



Beta-adrenergic signals regulate adipogenesis of mouse mesenchymal stem cells via cAMP/PKA pathway

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ABSTRACT

The adipogenic capacity of mesenchymal stem cells (MSCs) and the involvement of β -adrenergic signals in lipolysis and thermogenesis have been well established. However, little is known about the development of β -adrenergic receptor (β -AR) systems and the role of β -adrenergic signals in adipogenic differentiation of MSCs. In this study, we demonstrated that both the mRNA and protein levels of β 2- and β 3-AR were up-regulated following adipogenesis of mouse bone marrow derived MSCs. We also established that β -AR agonists negatively while antagonists positively affected MSC adipogenesis. Both the β 2- and β 3-AR were involved in MSC adipogenesis, with β 3-AR being the predominant subtype. The effect of β -ARs on MSC adipogenesis was at least partly mediated via the cAMP/PKA signaling pathway. These findings suggested that MSC is also a target for β -adrenergic regulation, and β -adrenergic signaling (major β 3-signaling) plays a role in MSC adipogenesis.

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1. Introduction

Mesenchymal stem cells (MSCs) have been the subject of intensive investigation over the past decades because of their multipotent ability to differentiate towards osteoblasts, chondrocytes, adipocytes, and even myocytes under defined conditions (Prockop, 1997; Dennis et al., 1999). Besides bone marrow, stem cells with mesenchymal potential also reside in other sources including skeletal muscle (Jankowski et al., 2002), periosteum (Fukumoto et al., 2003), adipose tissue (De Ugarte et al., 2003) and deciduous teeth (Miura et al., 2003). Investigation of MSCs *in vitro* offers a way to address critical questions on differentiation or cell fate determination by inducing them to a certain mature cell type. Adipocytes, deriving from MSCs *in vivo*, play critical roles in both normal physiology and the progression of various disease states (Meunier et al., 1971; Zhou et al., 1999). Thus, it is of considerable importance to understand the mechanism by which adipocyte differentiation is regulated.

The sympathetic nervous system (SNS) regulates several physiological functions via α - or β -adrenergic receptors (β -ARs) by the release of signaling molecules in the vicinity of target organs (Haskó and Szabó, 1998; Nonogaki, 2000; Bachman et al., 2002; Takeda et al., 2002). The importance of β -adrenergic signals in lipolysis and thermogenesis has been demonstrated in both *in vitro* and *in vivo* models (Germack et al., 1997; Margareto et al., 2001; Bachman et al., 2002). Noradrenaline, β -adrenergic agonists and specific β 3-adrenergic agonists were reported to promote thermogenesis and lipolysis, leading to increased energy expenditure and decreased fat stores (Atgié et al., 1998; Margareto et al., 2001; Canová et al., 2006). Conversely, addition of β -adrenergic antagonists reduced thermogenesis and/or lipolysis (Germack et al., 1997; Canová et al., 2006). Bachman et al. (2002) established that mice lacking the three known β -ARs had a reduced metabolic rate and developed massive obesity due entirely to failure of diet-induced thermogenesis.

In contrast to accumulating evidence for the β -adrenergic modulation of lipolysis and thermogenesis (Margareto et al., 2001; Bachman et al., 2002; Canová et al., 2006), however, little attention has been paid to the role of β -adrenergic signals in adipocyte differentiation. In the current study, the expression profile of β -ARs following adipogenic differentiation in mouse bone marrow derived MSCs was determined. Effects of β -adrenergic stimulation or suppression on MSC adipogenesis were evaluated. The involved β -AR subtypes were identified through observing the reversal ability of different β -antagonists on the effect of the β -agonist isoproterenol. Furthermore, the involvement of the cAMP/PKA

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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
β1-AR	NM.007419	TGGCTTACTGCGTTGTCTTG	TTTCCACTCGGGTCCTTG	135 bp
β2-AR	NM.007420	GGACAACCTCATCCCTAA	AGAGTAGCCGTTCCCATATA	169 bp
β3-AR	NM.013462	CAGTCCCTGCCTATGTTTG	TTCTTGATTCTCTGCTCT	165 bp
C/EBPα	NM.007678	TCCGTGCGTCTAAGATGAGG	TGAGTATCCAAGGCACAAGGT	130 bp
C/EBPβ	NM.009883	GCCAAGAAGACGGTGGACAAG	TGAACAAGTTCCCGAGGGTG	208 bp
PPARγ2	NM.011146	GACCACTCGCATTCCTTT	ACAGACTCGGCACATCAAT	264 bp
aP2	K02109	CCTTTGTGGGAACCTGGAA	TGTCGCTCTCGCGTGATTT	225 bp
ATL	AY894805	CTTGATGACCACTCTTCC	TGCTACCCGCTGCTCTTT	167 bp
GAPDH	NM.008084	GACTTCAACAGCAACTCCAC	TCCACCACCTGTTGCTGTA	125 bp

pathway was documented by the alteration of cAMP levels following β-agonists and/or antagonists treatment, and by the blockade of the PKA inhibitor H89 on the effect of isoproterenol.

2. Materials and methods

2.1. Chemicals and reagents

Dexamethasone, insulin, isoproterenol hydrochloride (ISO), propranolol hydrochloride (PH), epinephrine (EP), CGP20712A (CGP), ICI 18551 (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, peroxisome-proliferator-activated receptor gamma 2 (PPARγ2), GAPDH, β-tubulin, and the horseradish peroxidase (HRP)-labeled were from Santa Cruz (Santa Cruz, CA). Genomic DNA isolation kit, DNA quantification kit and cAMP assay kit were obtained from BioVision (BioVision, CA), Sigma (St. Louis, MO) and R&D systems (Minneapolis, MN), respectively. All other reagents were from domestic sources and of highest grade, unless otherwise stated.

2.2. Isolation, cultivation and adipogenic 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 (Zhou et al., 2007). 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% CO₂. 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 reaching subconfluence, the cells were detached by a mild treatment with 0.25% trypsin and 1 mM EDTA and subcultured. The third passage cells were used in the experiments described here. Adipogenic differentiation of MSCs was assessed in a medium supplemented with 0.1 μM dexamethasone and 10 μg/ml insulin as adipogenic stimuli (AS).

2.3. The expression of β-ARs in MSCs following adipogenic differentiation

MSCs were seeded in 6-well plates at a density of 4×10^4 /well and cultured for subconfluence. Quantitative real-time PCR (qRT-PCR) was performed to detect the expression of three β-AR subtypes in adipogenic 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 (Lindgren et al., 2004). qRT-PCR was performed by using a 7300 real time PCR system (Applied Biosystems) according to the manufacturer's protocol. Melt-curve analysis was performed to validate the used primer sets. The mRNA expression of β-ARs was calculated relative to GAPDH levels by the $2^{-\Delta\Delta Ct}$ method using 7300 system software. All experiments were repeated in triplicate.

Western blot analysis was performed to detect the protein expression of β-ARs in adipogenic MSCs (seeded in 6-well plates at a density of 4×10^4 /well and cultured for subconfluence) at the indicated intervals, as previously described (Zhou et al., 2007). 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). Moreover, the protein expression has been quantified by using Image-Quantity software (Bio-rad, USA).

2.4. Effects of ISO on adipogenesis of MSCs

MSCs were seeded in 24-well plates at a density of 1×10^4 /well and cultured for subconfluence. The effect of a general β-AR agonist (ISO) at concentrations of 0.001, 0.01, 0.1 or 1 μM on MSC adipogenesis was determined. On day 14 post-treatment, Oil red O staining was performed to detect the formed lipid droplets in

treated and control MSCs, as described by Li et al. (2003). The bound dye was dissolved with isopropyl alcohol and quantified by reading the absorbance at 510 nm. Parallel wells served for DNA isolation by using a standard kit (BioVision, CA). The adipogenic extent was normalized to total DNA content determined using the DNA quantification kit (Sigma, MO), and expressed as nmol Oil red O/μg total DNA.

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

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

MSCs were seeded in 24-well plates and 6-well plates for Oil red O staining and qRT-PCR analysis, respectively. To identify the involved β-adrenergic subtypes, 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 AS. 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 adipogenesis was also determined. The medium was replaced twice a week, agonist/antagonists were supplemented once medium was exchanged in the first 7 days, and AS was 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, the formed adipocytes were detected using Oil red O staining and quantification analysis. In addition, the mRNA levels of some adipocyte marker genes, including CCAAT/enhancer binding protein α (C/EBPα), C/EBPβ, PPARγ2 and adipocyte fatty acid binding protein (aP2), were analyzed by qRT-PCR after 1, 7 or 14 days treatment, using primers shown in Table 1. Total RNA extraction, reverse transcription and qRT-PCR were performed as above.

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

cAMP assay was performed as described by Takeda et al. (2002) with minor modifications. After 7-day induction with AS, cells 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 haemocytometer. cAMP content was expressed as pmol/10⁷ cells.

2.7. Western blot analysis of PPARγ2 expression

Western blot analysis was performed as above, using proteins extracted from MSCs (seeded in 6-well plates) incubated singly with ISO, or pre-incubated for 1 h with different antagonists (PH, CGP, ICI, SR or H89), in the presence of AS for 7 days. The medium was replaced twice a week and agonist/antagonists were supplemented once medium was exchanged. Thirty micrograms of protein from each sample served to detect the expression of PPARγ2 using β-tubulin as internal control.

2.8. Statistical analysis

Data represent means ± SD. Significant differences were determined by one-way ANOVA (Origin 6.0). A $p < 0.05$ was considered significant.

3. Results

3.1. β-ARs expressed in MSCs following adipogenic differentiation

The mRNA expression of β-ARs during MSC adipogenesis was analyzed by qRT-PCR. The result was presented in Fig. 1A. As seen, the mRNA levels of β1-AR increased and peaked at day 7, but

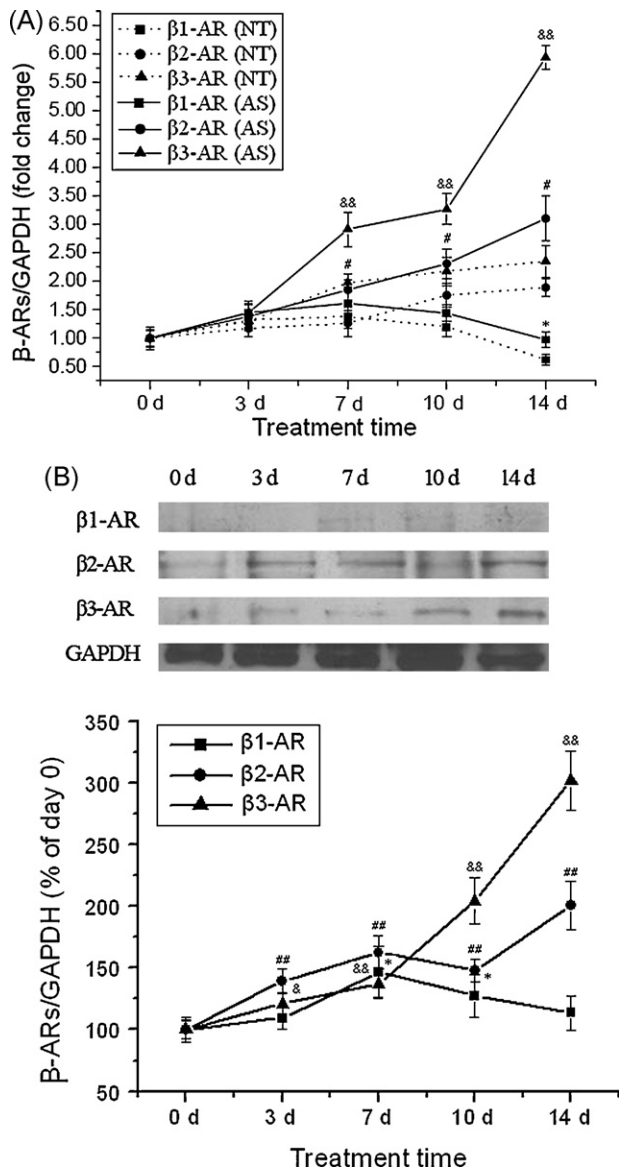


Fig. 1. The expression of the three β -AR subtypes in MSCs during adipogenic differentiation. (A) The time-course mRNA expression of $\beta 1$ -, $\beta 2$ - and $\beta 3$ -AR in cells treated with or without AS. The expression levels of each β -AR at day 0 were set to 1.00, and the fold-changes at other days were calculated relative to this. * $p < 0.05$; ** $p < 0.01$ the mRNA levels of $\beta 1$ -, $\beta 2$ - or $\beta 3$ -AR in AS treatments versus that in NT at corresponding days, respectively. Values were means $\pm$ SD of three separate experiments ($n = 3$). (B) The protein expression of β -ARs following adipogenic induction at the indicated intervals. GAPDH was used as internal control. The expression levels of each β -AR at day 0 were set to 100%, and the relative fold-changes at other days were calculated accordingly. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ the protein levels of $\beta 1$ -, $\beta 2$ - or $\beta 3$ -AR in different treatment days versus that at day 0, respectively. Similar result was obtained in other two experiments.

declined thereafter, falling to 62% and 98% of control by day 14 in NT and AS treated groups, respectively. In contrast, the levels of $\beta 2$ - and $\beta 3$ -AR were induced increasingly in both NT and AS treated groups. Following AS treatment, MSCs differentiated into adipocytes and expressed 2.1-fold and 4.9-fold more $\beta 2$ - and $\beta 3$ -AR, respectively.

We also determined the protein expression profiles of β -ARs in MSCs upon adipogenic induction. As shown in Fig. 1B, the expression of $\beta 1$ -AR was barely detectable, either before or post-induction. The protein expression of $\beta 2$ -AR was up-regulated after adipogenic induction, peaked early and showed no significant change at the latter four time points. Likewise, the $\beta 3$ -AR was

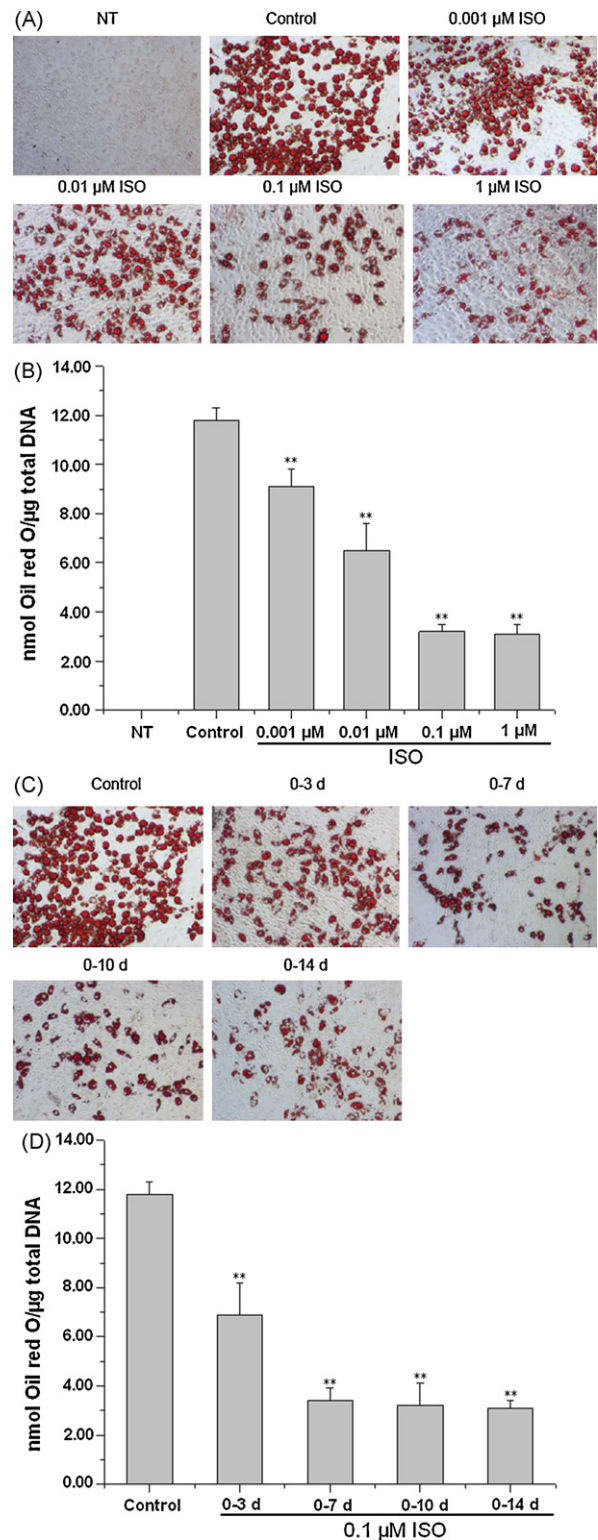


Fig. 2. Effects of ISO on MSC adipogenesis. (A) and (C) Adipocytes in cells treated with increasing concentrations of ISO or with 0.1 μ M ISO for different durations were identified by Oil Red O staining after 14 days. Images were from representative fields of view for each culture condition. (B) and (D) The bound Oil red O was dissolved with isopropyl alcohol and absorbance at 510 nm was measured, which was quantified to nmol Oil red O/ μ g total DNA. Values were means $\pm$ SD of three separate experiments ($n = 4$). ** $p < 0.01$ versus Control (AS treatment).

induced in response with AS treatment at the protein level, with day 14 showing maximal up-regulation.

3.2. ISO suppressed MSC adipogenesis in a dose- and time-dependent manner

The dose- and time-course effect of ISO on MSC adipogenesis was detected by Oil red O staining. Fig. 2A and B indicated that ISO ranging from 0.001 to 0.1 μ M inhibited the formation of adipocytes in a dose-dependent manner, as the stained Oil red O was decreased

by 23%, 45% and 73%, respectively. Notably, no distinct difference was seen between 0.1 μ M and 1 μ M treatment. Moreover, 0.1 μ M ISO was supplemented to AS-induced MSCs for different incubation durations. Fig. 2C and D showed that exposing MSCs to ISO for 3 days induced much less adipocytic cells and substantial less adipocytes were observed in cells treated for 7 days. A 7-day challenge with 0.1 μ M ISO reduced Oil red O intensity by up to 71%. However, prolonged exposure to ISO did not lead to further suppression on adipocyte formation. These data indicated that ISO exerted mainly its effect in the early differentiation stage.

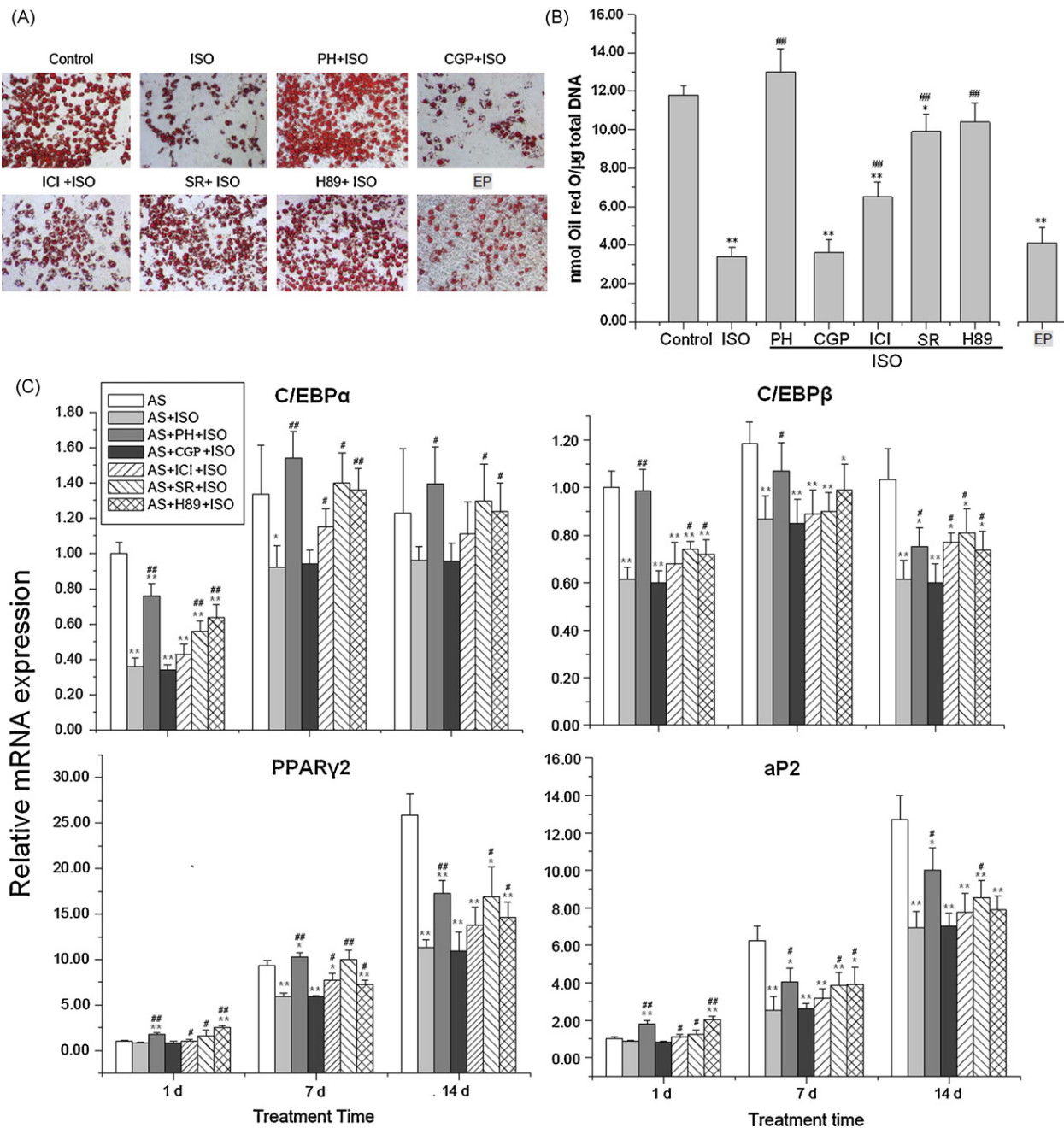


Fig. 3. Reversal of the inhibitory activity of ISO on MSC adipogenesis by different antagonists. (A) Adipocytes in different cultures were identified by Oil Red O staining after 14 days treatment. Treatment was performed as described in Section 2. Images were from representative fields of view for each culture condition. (B) The bound Oil red O was dissolved with isopropyl alcohol and absorbance at 510 nm was measured, which was quantified to nmol Oil red O/μg total DNA. Values were means $\pm$ SD of three separate experiments ($n=4$). * $p<0.05$, ** $p<0.01$ versus AS treatment; ### $p<0.01$ versus AS + ISO treatment. (C) The mRNA levels of C/EBPα, C/EBPβ, PPARγ2 and aP2 in MSCs treated with ISO alone or pre-treated with different antagonists at day 1, 7 and 14. The mRNA levels of each gene upon AS treatment at day 1 were set to 1.00, and the other values were expressed relative to this. Values were means $\pm$ SD of two separate experiments ($n=3$). * $p<0.05$, ** $p<0.01$ versus AS treatment; # $p<0.05$, ### $p<0.01$ versus AS + ISO treatment.

3.3. Reversal of the inhibitory activity of ISO by different antagonists

To identify the involved β -subtypes in MSC adipogenesis, the accumulated adipocytes in cultures exposing to ISO with or without pre-addition of various β -antagonists were compared. Fig. 3A and B showed that ISO inhibited the adipogenic extent by up to 71%, which could be restored by 114%, 37% and 77% with PH, ICI and SR, respectively, but not by CGP. Additionally, the PKA inhibitor H89 blocked most of the suppression of adipogenesis by ISO, indicating the PKA pathway may be involved. Moreover, ISO's effect can be mimicked by the natural β -AR ligand EP, in which the adipogenic extent was only 35% of the control.

To determine whether the β -AR ligands regulate MSC adipogenesis by affecting the expression of some key adipocyte marker genes, we examined the mRNA levels of C/EBP α , C/EBP β , PPAR γ 2 and aP2 in cells upon different treatments during adipogenic differentiation at day 1, 7 and 14, respectively (Fig. 3C). The mRNA expression of C/EBP α was reduced by 64%, 31% and 22% with ISO treatment at day 1, 7, and 14, respectively; whereas pre-treatment with PH or SR could overcome this inhibition to certain extent at all the indicated time points; pre-incubation with ICI only rescued ISO's effect at day 7; in contrast, CGP showed no reversal effect at any time. ISO suppressed the expression of C/EBP β by 27–41% at different time points; while pre-treatment with PH restored this suppression at all the times, with ICI only at day 14, and SR at day 1 and 14, but not with CGP at any times. PPAR γ 2 mRNA expression was significantly inhibited by ISO at day 7 and 14, but not at day 1. PH, ICI and SR reversed such inhibition at almost all the time points, except for ICI at day 14. The expression of aP2 was also down-regulated by ISO treatment at day 7 and 14, while PH or SR restored its expression at all the times, and ICI only exerted such effect at day 1. In addition, 1 μ M H89 exerted potent and highly reversal effect on ISO's inhibitory activity for all four genes at almost all the conditions, except for C/EBP β expression at day 7 and aP2 expression at day 14 (Fig. 3C).

3.4. cAMP production was modulated by β -AR agonists/antagonists in adipogenic MSCs

It has been demonstrated that β -ARs are G-protein coupled receptors that signal through the cAMP-dependent pathway (Benovic et al., 1988). To assess the biological relevance of β -adrenergic/cAMP signaling with MSC adipogenesis, we measured intracellular cAMP concentrations in cells treated with EP, and with ISO alone or in the presence of PH, CGP, ICI or SR. Results in Fig. 4A showed that EP and ISO increased cAMP levels by up to 1.8 and 2.2 folds of control, respectively. The stimulation of ISO could be counteracted by PH, ICI, or SR, but not by CGP. The reversal effect can be sequenced as PH > SR > ICI.

3.5. β -AR or PKA blockade rescued ISO inhibition of PPAR γ 2 protein expression

The protein expression of PPAR γ 2 was also determined. As shown in Fig. 4B, the expression of PPAR γ 2 was highly repressed by ISO addition, as compared to AS treatment. This inhibition was either totally or partially rescued by pre-culturing with PH, ICI or SR, with PH being more effective than ICI and SR. However, CGP addition showed no reversal effect on ISO's inhibitory activity. Moreover, pre-treatment with H89 can potently restore the expression of PPAR γ 2.

4. Discussion

Osteoporosis and the increased risk for fragility fractures are one of the characteristic manifestations of aging (Rodríguez et al.,

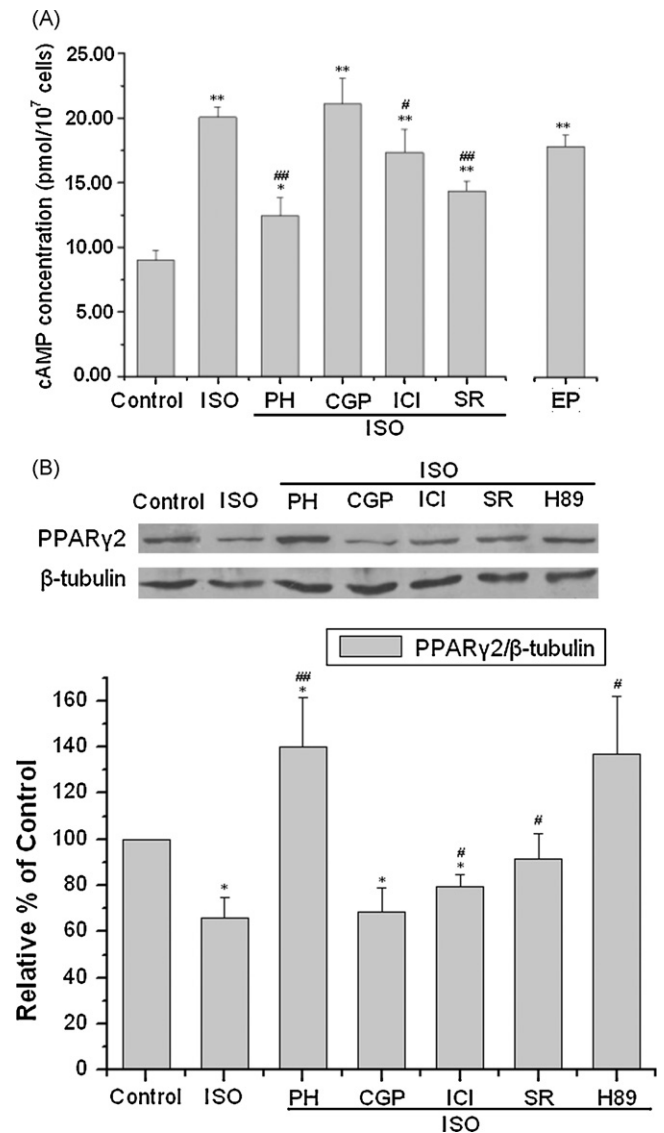


Fig. 4. (A) Intracellular cAMP concentrations in cells following agonists/antagonists treatment. * $p < 0.05$, ** $p < 0.01$ versus Control (PBS treatment); # $p < 0.05$, ### $p < 0.01$ versus ISO treatment. Values were means $\pm$ SD of two separate experiments ($n = 3$). (B) The protein expression of PPAR γ 2 upon agonists/antagonists treatment. β -Tubulin was used as internal control. The expression level of PPAR γ 2 upon AS treatment (Control) was set to 100%, and the other values were expressed relative to this. * $p < 0.05$, ** $p < 0.01$ versus ISO treatment. Similar result was obtained in other two experiments.

1999). Not only the decrease of osteoblasts, but also the increase of adipocytes in bone marrow may contribute to this bone-associated disease (Meunier et al., 1971; Koo et al., 1998). Besides stimulating bone formation and suppressing bone resorption, inhibition of adipogenesis may be of considerable significance for the prevention and treatment of osteoporosis (Nuttall and Gimble, 2000; Pei and Tontonoz, 2004). Obesity is also one of the most serious threats to modern people (Hotamisligil et al., 1993; Seals and Bell, 2004). Despite the phenotypic differences between MSCs from bone marrow and adipose tissue, those cell populations have been shown to have similar differentiation potentials (De Ugarte et al., 2003; Noël et al., 2008). Thus, the observations reported here may also be interesting for the understanding and prevention of obesity.

The expression of β -AR subtypes in undifferentiated MSCs has been studied (Takahada et al., 2009), which showed that mRNA expression was observed for β 2- and β 3-AR subtypes, but not for the β 1-AR subtype in mouse MSCs. However, the development of

β -AR systems during the process of MSC adipogenic differentiation is still unclear. In this study, the three β -AR transcripts were found to exist in primary MSCs. In MSC-derived adipocytes, the β 1-subtype represents the smallest population of β -ARs which was barely undetectable by western blot analysis, and showed minor alteration at the mRNA level. Although the β 2-AR expressed abundantly before induction, its modulation was much lower than the β 3-subtype during the adipogenic process. By contrast, while the expression of β 3-AR was slight before stimulation, it was up-regulated extensively upon adipogenic induction, especially at the late stage (Fig. 1). It is noted that this is the first report on the expression profile of β -ARs in MSCs following adipogenic differentiation. In fact, the expression alterations of particular β -AR subtypes during the adipose conversion in 3T3-L1 and 3T3-F442A cell lines have previously been elucidated (Guest et al., 1990; Fève et al., 1991; Hadri et al., 1996), whereas different cell lines own their respective characteristics. 3T3-L1 fibroblasts expressed equivalent levels of β 1- and β 2-ARs, while the β 2-subtype was predominantly expressed (β 1/ β 2 ratio, 5:95) after differentiating into adipocytes (Guest et al., 1990). In 3T3-F442A cells, the presence of transcripts homologous to the human β 3-AR gene was revealed in adipocytes but not in preadipocytes (Fève et al., 1991). Hadri et al. (1996) demonstrated that the β 1-AR mainly expressed and took action in early 3T3-F442A cells, while the β 3-subtype became detectable later and represented ~95% of total β -ARs in late differentiation stages.

To test the involvement of β -ARs in MSC adipogenesis, MSCs were treated with β -agonists or antagonists. ISO suppressed the development of adipocytes from MSCs with a trend of dose- and time-dependence (Fig. 2). The natural β -AR ligand, EP, also inhibited MSC adipogenesis (Fig. 3A and B). Moreover, a 7-day challenge with various β -antagonists including PH, ICI and SR showed more lipid positive cells over the control (data is shown in Supplementary Fig. 1). These data indicated that β -AR agonists negatively while antagonists positively affected MSC adipogenesis. Previous reports also showed that β -AR agonists were able to impair adipogenesis in adipose cells (Klaus et al., 2001) or tissues (Margareto et al., 2001). Additionally, since the β -AR agonists are lipolytic, the mRNA expression of the key lipolytic enzyme adipose triglyceride lipase (ATL) (Kershaw et al., 2006) in cells treated with ISO was determined (shown in Supplementary Fig. 2). As seen, the ATL mRNA level was markedly induced upon ISO treatment at day 1. However, it was significantly down-regulated both at day 7 and 14. This result suggested that ISO indeed induced lipolytic reaction in adipogenic MSCs at the very early stage (day 1), whereas this effect was counteracted possible due to the inhibition of adipogenesis by ISO as less adipocytes were formed at late stages (day 7 and 14).

To further identify the involved β -AR subtypes, the cells were treated with ISO alone or pre-incubated with different β -AR antagonists. Results indicated that the inhibitive effect of ISO could be totally or partially recovered by PH, ICI or SR, but not by CGP (Fig. 3, Fig. 4). The reversal potency can be sequenced as PH>SR>ICI, demonstrating that both β 2-AR and β 3-AR are involved in MSC adipogenesis, with β 3-AR as the dominant subtype. The alteration of adipocyte marker genes expression by β -AR ligands was consistent with previous observations that the expression of resistin and adiponectin was inhibited by the β -AR agonist (ISO) and was reversed by the β -AR antagonist (PH) in 3T3-L1 adipocytes (Fasshauer et al., 2001a,b). Antras et al. (1991) indicated that the rate of transcription of adipsin and lipoprotein lipase (LPL) was strongly reduced in ISO-treated 3T3-F442A adipocytes. Another study (Rehnmarmark et al., 1993) documented that C/EBP α and C/EBP β gene expression was under adrenergic control both *in vivo* and *in vitro* but the type of response was directed by the degree of differentiation of the cells. Also PPAR γ 2 mRNA level was down-regulated

by noradrenaline treatment in both proliferative and differentiating brown adipocytes (Lindgren et al., 2004).

Classically, β -adrenergic receptors are considered to be coupled to G-proteins that stimulate adenylate cyclase, leading to an increase in the level of cAMP (Benovic et al., 1988). In our study, cAMP levels in the adipogenic MSCs could be modulated by different β -AR agonists/antagonists (Fig. 4A). PKA is a downstream mediator of the cAMP pathway (Haskó and Szabó, 1998). The inhibitory effect of ISO could be abolished by the potent PKA inhibitor H89, as evidenced by the Oil red O staining, the expression of the marker genes, and the protein expression of PPAR γ 2 (Figs. 3 and 4B). These data implicated that β -ARs exert effects on MSC adipogenesis, at least in part, via the cAMP/PKA pathway. The effect of cAMP/PKA pathway on adipogenesis has been studied in different cell types, although the results are contentious (Antras et al., 1991; Rickard et al., 2006; Kim et al., 2010). Antras et al. (1991) showed that forskolin or 8-bromo-cAMP treatment markedly reduced the steady-state levels of adipsin mRNA in 3T3-F442A adipocytes. In human MSCs, intermittent treatment with forskolin inhibited adipocyte formation and, conversely, intermittent treatment with a PKA inhibitor produced extensive adipogenesis (Rickard et al., 2006). In contrast, CREB (cAMP-response element binding protein) has been shown to potentiate the transcription of C/EBP α , C/EBP β , PPAR γ 2, and adiponectin in 3T3-L1 cells (Reusch et al., 2000; Klemm et al., 2001; Kim et al., 2010). These differences are possibly due to the stage and duration in which stimulators/inhibitors are presented, and the stimulators/inhibitors concentration and the nature of the cell culture system may also have contributed to the discrepancies.

Based on previous studies and the observations reported here, we suggest a pathway for the intracellular signaling from adrenergic stimulation to down-regulation of adipocyte marker genes and suppression of MSC adipogenesis, as depicted in Fig. 5. cAMP level was increased after ISO treatment in adipogenic MSCs. It is likely that the ISO-induced decrease in C/EBP α , C/EBP β and PPAR γ 2 mRNA levels is mediated by PKA, since the effect was totally abolished by the PKA inhibitor H89. The down-regulated transcriptional factors may negatively affect the expression of the later marker aP2, and further suppress the maturation of adipocytes. Moreover,

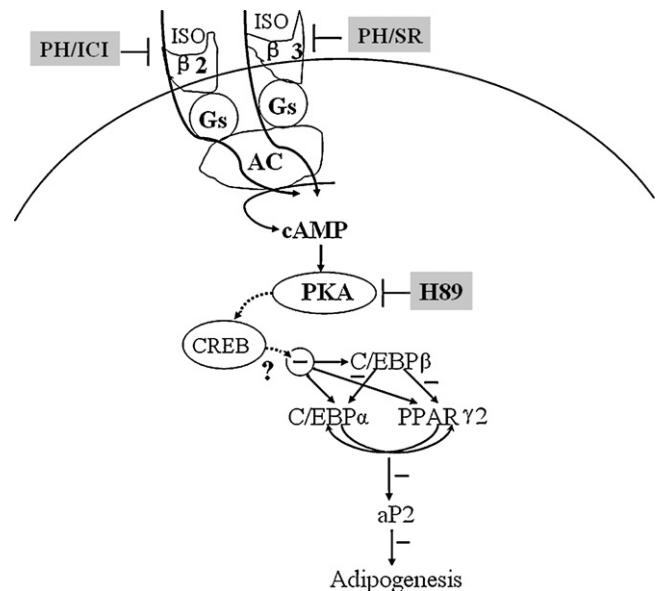


Fig. 5. Tentative pathway for the effect of β -adrenergic regulation on MSC adipogenesis. The detailed description was shown in Section 4. The signaling pathways suggested based on previous studies or the investigations reported here are shown in bold black arrows; mechanisms not demonstrated here or likely pathways are dotted.

the inhibitory effect of ISO can be blocked by PH, ICI or SR, and be mimicked by EP.

In conclusion, these data demonstrated for the first time that (i) both the mRNA and protein levels of β 2-AR and β 3-AR are up-regulated following adipogenesis of mouse MSCs; (ii) β -AR agonists negatively while antagonists positively affect MSC adipogenesis; (iii) both the β 2- and β 3-AR are involved in adipogenic differentiation of MSCs, with β 3-AR as the dominant one; (iv) the effect of β -ARs on MSC adipogenesis was at least partly mediated via the cAMP/PKA signaling. Assuming that these effects also occur *in vivo*, the inhibition of adipogenic differentiation of MSCs would be expected to contribute to prevent osteoporosis and obesity. However, the exact mechanisms underlying the regulation of β -ARs/cAMP/PKA signaling on transcriptional factors expression and the possible differences *in vivo* remain to be elucidated in future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2010.03.021.

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