β2- and β3-, but not β1-adrenergic receptors are involved in osteogenesis of mouse mesenchymal stem cells via cAMP/PKA signaling

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ABSTRACT

The osteogenic capacity of mesenchymal stem cells (MSCs) and the importance of β-adrenergic signals in bone formation and resorption have been well investigated. However, little is known about the development of β-adrenergic receptor (β-AR) systems and the role of β-adrenergic signals in osteogenic differentiation of MSCs, which is critically important in bone physiology and pharmacology. In this study, we demonstrated that both the mRNA and protein levels of β2- and β3-AR are up-regulated following osteogenesis of mouse MSCs. We also established that β-AR agonists negatively while antagonists positively affect MSC osteogenesis. Both β2- and β3-AR are involved in MSC osteogenesis, with β2-AR being dominant. The effect of β-ARs on MSC osteogenesis is partly mediated via the cAMP/PKA signaling. These findings suggest that MSC is also a target for β-adrenergic regulation and β-adrenergic signaling plays a role in MSC osteogenesis.

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Introduction

The bone is a specialized and dynamic organ that undergoes continuous regeneration, through the interplay of two functions: bone resorption by osteoclasts and bone formation by osteoblasts [1]. Several neuropeptides, neurohormones and neurotransmitters and their receptors are detectable in the bone and the bone marrow microenvironment, which locally control the development and differentiation of osteoblasts and osteoclasts [2,3]. Signals derived from the endocrine and autonomic nervous systems also exert potent effects on osteoclast and osteoblast development and differentiation [4–6]. The adrenergic receptors (ARs)[1] are one class of G-protein coupled receptors to receive signals from the nervous system, which could be categorized into α and β groups [7]. For the β-AR, it is classified into β1, β2, and β3 subtypes, which are coupled to the G-proteins for activation of adenylyl cyclase (AC) to stimulate cAMP formation [8].

Evidence for the functional expression of particular subtypes of β-ARs in undifferentiated mesenchymal stem cells (MSCs) [9,10], osteoblasts [11–14], and osteoclasts [14–17] is available in the literature. Takahata et al. [10] showed that mRNA expression was seen with β2- and β3-AR subtypes, but not with the β1-AR subtype in mouse MSCs. Previous studies [11–17] have shown that β2-AR is the major subtype expressed on osteoblasts and osteoclasts, via which the sympathetic nervous system (SNS) regulates bone formation and resorption. Bone formation rate, osteoblast number and mineralized surfaces were decreased in β-agonists treated animals and increased in antagonists treated animals [13,19–21]. Mice deficient for catecholamines, β-ARs or AC5 displayed impaired bone resorption, high bone formation rate or high bone mass [18,20,22]. Some clinical observations also found that patients treated with β-blockers showed an increased bone density and a reduced risk of fracture [23,24]. Moreover, pharmacological activation of β-receptors has been reported to cause bone resorption mediated by expression of osteoclast differentiation factors, such as receptor activator of NF-κB ligand (RANKL) [11,14–17].

The osteogenic capacity of MSCs has been well-established [25,26]. However, in contrast to accumulating evidence for the β-adrenergic modulation of bone formation and bone resorption, little attention has been paid to the role of β-adrenergic signals in osteogenic differentiation of MSCs, which is critically important in bone physiology and pharmacology. In this study, the expression profiles of the three β-AR subtypes were determined in mouse MSCs following osteogenic induction. The effect of β-adrenergic

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  [1] *Abbreviations used:* MSCs, mesenchymal stem cells; β-AR, β-adrenergic receptor; ISO, isoproterenol hydrochloride; EP, epinephrine hydrochloride; PH, propranolol hydrochloride; CGP, CGP20712A; ICI, ICI118551; SR, SR59230A; IBMX, 3-isobutyl-1-methylxanthine; AC, adenylate cyclase; PKA, protein kinase A; CREB, cAMP-response element binding protein.

stimulation or suppression on MSC osteogenesis was evaluated. The involved β-AR subtypes were identified through observing the reversal ability of different β-antagonists on the effect of isoproterenol, a general β-agonist. Furthermore, the involvement of the cAMP/PKA signaling was documented by the alteration of cAMP levels and PKA activity following agonists and/or antagonists treatments, and by the blockade of the PKA inhibitor H89 on isoproterenol's effect.

Materials and methods

Chemicals and reagents

Dexamethasome, L-ascorbic acid, β-glycerophosphate, isoproterenol hydrochloride (ISO), propranolol hydrochloride (PH), epinephrine hydrochloride (EP), CGP20712A (CGP), ICI118551 (ICI), SR59230A (SR), H89 and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO), cell culture reagents from Gibco (Gibco BRL, CA), and PCR reagents and primers from Invitrogen (Carlsbad, CA) or Applied Biosystems (Carlsbad, CA). Antibodies to β1-AR, β2-AR, β3-AR, bone morphogenetic protein 2 (BMP2), GAPDH, β-tubulin and the horseradish peroxidase (HRP)-labeled were from Santa Cruz (Santa Cruz, CA). A calcium assay kit, a cAMP assay kit and a non-radioactive cAMP-dependent PKA assay system were from BioAssay Systems (Hayward, CA), R&D systems (Minneapolis, MN) and Promega (Madison, WI), respectively. All other reagents were from domestic resources and of highest grade, unless otherwise stated.

Isolation, cultivation and osteogenic differentiation of mouse MSCs

Mouse MSCs were isolated from femur and tibia bone marrow from Kunming female mice (4–6 weeks old) as previously described [27]. The isolated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere with 5% CO_2. After 5 days, non-adherent cells were removed, the adherent cells were washed with PBS three times and resuspended in the complete culture medium, with medium replacement twice a week. When becoming near confluent, cells were detached and passaged. Experiments described here were performed using the third passage cells. Osteogenic differentiation of MSCs was assessed in the complete medium supplemented with 0.01 μM dexamethasone, 50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate as osteogenic stimuli (OS).

The expression of β-AR subtypes in MSCs following osteogenic induction

MSCs were seeded in 6-well plates and cultured for 50% confluence. Quantitative real-time PCR (qRT-PCR) was performed to detect the expression of three β-AR subtypes in osteogenic MSCs at

0, 3, 7, 10 and 14 days post-induction, using the primer sequences shown in Table 1. To eliminate the alteration resulting from culture, β-ARs expression in cells treated with vehicle (no treatment, NT) was also determined at the indicated intervals. Total RNA was extracted with TRIzol reagent following the supplier's instruction, and reverse transcription and qRT-PCR were performed as previously described [28,29]. qRT-PCR was performed by using a 7300 real-time PCR system (Applied Biosystems). The PCR protocol consisted of 40 cycles, as follows: denaturation at 94 °C for 15 s, annealing at 56 °C for 20 s, and amplification at 72 °C for 45 s. Expression of β-AR genes was calculated relative to GAPDH levels by the $2-\Delta\Delta Ct$ method using 7300 system software. All experiments were triplicated.

Western blot analysis was conducted to detect the protein expression of β-ARs in osteogenic MSCs (seeded in 6-well plates) at the indicated intervals, as previously described [30]. Fifty micrograms of protein from each sample served to measure the expression of the β-AR subtypes using GAPDH as internal control. The result was visualized by chemiluminescence method using an ECL kit (Amersham, Germany).

Effects of ISO on MSC osteogenesis

MSCs were seeded in 24-well plates and cultured for 50% confluence. The effect of a general β-AR agonist (ISO) at concentrations of 0.001, 0.01, 0.1 or 1 μM on MSC osteogenesis was determined. On day 14 post-treatment, alizarin red S (ARS) staining was performed to detect the mineral nodules in treated and control MSCs, as described by Stanford et al. [31]. Briefly, the cells were fixed with 95% ethanol for 30 min at room temperature (RT). The fixed cells were washed with PBS and stained for 10 min with 40 mM ARS (pH 4.2) at RT with rotation. After washing with distilled water five times and rinsing with PBS for 15 min, whole well photographs were taken with a camera (Olympus, Japan). Additionally, calcification was assessed as previously described [32]. In brief, the cultures were decalcified with 0.6 N HCl in PBS for 24 h at RT. The calcium content of the HCl supernatant was determined colorimetrically using a calcium assay kit (BioAssay Systems). After decalcification, the cultures were washed with PBS and solubilized with 0.1 N NaOH/0.1% SDS. The calcium content was normalized to total protein concentration determined using a BCA assay kit (Beyotime, China), and expressed as μmol Ca/mg protein.

To determine the time-course effect of ISO, 0.1 μM ISO was supplemented into the OS induction medium during the following intervals: 0–3 days, 0–7 days, 0–10 days and 0–14 days. After 14 days, ARS staining and calcium quantification were performed as described above.

Effects of pre-treatment with different antagonists on the activity of ISO

MSCs were seeded in 24-well plates for ARS staining and calcium quantification analysis, 6-well plates for qRT-PCR analysis,

Table 1
Sequences of the primers used in this study. The sizes of the PCR products are given in base-pairs (bp).

Genes	NCBI number	Forward primers (5′ – 3′)	Reverse primers (5′ – 3′)	Sizes (bp)
β1-AR	NM_007419	TGGCTTACTGGCTTGTCTTG	TTTCCACTCGGGTCCTTG	135
β2-AR	NM_007420	GGACAACCTCATCCCTAA	AGAGTAGCCGTTCCCATA	169
β3-AR	NM_013462	CAGTCCCTGCCTATGTTTG	TTCCTGGATTCCTGCTCT	165
Runx2	AF010284	CCGCACGACAACCGCACCAT	CGCTCCGGCCCACAAATCTC	289
BMP2	NM_007553	TGGCCCATTTAGAGGAGAACC	AGGCATGATAGCCCGGAGG	279
COL-I	NM _007742	CTGCCTGCTTCGTGTAAA	ACGTTCAGTTGGTCAAAGGTA	213
OCN	L24431	GAGCCCTTAGCCTTCCAT	GCGGTCTTCAAGCCATAC	297
GAPDH	NM_008084	GACTTCAACAGCAACTCCCAC	TCCACCACCCTGTTGCTGTA	125

respectively. To further confirm the role of β-ARs and the involved subtypes in MSC osteogenesis, MSCs were treated with 0.1 μM ISO alone or pre-treated for 1 h with 0.1 μM PH, a general β-AR antagonist, or CGP, ICI and SR, respectively the specific β1-, β2- and β3-AR antagonists, in the presence of OS. Additionally, 1 μM H89, a protein kinase A (PKA) inhibitor, was pre-exposed for 1 h before the supplement of 0.1 μM ISO. The effect of 0.1 μM EP, a natural β-AR ligand, on MSC osteogenesis was also determined. The medium was replaced twice a week, agonist/antagonists were supplemented once medium was exchanged in the first 7 days, and OS was singly added to the culture medium for an additional 7 days. All the concentrations were optimized from pre-experiments and the same doses were used in the following experiments. On day 14 post-treatment, mineralization intensity was determined using ARS staining and calcium content analysis.

In addition, the mRNA levels of selected osteoblast marker genes, including BMP2, runt-related transcription factor 2 (Runx2), type I collagen (COL-I) and osteocalcin (OCN), were analyzed by qRT-PCR after 1, 7 or 14 days treatment with ISO in itself or pre-exposure with different β-antagonists, using primers shown in Table 1. Total RNA extraction, reverse transcription and qRT-PCR were performed as described above.

Measurement of cAMP concentration in cells upon β-AR agonists/ antagonists treatment

cAMP assay was performed as described by Takeda et al. with minor modifications [13]. After 7-day induction with OS, MSCs seeding in 12-well plates were initially incubated with serum-free medium containing 100 μM IBMX for 15 min, then PBS, EP, and ISO alone or in combination with PH, CGP, ICI or SR, were added for an additional 10 min at 37 °C. Intracellular cAMP concentration was measured by immunoassay, following the manufacturer's instructions (R&D Systems). Parallel wells were analyzed for cell number count by using a haemacytometer. cAMP content was expressed as pmol/10^7 cells.

Analysis of PKA activity

MSCs seeding in 6-well plates were treated with ISO alone or pre-treated for 1 h with different antagonists (PH, CGP, ICI, SR, or H89) in the presence of OS. The medium was replaced twice a week and agonist/antagonists were supplemented once medium was exchanged. At day 7, cultures were rinsed with cold PBS and protein was extracted using the suggested extraction buffer. The suspension was homogenized and centrifuged 5 min at 14,000g, after which the supernatant was collected. Two micrograms of PepTag® A1 Peptide were incubated as described in the standard PKA assay (Promega) with aliquots of supernatant in a final volume of 25 μl for 30 min at RT. The reactions were stopped by heating at 95 °C for 10 min. The samples were separated on a 0.8% agarose gel at 100 V for 15 min. Phosphorylated peptide migrated toward the anode (+), while nonphosphorylated peptide migrated toward the cathode (−). The gel was photographed on a UV trans-illuminator.

Western blot analysis of BMP2 expression

Cell treatment was the same as that for PKA activity analysis. Thirty micrograms of protein from each sample served to detect the expression of BMP2 using β-tubulin as internal control.

Statistical analysis

Data were presented as means ± SD. Significant differences were determined by one-way ANOVA (Origin 6.0). A $p < 0.05$ was considered significant and highly significant at $p < 0.01$.
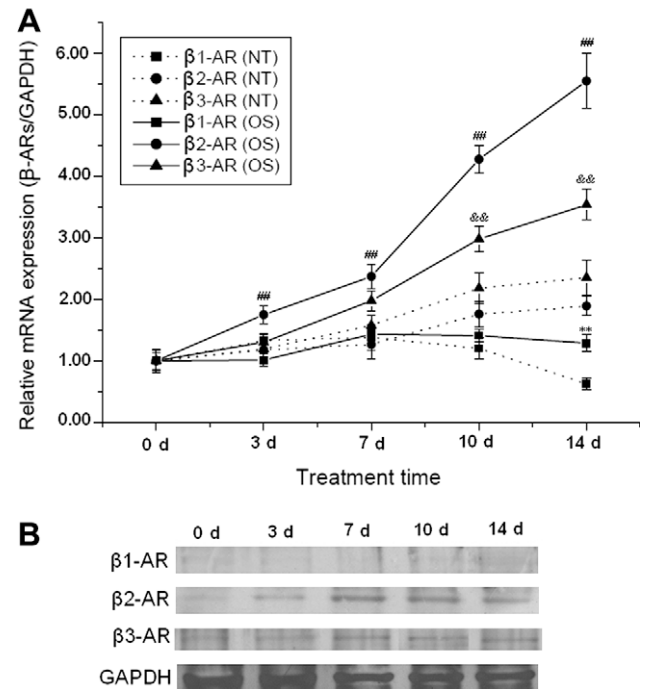

Fig. 1. The time-course expression of β-ARs during MSC osteogenesis. (A) The time-course mRNA expression of β-AR subtypes in cells treated with or without OS. The expression levels of each β-AR subtype at day 0 were set to "1.00", and the fold-changes at other days were calculated relative to this. Values were means ± SD of three separate experiments ($n = 3$). $^{**}p < 0.01$, $^{##}p < 0.01$, $^{\&\&}p < 0.01$ β1-, β2- or β3-AR expression in OS treatments versus NT at corresponding days, respectively. (B) The protein expression of β-ARs in cells following osteogenic induction at the indicated intervals. GAPDH was used as internal control. Similar result was obtained in other two experiments.

Results

β-ARs expressed in MSCs upon osteogenic induction

The mRNA expression of three known β-ARs during MSC osteogenesis was analyzed by qRT-PCR. As shown in Fig. 1A, the mRNA expression of β1-AR had minor alterations either in cells treated or untreated with OS. The expression of β2-AR was up-regulated substantially following OS induction (OS groups) compared with corresponding control cultures (NT groups), which was increased by approximately 4.5-fold after 14 days induction. The expression of β3-AR gene was also increased after 10 days or 14 days inducement, with 2.5-fold up-regulation at day 14.

The protein expression profile of β-ARs in MSCs upon osteogenic induction was presented in Fig. 1B. The expression of β1-AR was barely detectable either before or post-induction. The protein expression of β2-AR was markedly up-regulated following osteogenic induction, but no further increase was seen after 7 days induction. The β3-AR was also induced upon OS treatment, but its alteration extent was relatively low.

ISO suppressed MSC osteogenesis in a dose- and time-dependent manner

The dose- and time-course effect of ISO on MSC osteogenesis was detected by ARS staining and calcification determination. Fig. 2A showed that mineral matrix nodules were barely detectable in NT groups, while substantial stained nodules were seen in OS groups. ISO inhibited the mineral intensity in a dose-dependent manner. The calcium content was decreased by 28.2%, 44.3%, 55.4% and 56.6% with ISO ranging from 0.001 to 1 μM, respectively
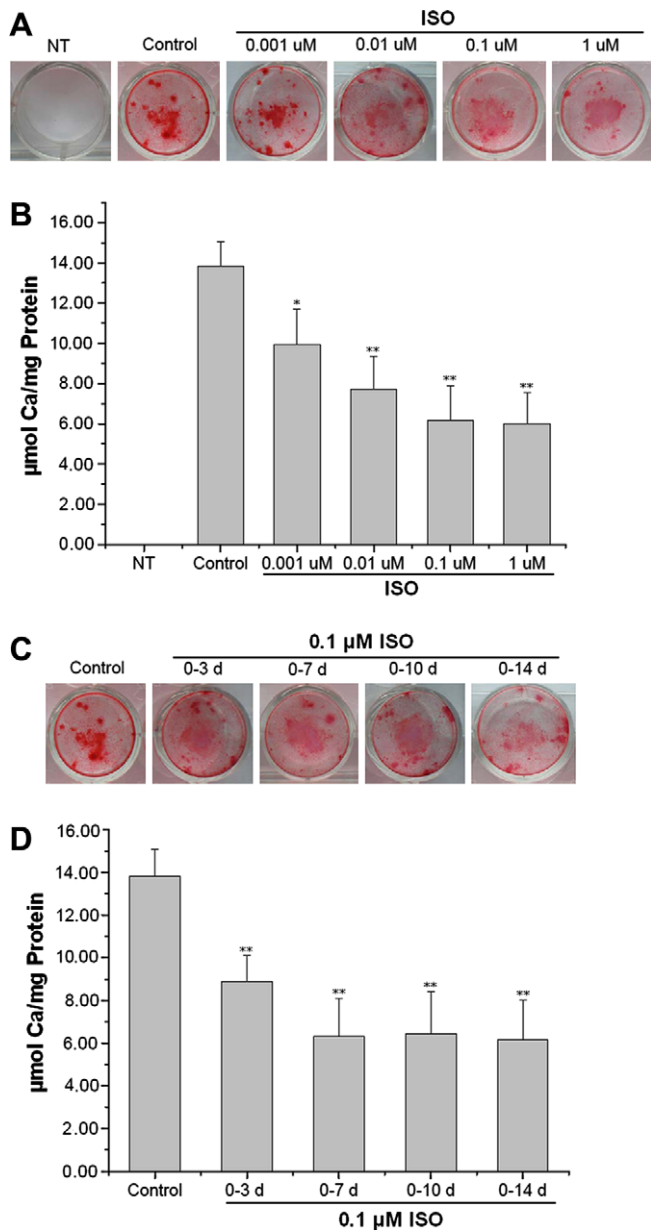
A

B

C

D

Fig. 2. Effects of ISO on MSC osteogenesis. (A, C) The formed mineral nodules in cells treated with increasing concentrations of ISO or with 0.1 μM ISO for different durations were identified by ARS staining at day 14 post-induction. (B, D) The calcium content in cells treated with increasing concentrations of ISO or with 0.1 μM ISO for different durations. Values were means ± SD of three separate experiments ($n = 4$). $^*p < 0.05$, $^{**}p < 0.01$ versus Control (OS treatment).

(Fig. 2B). Moreover, 0.1 μM ISO was supplemented to OS-induced MSCs for different incubation durations. Our study revealed that exposing MSCs to ISO for 3 days resulted in decreased nodules and substantially less nodules in cells treated for 7 days. Correspondingly, a 7-day exposure with ISO decreased the calcium content by up to 54.4%. However, no further suppression was observed when exposing the cells for even 10 or 14 days (Fig. 2C and D). These data indicated that ISO might mainly exert its effect in the early differentiation stage.

Reversal of different antagonists on the inhibitory activity of ISO

The mineralization intensity in cultures either singly with ISO or pre-incubated with selected inhibitors was measured by ARS staining and calcium quantification analysis. As shown in Fig. 3A,

ISO significantly inhibited the formation of mineral nodules, while PH, ICI, or SR could reverse this inhibition to certain extent. The calcium content was decreased by 54.4% following ISO treatment, whereas it could respectively be reversed to 107.1%, 78.7% and 65.4% of control by PH, ICI, and SR, but not by CGP (Fig. 3B). In addition, H89, the PKA inhibitor, could totally rescue the suppression of mineralization by ISO to the control level, indicating that the PKA pathway may be involved. Moreover, ISO's effect can be mimicked by EP, in which the mineralization extent was only 46.9% of control (Fig. 3A and B).

To determine whether the β-AR ligands regulate MSC osteogenesis by affecting the expression of some key osteogenic marker genes, we examined the mRNA levels by qRT-PCR of BMP2, Runx2, COL-I and OCN in cells upon different treatments at day 1, 7 and 14, respectively (Fig. 3C). ISO decreased the mRNA levels of BMP2, while pre-treatment with PH or ICI could totally overcome this inhibition to the extent similar to or exceeding its expression as controls at all the indicated days. SR only rescued its effect at day 7, whereas CGP showed no significant reversal at any time. The expression of Runx2 was inhibited significantly by ISO; at day 1, only pre-treatment with PH restored this inhibition; at day 7, PH and ICI pre-exposure abolished the effect of ISO by 82.4% and 45.2%, respectively; at day 14, PH, ICI or SR all could partially rescue ISO's inhibitory effect. The expression of COL-I was also suppressed by ISO, and this suppression was abolished by pre-treatment with either PH or ICI, while neither CGP nor SR exerted significant reversal effect. By contrast, PH, ICI or SR could all reverse the reduction on OCN expression by ISO to certain extent. In addition, H89 exerted potent and highly reversal effect on ISO's inhibitory activity for all of the four genes at all the conditions, except for Runx2 expression at day 1 (Fig. 3C).

cAMP production and PKA activity were modulated by agonists/antagonists in osteogenic MSCs

It has been demonstrated that β-ARs are G-coupled receptors that signal through the cAMP-dependent pathway, and PKA is one of the downstream mediators of this pathway [8]. To assess the biological relevance of β-ARs with MSC osteogenesis, we measured the intracellular cAMP concentration and PKA activity in cells following different treatments. Results in Fig. 4A showed that EP and ISO increased cAMP levels to 2.19 and 1.94 folds of control, respectively. The stimulation of ISO could be counteracted by PH, ICI, or SR, but not by CGP. The reversal effect could be ranked as PH > ICI > SR. Similarly, PKA activity was increased by ISO addition, as compared to OS alone treatment. Both PH and H89 pre-exposure substantially blocked the activation of PKA activity by ISO. ICI and SR, but not CGP, also showed some reversal activity (Fig. 4B).

β-AR or PKA blockade rescued ISO inhibition of BMP2 protein expression

The trigger for MSC differentiation into osteoblasts begins with BMPs, and BMP2 is the most important subtype [1]. Thus the protein expression of BMP2 was also determined. As shown in Fig. 4C, the expression of BMP2 was highly suppressed by ISO addition, as compared to OS treatment. This inhibition was totally or partially rescued by pre-culturing with PH, ICI or SR, but not by CGP, with PH and ICI being more effective than SR. Moreover, pre-treatment of H89 could potently restore the expression of BMP2.

Discussion

Osteoporosis and the associated susceptibility to fractures are one of the major health threats to the aging people [33,34]. Despite
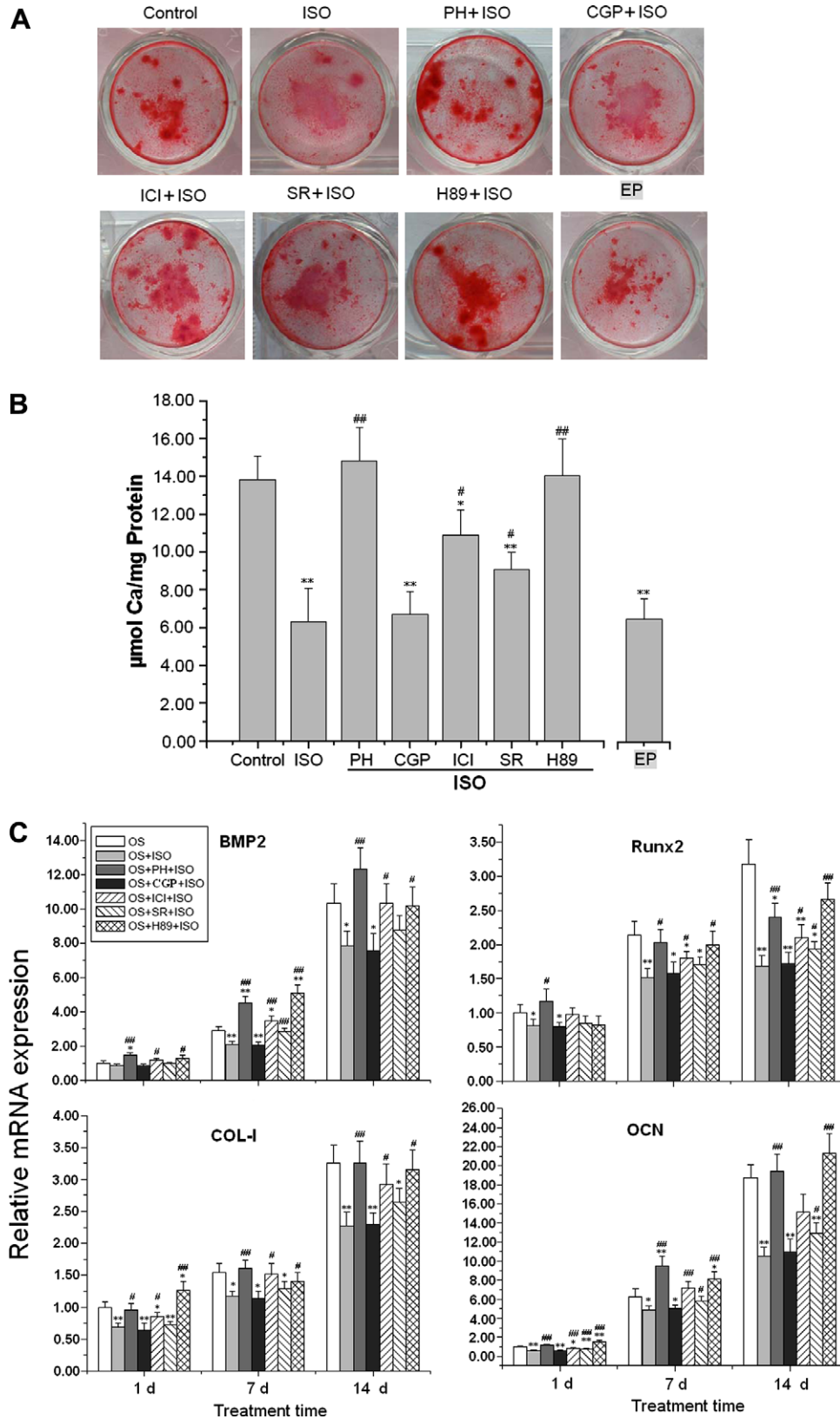
Fig. 3. Reversal of different antagonists on the inhibitory activity of ISO on MSC osteogenesis. (A) The formed mineral nodules in different treatments were identified by ARS staining after 14 days induction. Treatment was performed as described in Method part. (B) The calcium content in different cultures at day 14 post-treatment. Values were means ± SD of three separate experiments ($n = 4$). $^*p < 0.05$, $^{**}p < 0.01$ versus Control (OS treatment); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus OS + ISO treatment. (C) The mRNA levels by qRT-PCR of BMP2, Runx2, COL-I and OCN in MSCs treated with ISO alone or pre-treated with different antagonists at days 1, 7 and 14. The expression levels of each gene upon OS treatment at day 1 were quantified to "1.00", and other values were expressed relative to this. Values were means ± SD of two separate experiments ($n = 3$). $^*p < 0.05$, $^{**}p < 0.01$ versus OS treatment; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus OS + ISO treatment.
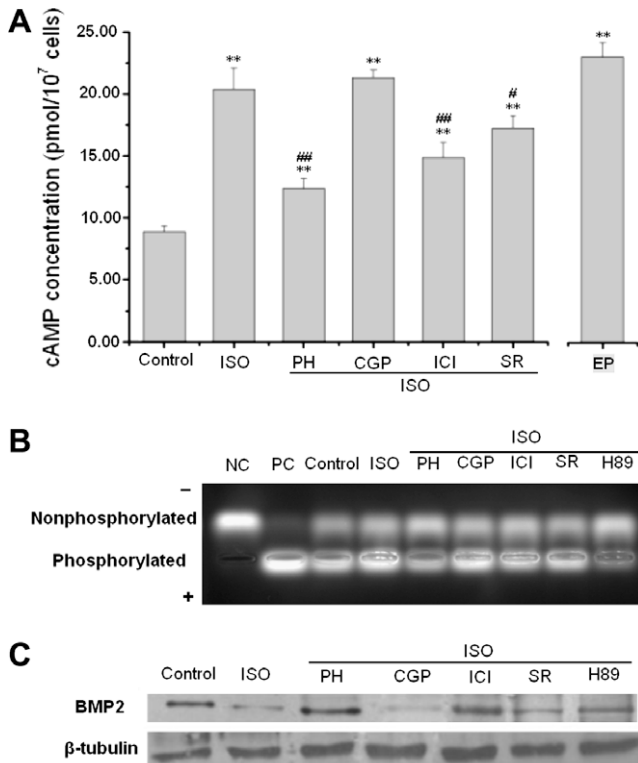
Fig. 4. (A) Intracellular cAMP concentrations in cells following agonists/antagonists treatment. $^{**}p < 0.01$ versus Control (PBS treatment); $^{#}p < 0.05$, $^{##}p < 0.01$ versus ISO treatment. Values were means ± SD of two separate experiments ($n = 3$). (B) PKA activity in cells upon agonists/antagonists treatment. NC: ddH$_2$O, PC: 10 ng of PKA, Control: OS treatment. Similar result was obtained in other two experiments. (C) The expression of BMP2 at the protein level in cells upon agonists/antagonists treatment. β-tubulin was used as internal control. Similar result was obtained in other two experiments.

recent successes with drugs that inhibit bone resorption, the development of modalities to promote local or systemic bone formation is an important issue in the treatment of fractures or osteoporosis, especially to those patients who have lost substantial skeletal bone mass [35–37]. The increase in bone mass may result from an increase in the number of osteoblasts either by formation of new osteoblasts from bone lining cells and/or by stimulating proliferation of mature osteoblasts [24,29,38]. MSCs serve as a source of osteochondral progenitors that invade the bone site, proliferate, and differentiate into cartilage and bone [38,39]. Thus, besides bone cells, MSCs may also be a target for the discovery of bone anabolic agents.

Although the regulation of SNS on bone turnover through the β2-receptors expressed on osteoblasts and osteoclasts has been widely demonstrated [11–17], little is known about the development of β-AR systems during the osteogenic differentiation of MSCs. In this study, all three β-AR subtypes were found on primary MSCs, and both the mRNA and protein expression levels were regulated during MSC osteogenesis (Fig. 1). It is noteworthy to see the substantial increase in qRT-PCR for β2-AR during the day 7 to 14 period with no increase in protein beyond day 7, which suggests that the changes in protein expression are not always a fully reflection of changes at the level of mRNA. Such discrepancies between the mRNA and protein measures have been found in many studies [40,41]. To our knowledge, this is the first report on the expression profile of β-AR subtypes in MSCs following osteogenic differentiation. The presence of β-ARs on MSCs and the modulation of β-ARs both at the gene and protein levels suggested that β-ARs may play a role in MSC osteogenesis.

To clarify the involvement of β-ARs in MSC osteogenesis, we treated MSCs with some β-agonists or antagonists. ISO suppressed the mineralization of MSCs in a dose- and time-dependent manner (Fig. 2). The natural β-AR ligand, EP, also inhibited MSC osteogenesis (Fig. 3A and B). Moreover, a 7-day challenge with various β-antagonists including PH, ICI and SR showed more intense staining nodules and increased calcium content than the control (data is shown in Supplementary Fig. 1). These data indicated that β-AR agonists negatively while antagonists positively affect MSC osteogenesis. Previous studies have shown that treatments with sympathetic agents resulted in decreased bone formation rate and bone mass in mice [13,21]. Combining with our results, the decreased bone formation rate is due to not only the decreased bone growth rate [13,21], but the weakened MSC osteogenic potency may also have contributed.

To identify the involved β-AR subtypes, we treated MSCs with ISO alone or pre-treated with different β-antagonists. Results indicated that the effect of ISO could be totally or partially recovered by PH, ICI or SR, but not by CGP (Figs. 3 and 4). The reversal potency can be ranked as PH > ICI > SR. This tendency is in accordance with the higher expression level and the higher up-regulation extent of β2-AR than that of β3-AR in the process of MSC osteogenesis (Fig. 1). These results demonstrated that both β2-AR and β3-AR are involved in MSC osteogenesis, with β2-AR being the dominant subtype.

Classically, stimulation of β-ARs leads to activation of G-proteins which, in turn, activate AC and cAMP production [7,8]. In our study, cAMP levels in the osteogenic MSCs could be modulated by different agonists/antagonists (Fig. 4A). PKA is a downstream mediator of the cAMP pathway [8]. The effect of ISO could indeed be abolished by the potent PKA inhibitor, H89 (Figs. 3 and 4B, C). These data implicated that β-ARs exert effects on MSC osteogenesis, at least in part, via the cAMP/PKA signaling. The effect of cAMP/PKA signaling on osteogenesis has been studied in different cell types, although the results are contentious [28,29,42–45]. Turksen et al. [42] reported that the AC activator forskolin increased bone nodule formation in fetal rat calvarial cells at low concentrations but inhibited it at higher concentrations. Forskolin inhibited osteoblast phenotypic characteristics [43,44]; but agents that reduce or
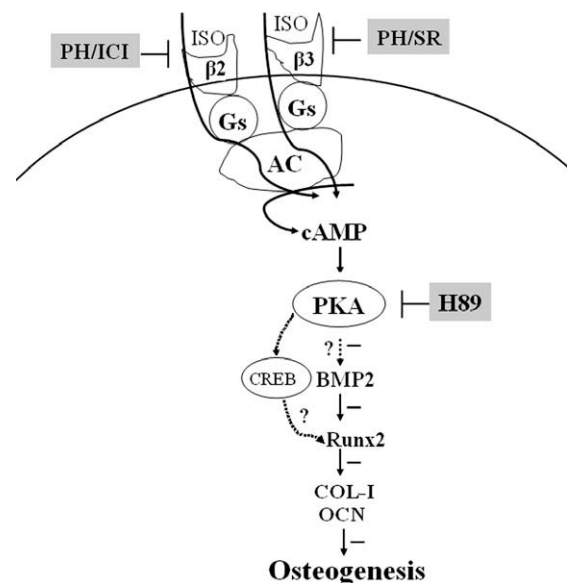

Fig. 5. Tentative pathway for the effect of β-adrenergic regulation on MSC osteogenesis. The detailed description is shown in Discussion part. The signaling pathway suggested based on previous studies or the investigations reported here is shown in bold black arrows; mechanisms not demonstrated here or likely pathways are dotted.

block AC or cAMP activity promoted osteoblast differentiation [44]. Li et al. [28] found that dibutyryl cAMP (d-cAMP) suppressed the expression of Runx2 in chick upper sternal chondrocytes, and H89 blocked the suppression of Runx2. In contrast, opposite results were reported from some other studies [29,45]. D-cAMP or forskolin treatment of calcifying vascular cells stimulated osteoblast-like differentiation [45]. A recent study [29] demonstrated that treatment of human MSCs with d-cAMP stimulates the expression of a set of target genes ultimately resulting in osteogenic differentiation *in vitro* and bone formation *in vivo*. These differences likely depend on the stage and duration in which stimulators/inhibitors are presented, but the stimulators/inhibitors concentration and the nature of the cell culture system may also have contributed to the conflicting observations.

Based on previous studies and the observations reported here, we suggest a pathway for the intracellular signaling from adrenergic stimulation to repression of osteogenic marker genes expression and inhibition of MSC osteogenesis, as depicted in Fig. 5. cAMP level was increased after ISO treatment in osteogenic MSCs. It is likely that the ISO-induced decrease in BMP2 and Runx2 mRNA levels is mediated by PKA, since the effect was totally abolished by the PKA inhibitor H89. The down-regulated BMP2 and Runx2 may negatively affect the expression of later markers COL-I and OCN, and further inhibit the maturation of osteoblasts. Moreover, the inhibitory effect of ISO can be blocked by PH, ICI or SR, and be mimicked by EP.

Overall, these data demonstrated for the first time that (i) both the mRNA and protein levels of β2-AR and β3-AR are up-regulated following osteogenesis of mouse MSCs; (ii) β-AR agonists negatively while antagonists positively affect MSC osteogenesis; (iii) both the β2- and β3-AR are involved in MSC osteogenesis, with the β2-AR being dominant; (iv) the effect of β-ARs on MSC osteogenesis is at least partly mediated via the cAMP/PKA signaling pathway. This study on the functional role of β2- and β3-AR in MSC osteogenesis may provide some information for the discovery and development of bone anabolic agents via β-adrenergic regulation using MSCs as a target, which may have great potential for the treatment of fractures or metabolic bone disorders such as osteoporosis. On the other hand, the exact mechanisms underlying the regulation of β-AR/cAMP/PKA signaling on BMP2 and/or Runx2 expression and the possible differences between mouse and human MSCs remain to be elucidated in future studies.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2010.01.016.

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