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Long-term effects of tripeptide Ile-Pro-Pro on osteoblast differentiation in vitro

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Abstract

Bone mineralization is a result of the function of bone-forming osteoblasts. Osteoblast differentiation from their precursors is a carefully controlled process that is affected by many signaling molecules. Protein-rich food-derived bioactive peptides are reported to express a variety of functions in vivo. We studied the long-term in vitro effect of bioactive tripeptide Ile-Pro-Pro (IPP) on osteoblasts differentiated from human mesenchymal stem cells. Osteoblast bone alkaline phosphatase activity (bALP), bone-forming capacity and gene expression were investigated. Treatment with 50 μM IPP had no effect on bALP activity, but osteoblast mineralization was increased. Gene expression of β-catenin, Cbfa1/Runx2, PTHrP, CREB-5, osteoglycin, osteocalcin, caspase-8, osteoprotegerin (OPG) and RANKL was analyzed by quantitative real-time PCR on Days 13, 17 and 20 of culture. The results indicate that IPP increased mineral formation due to enhanced cell survival and matrix formation. In addition, IPP reduced the RANKL/OPG ratio. Bioactive peptides, such as IPP, could be one method by which a protein-rich diet promotes bone integrity.

Keywords: Osteoblast differentiation; Bioactive peptide; IPP; hMSC

1. Introduction

Bone formation occurs during embryonic development and postnatal growth and in adulthood during bone remodeling to support calcium homeostasis and in response to physical forces [1]. The development of bone marrow-derived progenitor cells into bone-forming osteoblasts is a strictly controlled process that involves cell recruitment, proliferation and differentiation. Enhanced osteoblast development aids in bone formation and reduces fracture incidence.

The literature reports an overall beneficial relationship between high dietary protein and bone health [2–8]. Protein's osteotrophic effects are partly explained by enhanced IGF-I production and action [9]. IGF-I is an essential mediator of tissue anabolism [10]. However, in a rodent model, a protein-restricted diet with normalized IGF-I by infusion failed to promote growth, indicating adequate protein to have effects beyond IGF-I [9]. Bioactive peptides are short chains of amino acids produced in the hydrolysis of proteins. Food-derived bioactive peptides are claimed to be health-enhancing components that can be used in functional food and pharmaceutical preparations [11].

In our previous study [12], we showed that short-term (24 h) treatment with tripeptides Ile-Pro-Pro (IPP), Val-Pro-Pro and Leu-Lys-Pro induces human mesenchymal stem cell (hMSC) proliferation and osteoblast gene expression. IPP was especially efficient in up-regulating genes involved in cell growth, differentiation and signaling. Based on these results, our hypothesis was that long-term treatment with IPP would direct cell differentiation towards the osteogenic lineage and enhance osteoblast function, leading to increased bone formation and mineralization. Hence, in the present study, we concentrated on the long-term (from Day 1 to cell culture mineralization) in vitro effects of IPP on hMSC-differentiated osteoblasts. We assessed total protein content, bone-specific alkaline phosphatase (bALP) activity and cell culture mineralization, and

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conducted qRT-PCR for nine specific genes. The results show that continuous long-term treatment with 50 μM IPP did not affect osteoblast bALP activity, but osteoblast bone formation was enhanced. The increased mineral formation could be due to enhanced cell survival and matrix formation. Furthermore, the RANKL/osteoprotegerin (OPG) ratio was reduced, which could lead to decreased bone resorption. These findings support protein degradation products, such as bioactive peptide IPP, contributing to the favorable effects of dietary protein on bone.

2. Materials and methods

2.1. Cell culture

Human mesenchymal stem cells (Poietics, Cambrex Bio Science, Walkerville, MD, USA) purified from human bone marrow were seeded at 3100 cells/cm^2 and cultured in Poietics mesenchymal stem cell growth medium with mesenchymal cell growth supplements (Cambrex Bio Science) at 37°C in a humified atmosphere with 5% CO_2 on 24- and 96-well plates or in 10-ml plastic dishes. The experiments were conducted on two separate cell batches, on passage numbers 3–5. Differentiation of the hMSC into osteoblasts was induced after 1 day of seeding. The medium was then substituted with fresh growth medium (α-MEM, 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin), an osteoblast differentiation medium (α-MEM, 10% FBS, 10^{-8} M Dex, 50 μg/ml L-ascorbic acid, 10 mM β-GP, 100 U/ml penicillin, 100 U/ml streptomycin) or an osteoblast differentiation medium supplemented with 50 μM IPP tripeptide. After 12–14 days of differentiation, the osteoblastic phenotype was confirmed by the amplification of osteocalcin with qRT-PCR (Table 1) and by alkaline phosphatase staining (Sigma histological alkaline phosphatase kit no. 86).

2.2. IPP tripeptide

The peptide was synthesized using 9-fluorenylmethyloxycarbonyl chemistry and purified by HPLC reverse-phase columns. Synthesis was carried out at the Core Facility for Synthetic Peptides (Division of Biochemistry, Department of Biological and Environmental Sciences), University of Helsinki, Finland. The peptide was dissolved in sterile water prior to administration.

2.3. Total protein content

Total protein content was assessed from cells grown on the 24-well plates according to the Bradford [13] method on Days 3, 7, 12, 14, 17, 21 and 34 of culture. Bovine serum albumin (Sigma) was used as a standard.

2.4. Alkaline phosphatase activity

Bone-specific alkaline phosphatase activity was measured at the same seven time points as total protein content, with an OCTEIA Ostase BAP immunoenzymetric assay (Immunodiagnostic Systems Limited, Boldon, UK). Prior to measurement, cells were washed with phosphate buffered saline and frozen (−70°C) with 300 μl of Tris-Triton (0.1 M Tris base, 0.2% Triton X-1000). After thawing, the cells were centrifuged (12,000×*g*, 5 min). Supernatant was used for the analysis. ALP activity was corrected with the total protein content.

Table 1
Nucleotide sequences of primers used for quantitative RT-PCR detection

Gene	Primer sequence	Product size (bp)	Reference
β-Actin	F: AGG CCA ACC GCG AGA AGA TGA CC R: GAA GTC CAG GGC GAC GTA GCAC	350	[42]
β-Catenin	F: TTC TGG TGC CAC TAC CAC AGC R: TGC ATG CCC TCA TCT AAT GTC	216	[43]
Caspase-8	F: AGG AGG AGA TGG AAA GGG AAC TT R: ACC TCA ATT CTG ATC TGC TCA CTT CT	108	[44]
Cbfa1/Runx2	F: TGA GAG CCG CTT CTC CAA CC R: GCG GAA GCA TTC TGG AAG GA	266	Primer 3
CREB-5	F: GCT TTG GTG CTT TTC TCC AG R: GGT GAC ACC ACA GCA CAA AC	245	Primer 3
OPG	F: GGC AAC ACA GCT CAC AAG AA R: CTG GGT TTG CAT GCC TTT AT	241	[45]
Osteocalcin	F: ATG AGA GCC CTC ACA CTC CTCG R: GTC AGC CAA CTC GTC ACA GTCC	255	[46]
Osteoglycin	F: TTG ATG CTG TAC CAC CCT TAC R: ATT CCA GGG CAT TAT GGT CC	348	Primer 3
RANKL	F: AGA GCG CAG ATG GAT CCT AA R: TTC CTT TTG CAC AGC TCC TT	180	Primer 3
PTHrP	F: GTC TCA GCC GCC GCC TCA A R: GGA AGA ATC GTC GCC GTA AA	93	[47]

All primer sequences used were designed as described previously, except primer pairs for Cbfa1/Runx2, CREB-5, osteoglycin and RANKL, which were designed with Primer 3 software for the present study.

2.5. Mineralization

Cell culture mineralization was assessed by Alizarin red S staining of cells grown on the 96-well plates [14]. After staining, the dye was eluated with a solution of 10% cetylpyridinium chloride and 10 mM sodium phosphate (pH 7.0, 15 min, RT). Absorbance was measured with a spectroscopic plate reader (Multiskan Ex, Thermo Labsystems, Helsinki, Finland) at 590 nm.

2.6. Total RNA isolation

Total RNA from the hMSC-differentiated osteoblasts (control) and from osteoblasts treated with 50 μM IPP was extracted from 10-ml plastic cell culture dishes on Days 13, 17 and 20 using a RNeasy Protect mini kit (Qiagen, Hilden, Germany). The RNA concentration was measured at 260 nm by a spectrophotometer (SmartSpec 3000 Biorad, Hercules, CA, USA).

2.7. Quantitative real-time PCR

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for β-actin, β-catenin, caspase-8, core binding factor alpha 1/runt-related transcription factor 2 (Cbfa1/RUNX2), cAMP response element binding protein (CREB-5), OPG, osteocalcin, osteoglycin, parathyroid hormone-related peptide (PTHrP) and osteoclast growth factor receptor activator of NF-κB ligand (RANKL) was performed in a real-time quantitative PCR (Mx3000P, Stratagene, La Jolla, CA, USA). The genes of interest were chosen on the basis of our previous microarray study results to represent cell growth, differentiation and signaling [12].

cDNA was synthesized from 1.5 μg of total RNA by a method described earlier [15]. Each PCR amplification was performed according to the manufacturer's instructions using a Brilliant SYBR Green QPCR Master mix kit (Stratagene) and cDNAs as templates in a 25-μl reaction volume containing 200 nM of the gene-specific primer (Table 1). Fluorescence data were collected during the annealing step and analyzed with Mx3000P software. Amplification was obtained by denaturing at 95°C for 10 min, followed by 45 cycles of denaturing at 95°C for 30 s, annealing at 58°C for 1 min and extension at 72°C for 30 s. For dissociation measurement, the amplification cycle was followed by 1 min at 95°C and 30 s at 55°C. The temperature was then returned to 95°C.

A dilution series of cDNA from human brain total RNA (BD, Biosciences, Clontech, Palo Alto, CA, USA) was used as a calibration standard, and data were normalized by the amplification result of β-actin. All reactions were run in triplicate, and the mean value was used to calculate the ratio of target gene/β-actin expression in each sample.

2.8. Statistical analysis

Statistical analysis was performed with GraphPad Prism software (version 3.03). All experiments were repeated at least twice, and qualitatively identical results were obtained. Results are expressed as means±S.D. based on triplicate-independent samples. The data were analyzed using either Student's unpaired *t* test or ANOVA, followed by Tukey's post hoc test. Results were considered significant at the 95% significance level ($P<05$).

3. Results

3.1. Total protein content

Total protein content increased in all groups from Day 3 (hMSC 0.005±0.007 mg/ml, control 0.00 mg/ml, IPP 0.01±0.012 mg/ml) to Day 34 (hMSC 0.22±0.071 mg/ml, control 0.38±0.076 mg/ml, IPP 0.35±0.035 mg/ml) (Fig. 1A). Total protein content did not differ between groups.

3.2. Alkaline phosphatase activity

Bone alkaline phosphatase activity increased from Day 7 onwards in control and IPP-treated cells grown in an osteoblast differentiation medium, but not in cells grown in a mesenchymal cell growth medium (Fig. 1B). bALP activity further increased up to Day 21 (control 303.2±24 μg/mg, IPP 295.6±26 μg/mg) and remained high until Day 34 (control 206.0±0.54 μg/mg, IPP 237.7±13.8 μg/mg). bALP activity did not differ between IPP-treated osteoblasts and controls.

3.3. Mineralization

IPP (50 μM) treatment increased osteoblast mineralization ($P<05$). Mineralization results are presented in Fig. 1C. Cell culture mineralization was also confirmed by visual inspection and the observation of heavily dyed distinct peak-like increases on the plate. This bone-like matrix formation was not observed in cells grown with plain growth medium, nor did these cells dye with Alizarin red S.

3.4. Quantitative real-time PCR

A set of IPP-regulated genes were analyzed by quantitative RT-PCR on Days 13, 17 and 20. The qRT-PCR results are shown in Fig. 2. Control cell β-catenin expression did not differ within groups over the time period studied. IPP treatment elevated the expression on Day 17 (Fig. 2A). Cbfa1/Runx2 expression peaked on Day 17 in control cells and was higher than in IPP-treated cells, where the Cbfa1/Runx2 expression continued to rise until Day 20 (Fig. 2B). PTHrP expression was reduced in control cells on Day 17 and increased back to Day 13 levels by Day 20. In IPP-treated cells, the PTHrP expression remained at a constant level and was less than in control cells on Days 13 and 20 (Fig. 2C). CREB-5 expression was elevated in control cells from Day 13 to Day 17, decreasing by Day 20. In IPP-treated cells, CREB-5 remained low on Day 17, rising by Day 20 to the same level as in control cells (Fig. 2D). Control cell osteoglycin expression remained constant over the study period, but was increased in IPP-treated cells on Days 13 and 17, falling to the control cell level by Day 20 (Fig. 2E).

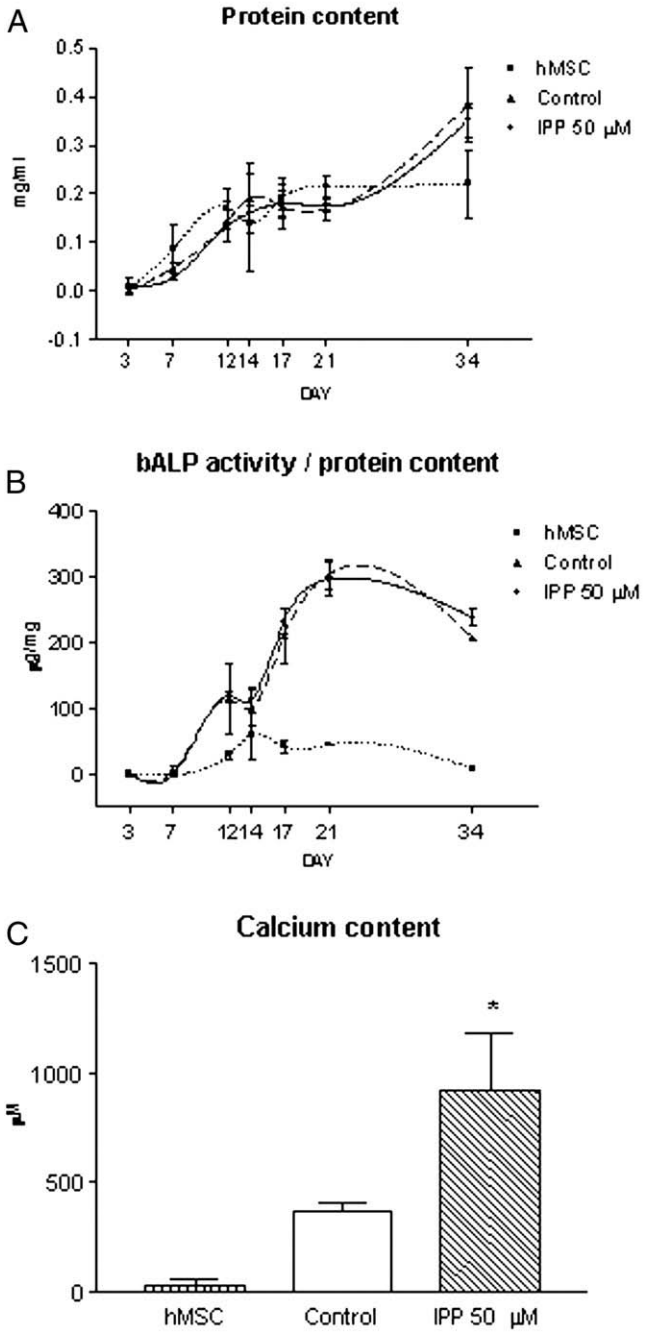

Fig. 1. Effects of 50 µM IPP on osteoblast differentiation. Protein content and bALP activity protein content of hMSC and hMSC-differentiated osteoblasts after 3, 7, 12, 14, 17, 21 and 34 days of culture, expressed as means±S.D. (A) Total protein content (mg/ml) did not differ between groups over the study period. (B) bALP activity was increased in hMSC cells grown in osteoblast differentiation medium from Day 7 onwards. IPP-treated cells did not differ from the osteoblast control cells in bALP activity. (C) Alizarin Red S staining of mineralized osteoblast and hMSC cultures. IPP (50 µM) increased osteoblast mineralization ($P<.05$) Means±SD.

Osteocalcin expression was reduced in control cells from Day 13 to Day 17, rising again by Day 20. IPP-treated cells had a similar expression pattern (Fig. 2F). Caspase-8 expression was induced in control cells on Day 17 and reduced by Day 20, but not to the Day 13 level. IPP-treated cells had a similar expression pattern, but the increase on Day 17 was less than in control cells (Fig. 2G). OPG expression remained at a constant level in control cells over the study period. In IPP-treated cells, OPG expression was less than in control cells on Day 13 increasing to the same level by Day 17 (Fig. 2H). RANKL expression did not differ within or between treatments over the study period (Fig. 2I). The control cell RANKL/OPG ratio increased on Day 17, whereas IPP treatment prevented the rise, maintaining a constant RANKL/OPG ratio throughout the study (Fig. 2J).

4. Discussion

This in vitro study was conducted to clarify the long-term effects of tripeptide IPP on osteoblast differentiation and function. The results show that IPP increases matrix formation-related osteoglycin expression and matrix mineralization, and reduces apoptosis-related caspase-8 expression. IPP had no effect on the activity of osteoblast differentiation marker bALP.

bALP serves as a useful marker of the transition from the proliferative period to matrix maturation in osteogenesis [16,17]. In our study, bALP activity started to rise in control and IPP-treated cells after 1 week of culture. bALP activity doubled after 2 weeks and continued to rise up to nearly sevenfold by 3 weeks of culture. This result is in line with previous studies [16,18,19]. Our hypothesis was that IPP enhances osteoblast differentiation, but this was not supported by the bALP activity measurement, as no difference was found between control and IPP-treated cells. However, osteoblast differentiation is not regulated by a single set of factors, and all genes need not be expressed similarly in order to up-regulate osteoblast markers [20,21]. Although no difference between bALP activities was present to verify our hypothesis of increased osteoblast differentiation due to IPP, long-term treatment with IPP did enhance other markers related to osteoblast function.

The three principal periods in osteoblast development are proliferation, extracellular matrix maturation and mineralization [16,18,22,23]. Each period of osteoblast development has specific modifications in gene expression, and these can be used to follow osteoblast development [16]. The RT-PCR results here are from culture Days 13, 17 and 20; hence, the results represent the later stages of osteoblast differentiation, i.e., matrix formation and mineralization. The RT-PCR genes examined included cell signaling (β-catenin, Cbfa1/Runx2, CREB-5, OPG, RANKL), matrix formation (osteoglycin, osteocalcin), cell death (caspase-8) and hormone-related (PTHrP) genes.

In our previous study, a short-term treatment with IPP up-regulated genes involved in cell differentiation, growth and signal transcription [12]. In the present study, β-catenin, a transcriptional co-activator [24], was up-regulated on Day 17, and at the same time transcription factor Cbfa1/Runx2

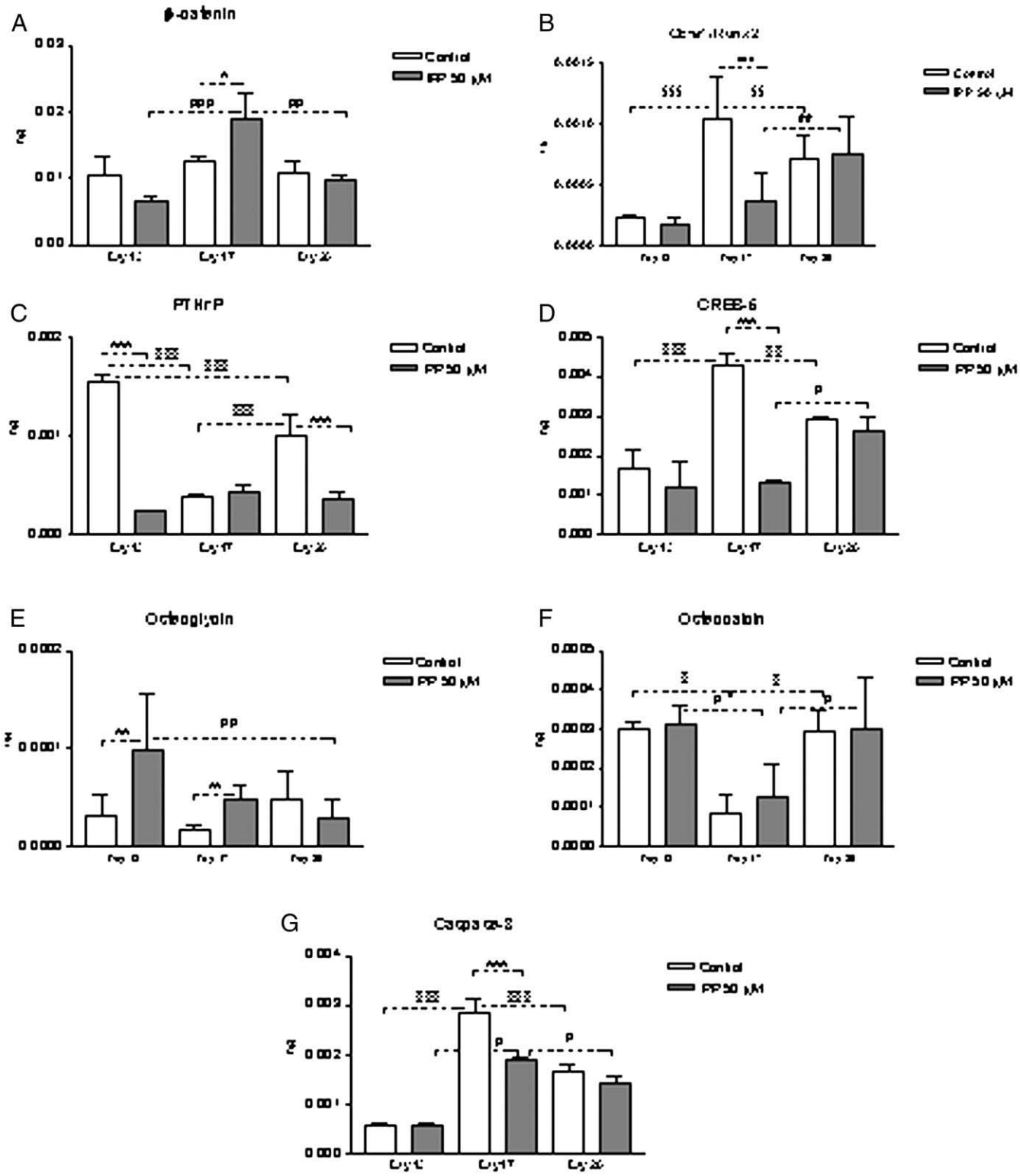

Fig. 2. mRNA expression on Days 13, 17 and 20 in hMSC-differentiated osteoblasts (control) and osteoblasts cultured with 50 μM IPP. The qRT-PCR results represent means±S.D. ANOVA followed by Tukey's post hoc: control vs. IPP, *P<.05, **P<.01, ***P<.001; control vs. control over time, $^{§}P$<.05, $^{§§}P$<.01, $^{§§§}P$<.001; IPP vs. IPP over time, $^{\#}P$<.05, $^{\#\#}P$<.01, $^{\#\#\#}P$<.001.

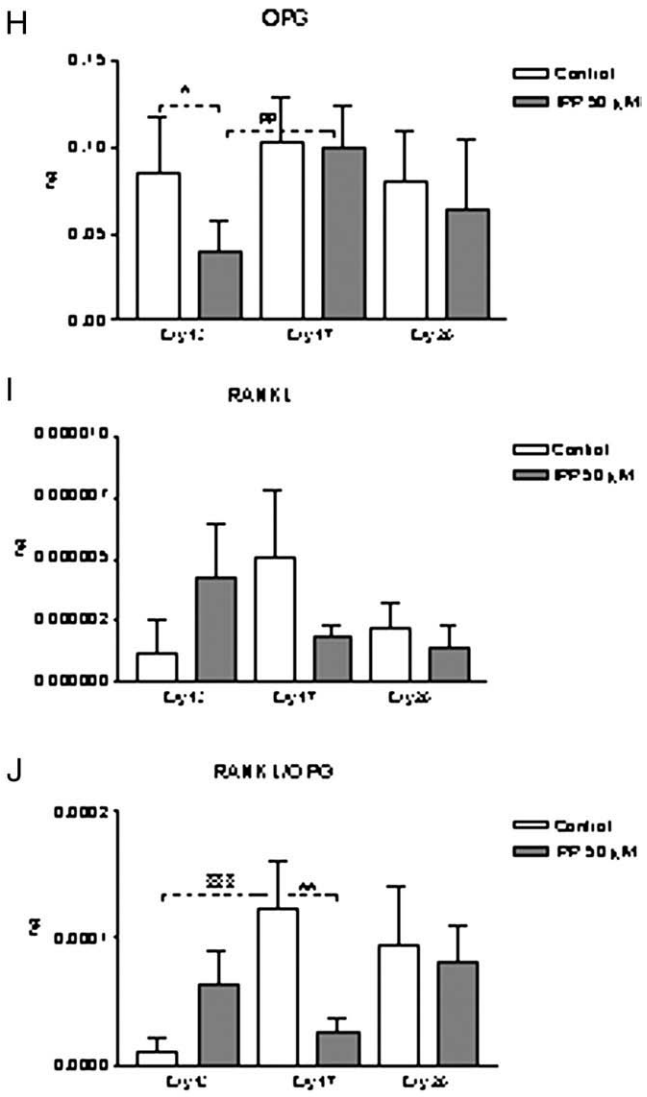

Fig. 2 (*continued*).

was reduced. Cbfa1/Runx2 is the most extensively studied transcription factor in osteogenesis, and it plays an essential role in osteoblast differentiation and bone formation [25,26]. Our hypothesis was that IPP enhances osteoblast differentiation, but neither bALP activity nor Cbfa1/Runx2 was up-regulated. The drop in Cbfa1/Runx2 expression could be a result of increased β-catenin expression, as the Wnt/β-catenin pathway represses Cbfa1/Runx2 via lymphoid enhancer factor-1 [27]. The Wnt/β-catenin pathway regulates bone development through different mechanisms at different stages of life [24]. In the early stages of osteoblastogenesis, the Wnt/β-catenin pathway stimulates Cbfa1/Runx2 expression [28], but Day 17 falls into a later stage of osteoblast differentiation. An earlier time point, such as Day 7, might have shown stimulation of Cbfa1/Runx2 expression. Furthermore, human osteoblast differentiation is associated primarily with increased Cbfa1/Runx2 activity, without a change in mRNA or protein levels [29]. In this study, we did not look at Cbfa1/Runx2 activity, only expression at the mRNA level; the effect of IPP on Cbfa1/Runx2 activity might differ from that of Cbfa1/Runx2 mRNA expression.

In the later stages of osteoblast development, the Wnt/β-catenin pathway down-regulates RANKL expression and controls osteoclast differentiation [30,31]. In our study, a significant decrease occurred in the RANKL/OPG ratio in IPP-treated cells on Day 17; β-catenin expression was simultaneously increased. The reduction in the RANKL/OPG ratio in IPP-treated cells was a result of increased OPG expression and a slight decrease in RANKL expression from Day 13 to Day 17. On Day 13, the OPG was reduced in IPP-treated cells, but the RANKL/OPG ratio did not differ between treatments. In osteoclast differentiation, the ratio of these two ligands is important, as they compete for RANK receptor. A decline in the RANKL/OPG ratio could result in reduced osteoclastogenesis and bone resorption, favoring bone formation. In an earlier study by Narva et al. [32], IPP had no direct effect on osteoclasts, but our result suggests that in an osteoblast–osteoclast co-culture IPP diminishes osteoclast formation and function via a reduced RANKL/OPG ratio.

Matrix formation precedes mineralization. Osteoblasts synthesize matrix proteins and then facilitate matrix mineralization. Osteoglycin is a small leucine-rich proteoglycan found in the extracellular matrix of bone. In vitro, osteoglycin expression exhibits stage dependence and is high during extracellular matrix production, decreasing with osteoblast differentiation [33,34]. Osteocalcin, another matrix protein, is present in osteoblasts from Day 7 of the differentiation process [25,26], but its appearance also correlates with transition to the mineralization phase [16,18]. In our study, control cell osteoglycin expression remained constant, while IPP up-regulated it on Days 13 and 17. Osteocalcin had a similar expression pattern in control and IPP-treated cells. The expression was high on Days 13 and 20, but lower on Day 17.

Matrix accumulation and mineralization activate an oxidative stress response in osteoblasts [18]. The oxidative stress causes DNA damage and cell death. Our results show a marked increase in caspase-8 mRNA on Day 17. IPP significantly lowered this increase. The increase in caspase-8 mRNA could be a response to the oxidative stress caused by matrix accumulation. Caspase-8 is an initiator caspase, which in turn activates caspase-3, the true executioner leading to cell apoptosis. In addition to reducing caspase-8 on Day 17, IPP simultaneously increased β-catenin mRNA. In osteoblasts, β-catenin exhibits anti-apoptotic effects [24,35]. A reduction in osteoblast apoptosis could enhance bone formation [36]. Our mineralization results confirmed that IPP increases mineralization. Increased mineralization with IPP has previously been shown by Narva et al. [32] with mouse bone marrow-derived osteoblasts.

Mesenchymal cells differentiating into osteoblasts produce PTHrP at an early stage, with decreasing levels as cells mature [37]. Within the skeletal microenvironment, PTHrP

propels pluripotential bone marrow stromal cells towards the osteogenic lineage and also exerts anti-apoptotic effects [38]. In addition to promoting osteoblastogenesis and osteoblast survival, PTHrP increases osteoblast RANKL production, hence promoting osteoclastogenesis [37]. The ability of PTH to drive changes in gene expression is dependent upon activation of such transcription factors as CREB [39]. In response to PTH, CREB is phosphorylated and activates transcription. In our study, control cell PTHrP mRNA was up-regulated on Day 13, with CREB-5 and RANKL mRNA up-regulation following on Day 17. IPP abolished these changes. We previously found short-term (24-h) treatment with IPP to increase PTHrP and CREB-5 expression [12], and now we show long-term IPP exposure reduces both. Kulkarni et al. [40] demonstrated exogenous PTH to increase β-catenin levels and to stimulate the canonical Wnt signaling pathway in vitro and in vivo. In our study, endogenous PTHrP did not influence β-catenin levels, and vice versa.

Many factors affect the expression of the osteoblast phenotype in culture, i.e., cell source, culture medium, culture time and the presence of compounds that influence cell proliferation and differentiation [41]. Variation in culture conditions may cause deviation in results. The origin of the cells is also a confounding factor. A cell line prepared from tumor tissue may not correctly reflect osteogenic changes in normal human osteoblasts; hence, we used hMSCs and osteoblasts differentiated from these. Although direct comparison of different in vitro studies is difficult, general guidelines apply to all osteoblast differentiation. Based on the results presented here, we can conclude that continuous treatment with tripeptide IPP enhances osteoblast gene expression in favor of bone formation and increases mineralization. IPP's effects seem to be related to or transmitted through β-catenin, as shown by several temporal associations as well as literature linking the same genes to β-catenin [24,27,30,31,35]). Long-term treatment with IPP had no effect on bALP activity or osteocalcin expression. These results indicate that IPP does not have an impact on osteoblast differentiation, instead influencing osteoblast function. Factors that increase the number or function of osteoblasts can have an effect on bone formation, also contributing to bone mineral mass development in vivo. This was an in vitro experiment, and the in vivo effects of IPP on osteoblasts might differ since eating frequency drives food consumption and protein degradation products, such as bioactive peptides, are available periodically, not continuously as in this study. Intermittent but frequent exposure to IPP could have a different, even more ample effect on osteoblast function.

Acknowledgment

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