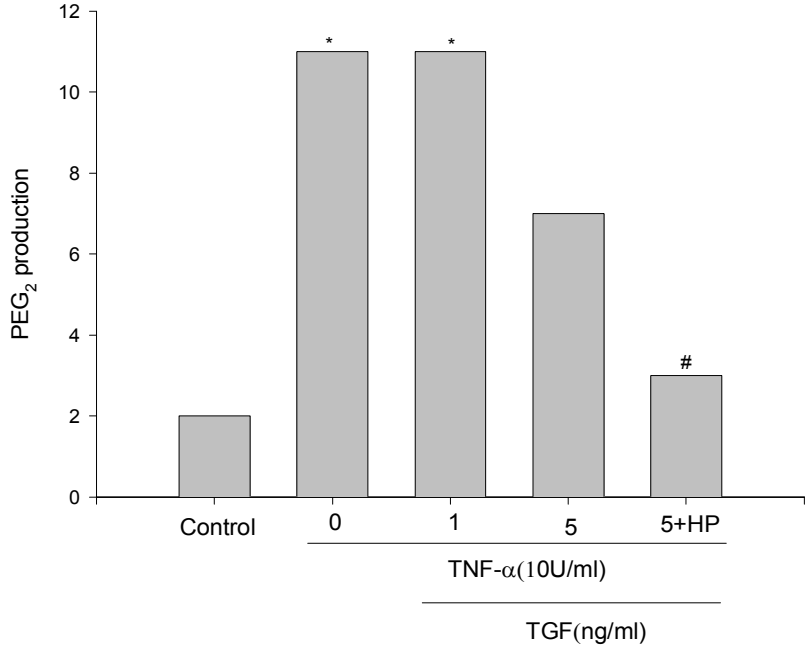
10+HP
IL-1β(10ng/ml)
TGF-β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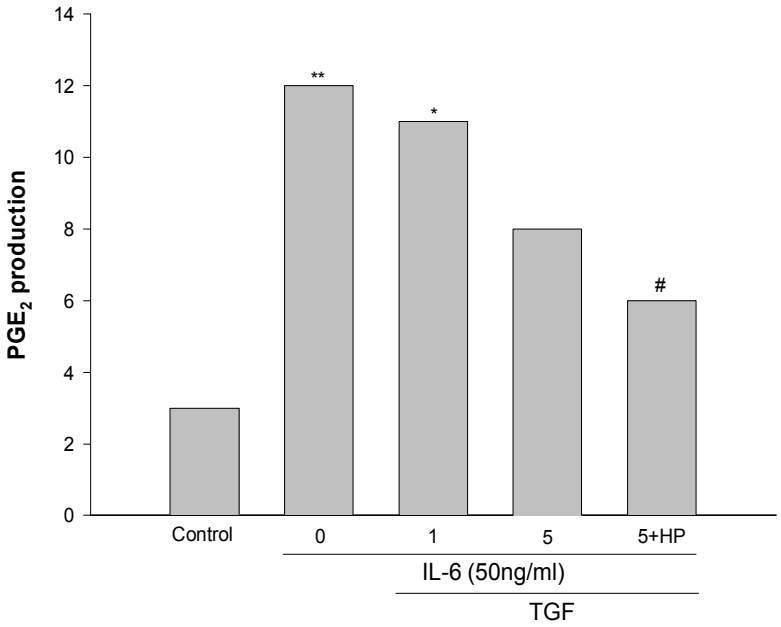

Fig. 3. Inhibitory effect of TGF-β and a combination of TGF-β plus(+) HP on IL-1β-induced PGE_2 production.

Effect of TGF-β, at different concentrations, in the absence or presence of IL-1 β (10 ng/ml), in 6 h cultures, was assayed to see the PGE_2 production level in mouse osteoblasts (N=6).

Mean±SD of triplicate experiments representing one of three separate experiments with similar results.
*, significantly different from untreated control cells (P<0.01),
**, significantly different (P<0.001).
#, significant different from 5 or 10ng/ml TGF-β and TGF-β+ HP.

4. HP inhibits PTH-induced bone resorption in vitro.

The effect of the Src inhibitor HP on bone resorption was evaluated in vitro in the fetal rat long bone organ culture system described previously[12]. Fetal rat long bones were cultured in the presence of 10 nmol/L human PTH-(1-34) in the presence or absence of HP over the concentration range 0.1-100 μg/ml. Bone resorption was assessed as the percentage release of ^{45}Ca into the culture medium at day 5 of culture. In this system, bone resorption is stimulated with PTH and measured by the release of ^{45}Ca into the medium from fetal long bones prelabeled with ^{45}Ca. As the results in Fig. 4 show, HP inhibited the PTH-stimulated release of ^{45}Ca in a concentration-dependent manner with an apparent IC50 value of 17 μg/ml. This value is similar to that obtained for the inhibition of bone resorption with isolated osteoclasts (IC50 value of 23 μg/ml in our preliminary data). We could also show that HP did not affect signaling by several growth factors in osteoblastic cells.

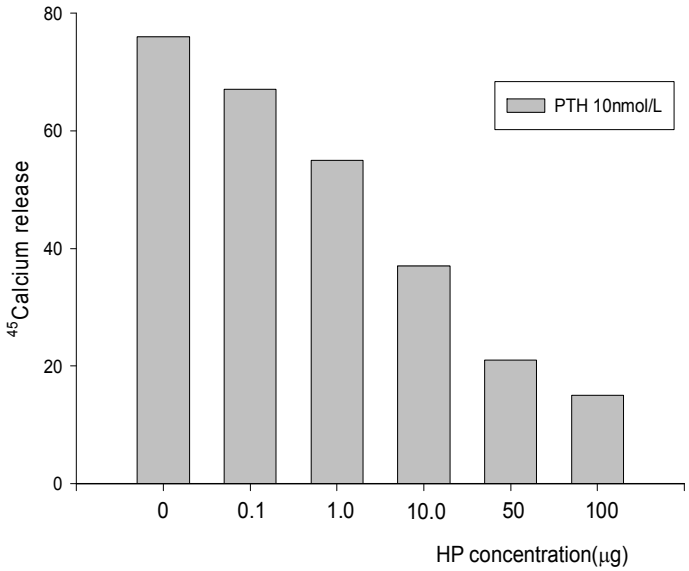

Fig. 4. HP inhibits bone resorption in the fetal long bone organ culture system.

Fetal rat long bones were cultured in the presence of 10 nmol/L human PTH-(1-34) in the presence or absence of HP over the concentration range 0.1-100 μg/ml. Bone resorption was assessed as the percentage release of ^{45}Ca into the culture medium at day 5 of culture. Four experiments are done and the results from a representative experiment are shown as percent release over control for six long bones per group (mean ± SEM, n = 6). Open circles show ^{45}Ca release in nontreated controls and PTH-treated samples; closed circles show ^{45}Ca release in samples treated with PTH and HP.

Ⅳ. Discussion

We demonstrate that HP can inhibit bone resorption in vitro and in vivo. We have demonstrated IL-6 enhances COX-2 mRNA level as well as synergistically potentiates IL-1 βinduced COX-2 mRNA level, which was also reflected by increased PGE_2 production. Interleukin-1 together with IL-6 and TNF-α is thought to play a major role in the ovariectomy-induced bone loss[11]. Thus, based on the inhibition of IL-1-induced hypercalcemia with HP, it was expected that the ovariectomy-induced bone loss would be affected as well, but it was possible that the effect on bone mass could be obscured by unfavorable pharmacokinetics or toxic effects of the compound. Taken as a whole, our data show that HP may be effective in preventing bone loss in animal models, and may be useful for the treatment of conditions associated with bone loss in humans.

In conclusion, it was indicated that HP can inhibit bone resorption in vitro and in vivo.

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