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ARTICLES

β_2-Adrenergic Receptors Expressed on Murine Chondrocytes Stimulate Cellular Growth and Inhibit the Expression of Indian Hedgehog and Collagen Type X

Lick Pui Lai and Jane Mitchell*

Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada

Abstract The sympathetic nervous system has been demonstrated to have a role in regulating bone remodeling through β-adrenergic receptors (β-AR) expressed on osteoblasts. Studies using β_2-adrenergic receptor agonists in vivo have also suggested an effect on endochondral bone development; however, it was not clear if this effect was mediated through osteoblasts or chondrocytes. To more thoroughly examine the role of β-AR in chondrocytes we characterized the expression and signal transduction systems activated by β-AR in growth plate chondrocytes prepared from ribs of embryonic E18.5 mice. Using RT-PCR and immunohistochemistry we found that the chondrocytes expressed only β_2-AR. The receptors were coupled to stimulation of adenylyl cyclase, phosphorylation of the cyclic AMP response element binding protein (CREB) and extracellular signal-regulated kinase (ERK1/2). Stimulation of ERK1/2 was transient and limited by the concomitant stimulation of the mitogen-activated protein kinase phosphatase (MKP-1). Isoproterenol stimulated the growth of chondrocytes as assessed by increased incorporation of [^{3}H]-thymidine into the cells. The cellular expression of two markers of chondrocyte differentiation, Indian hedgehog, expressed in pre-hypertrophic cells and collagen type X, expressed in hypertrophic chondrocytes, were both significantly inhibited after incubation with isoproterenol. Collectively, these findings demonstrate regulation of chondrocytes through β_2-AR expressed on the cells that stimulate their growth and inhibit their differentiation, indicating that the sympathetic nervous system may be an important regulator of embryonic cartilage development. J. Cell. Biochem. 104: 545–553, 2008. © 2007 Wiley-Liss, Inc.

Key words: chondrocytes; growth plate; β_2-adrenergic receptors; Indian hedgehog; collagen type X; cell growth

The sympathetic nervous system has been shown to be an important regulator of bone remodeling mediating the antiosteogenic effect of leptin. The link between leptin and the sympathetic system was identified using several mouse models. Leptin-deficient mice have a high bone mass phenotype [Ducy et al., 2000], as do mice deficient of β_2-adrenergic receptors (β_2-AR) or normal mice treated with β-blockers. Leptin infusion rescued the high bone mass phenotype in leptin-deficient mice but not in β_2-AR-deficient mice [Ducy et al., 2000; Takeda et al., 2002]. Subsequent studies showed that β_2-AR expressed on osteoblasts directly inhibit osteoblast proliferation and indirectly increase osteoclast differentiation. These two effects are mediated by two different signaling pathways. β_2-AR activation in osteoblasts causes PKA phosphorylation of CREB, which activates both AP-1 factors and the clock gene. Together these transcription factors coordinate the rate of osteoblast proliferation [Fu et al., 2005]. At the same time the PKA activation of ATF4, another member of the CREB family of transcription factors, upregulates RANKL transcription and secretion, that consequently induces osteoclast activity [Elefteriou et al., 2005]. The final outcome of β_2-AR activation in osteoblasts is a significant loss of bone mineral density.

The effect of leptin and the sympathetic nervous system on bone development is still not clear. Mice deficient in leptin show reduced long bone growth that can be recovered with leptin administration [Steppan et al., 2000].

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*Correspondence to: Dr. Jane Mitchell, Department of Pharmacology, 1 King's College Circle, Room 4342, Toronto, Ontario, Canada M5S 1A8.
E-mail: jane.mitchell@utoronto.ca

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Leptin receptors have been reported on rabbit chondrocytes [Nakajima et al., 2003] and leptin can stimulate the growth and differentiation of chondrocytes in mouse condyles in culture [Maor et al., 2002]. In addition to the direct effects of leptin on chondrocytes, these cells may also be subject to indirect regulation from leptin through the sympathetic nervous system as seen for osteoblasts. A study using 8-week-old male rats treated for 4 weeks with a selective β_2-AR agonist, clenbuterol, found that β_2-AR activation shortened bone length probably due to decreased longitudinal bone growth in addition to its effect of decreasing bone mineral density and bone mineral content of the femur and tibia [Kitaura et al., 2002]. The effects of β_2-AR activation on bone development could be mediated by an effect on chondrocytes since the rate of chondrocyte proliferation and hypertrophic differentiation determine the growth rate and length of bones, and they are regulated by multiple hormones as well as local factors [reviewed in Olsen et al., 2000; Kronenberg, 2003].

The aim of this study was to investigate if growth plate chondrocytes (GPC) express β-AR and what role they may play in regulation of endochondral bone development. We demonstrate here that mouse chondrocytes express β_2-AR, and demonstrate that these receptors are functionally coupled to two different signaling cascades; adenylyl cyclase and the ERK1/2 mitogen-activated protein kinases. Stimulation of the β_2-AR on chondrocytes resulted in stimulation of growth and inhibition of chondrocyte differentiation marker genes collagen type X (Col X) and Indian hedgehog (Ihh).

MATERIALS AND MATERIALS

Reagents

Isoproterenol (ISO), propranolol (PRO), metoprolol, salbutamol (Sal), and BRL-37344 were all purchased from Sigma (Oakville, ON, Canada). Rat PTH (1–34) was purchased from Bachem (King of Prussia, PA). Cell culture media and reagents were purchased from Gibco BRL/Invitrogen (Burlington, ON, Canada). Human β-AR expression plasmids were obtained from UMR (University of Missouri, Rolla, MO). Polyclonal antibodies specific for pERK1/2, and ERK1/2 were from Cell signaling (Dancers, MA), pCREB and CREB from Sigma, and MKP1 from Santa Cruz Biotechnology (Santa Cruz, CA).

Primary Cell Preparation and Cell Culture

Timed pregnant CD1 mice at E18.5 were purchased from Charles River Laboratories (St Constant, QC, Canada). Animals were treated in accordance with guidelines of the University of Toronto animal care committee. Primary chondrocytes isolated from the fetal mouse ribs according to Lefebvre et al. [1994], were plated at 150,000 cells/cm^2 in monolayer culture, and maintained in DMEM (high glucose) containing 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 mg/ml amphotericin B, and 10% fetal bovine serum. Cells were treated on day 2, and cell lysates and RNA were prepared on day 2 or day 3.

Alcian Blue and Alkaline Phosphatase Staining

Primary chondrocytes grown in monolayer culture were washed with PBS, and fixed with 3% formaldehyde for 15 min. The cells were then washed with PBS and stained with 3% (w/v) alcian blue in 3% acetic acid for 30 min followed by rinsing with 3% acetic acid and finally washing in running tap water for 5 min. For alkaline phosphatase staining, cells were first washed with PBS twice and fixed with 10% formalin for 2 h. After being washed twice with water (pH 8) and incubated with water (pH 8) for 15 min, cells were stained with a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.5% *N*,*N*-dimethylformamide, 2 mM $MgCl_2$ and 0.6 mg/ml Red Violet LB salt (Sigma) in 0.1 M Tris/HCl (pH 8.3) in the dark at room temperature for 45 min, followed by observation using phase-contrast microscopy [Stanton et al., 2003].

UMR-106-01 Cell Culture and Transient Transfection

UMR-106-01 osteosarcoma cells that do not express endogenous β-AR were used to express cDNAs encoding each of the β-AR subtypes for controls in PCR reactions. Cells were grown in 50% DMEM:50% F-12 medium containing 1 U/ml penicillin, 1 μg/ml streptomycin, and 0.25 μg/ml amphotericin B and supplemented with 5% fetal bovine serum. Cells were grown to 60–70% confluence in 6-well plates over 24 h and transfected with β_1-AR, β_2-AR, β_3-AR expression vectors or vector alone

(pcDNA 3.1^+) using Lipofectamine reagent (Gibco Invitrogen). One microgram of DNA and 4 μl of transfection reagent were used for each well in serum-free media. Total RNA was extracted 24 h after transfection.

RNA Isolation and RT-PCR

Total RNA was extracted using Trizol reagent (Gibco Invitrogen), as specified by the manufacturer, and subsequently digested with DNase 1 (GE healthcare, Baie d'Urfe', QC, Canada) to remove any contaminating genomic DNA. For each reverse transcription reaction (RT), 1 μg of RNA was reverse-transcribed by MMLV-reverse transcriptase in a total volume of 20 μl. One microliter samples of the RT reactions were amplified by PCR using Titanium *Taq*™ DNA polymerase (Clontech, Mountain View, CA), using specific primers for β_1-, β_2, or β_3-AR. The sequences are: β_1-AR forward: CCG CTG CTA CAA CGA CCC CAA G; β_1-AR reverse: AGC CAG TTG AAG ACG AAG AGG CG; β_2-AR forward: GGT TAT CGT CCT GGC CAT CGT GTT TG; β_2-AR reverse: TGG TTC GTG AAG TCA CAG CAA GTC TC; β_3-AR forward: ATG GCT CCG TGG CCT CAC; β_3-AR reverse: CTG GCT CAT GAT GGG CGG. 18S rRNA was amplified by Quantum RNA™ 18S internal standards (Applied Biosystems, Foster City, CA) in each PCR, and was used as an internal control. PCR products were separated by electrophoresis on agarose gels, and visualized by Storm imaging system (Molecular Dynamics, Sunnyvale, CA) after Vistra Green staining (GE Healthcare).

Real-Time PCR

Real-time PCR amplification was performed using the 7500 Real-time PCR System (Applied Biosystems). For each reaction, 1 μl of the total RT product was amplified using the Power SYBR green PCR master mix in a total of 25 μl reaction volume according to the manufacturer's standard cycling conditions (50°C for 2min, 95°C for 10 min, 40× (95°C for 15 s, 60°C for 1 min). Primers specific for Col X, Ihh and GAPDH were designed using the Primer express software (v.3) (Applied Biosystems). The sequences are: Col X forward: TGC TCC TGG GCA GAG GAA; reverse: AAA TCA CTG GTG CTG GGA GAT G; Ihh forward: GGC TTC GAC TGG GTG TAT TAC G; reverse: GGC CGA ATG CTC AGA CTT GA; GAPDH forward: CAT GGC CTT CCG TGT TCC TA; reverse: GCG GCA CGT CAG ATC CA. GAPDH was used as an internal control for the quality and quantity of each cDNA. Analysis of the real-time PCR results was done by the $2^{\Delta\Delta C_t}$ method for the relative quantification of Col X and Ihh, where CT is the threshold cycle at which a significant increase in fluorescent signal is first detected.

Cell Lysate Preparation and Western Blotting

Primary chondrocytes were washed with ice-cold PBS and then lysed in buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 20 mM NaF, 3 mM Na_3VO_4, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1% Nonidet P-40, on ice for 30 min. Lysates were cleared by centrifugation at 12,000*g* for 15 min at 4°C. Protein concentrations of the cleared lysates were determined by the Amido Black protein assay using bovine serum albumin as a standard [Schaffner and Weissmann, 1973]. Cell lysates were separated by electrophoresis on 11% SDS–polyacrylamide gels, transferred to nitrocellulose membranes and blocked with Tris-based buffer containing 0.1% Tween (TBST) with 5% milk for 1 h. For determination of protein expression, nitrocellulose membranes were then incubated with specific polyclonal antibodies (pERK1/2, ERK1/2, pCREB, CREB: 1:1,000; MKP1: 1:500) diluted in TBST with 1% BSA overnight at 4°C, followed by incubation with secondary antibody (Cell Signaling) diluted (1:2,000) in TBST with 5% milk for 1 h. The nitrocellulose membranes were finally incubated with ECL enhanced chemiluminesence solution (GE Healthcare), and exposed to Kodak X-OMAT films. Protein band intensities were quantitated by scanning densitometry using ImageQuant™ software (Molecular Dynamics).

Adenylyl Cyclase Assay

Adenylyl cyclase activity was measured in lysed cells as previously described [Jacobowitz et al., 1994]. Briefly, 3 days after plating, chondrocytes were lysed in hypotonic solution containing 10 mM HEPES pH 8.0, 2 mM EDTA, and 100 μM AEBSF for 15 min at 37°C. Assay buffer (100 μM ATP (containing approximately 10^6cpm [α-^{32}P] ATP), 12 mM $MgCl_2$, 1 mM cAMP, 10 μg/ml creatine kinase, 20 mM creatine phosphate, 2 mM EGTA, 500 μM isobutylmethylxanthine) was added to the cell lysate along with the indicated agonists or antagonists

for 30 min. [^{32}P]-cAMP formed in each sample was isolated according to Salomon et al. [1974], and measured in a scintillation counter.

[^{3}H]-Thymidine Incorporation

Chondrocytes plated in 24-well plates were incubated for 24 h in the presence or absence of 1 μM ISO, during the final 4 h of incubation 1 μCi of [^{3}H]-thymidine was added to each well. At the end of the incubation period medium was removed and the cells were rinsed twice in PBS followed by precipitation in 10% trichloroacetic acid for 10 min. The precipitated cells were rinsed twice more in PBS and finally solubilized in 0.5 ml of 2N NaOH. 0.3 ml of the solubilized cells were used to assess the amount of [^{3}H]-thymidine incorporated into cellular DNA and the remaining 0.2 ml were used to assess the total amount of cell protein in each well using the amido black assay as outlined above.

Immunohistochemistry

Primary chondrocytes were cultured on Nunc Lab-Tek chambered slides (Fisher Scientific, Napean, ON, Canada) for 2 days followed by fixation in methanol:acetone (50:50) for 2 min followed by three rinses in PBS. Fixed cells were stained with a β_2-AR-specific rabbit polyclonal antibody (Santa Cruz Biotechnology) using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Labeled cells were visualized by DAB staining and cell nuclei were counter stained with hematoxylin QS (Vector Laboratories). Cells were imaged using an Olympus 1X70 light microscope.

Presentation of Data

All data shown are representative of at least three experiments from different groups of animals. Quantitative data were plotted as mean ± SE of the values obtained from three individual experiments, and analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test.

RESULTS

β_2-AR Expression on Primary Growth Plate Chondrocytes

GPC isolated from fetal mouse (E18.5) ribs and plated in monolayer culture maintained a chondrocytic phenotype as shown by the positive staining of Alcian blue (Fig. 1A). Some

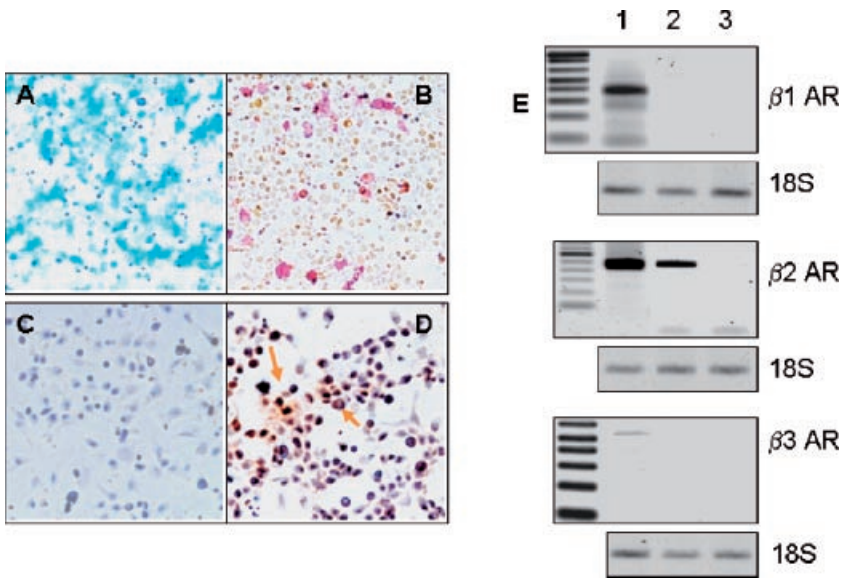

Fig. 1. β_2-AR expression on primary growth plate chondrocytes. Primary chondrocytes in monolayer culture were fixed and then stained for various proteins as outlined in Materials and Methods Section. Proteoglycans secreted by the cells are stained blue using Alcian blue (**A**). Alkaline phosphatase produced by the cells is shown as the red stain (**B**). Immunohistochemistry was performed using either non-immune rabbit serum (**C**) or a polyclonal antibody selective for β_2-AR (**D**) followed by a biotinylated goat anti-rabbit secondary antibody using an ABC kit, followed by counterstaining with hematoxylin. Immunoreactive proteins are detected by the brown DAB staining (orange arrows, magnification 200×). RT-PCR was performed with specific primers for β_1-AR, β_2-AR or β_3-AR using RNA from positive controls: UMR-106-01 cells transfected with β_1-, β_2-, or β_3-AR expression vector (**lane 1**), growth plate chondrocytes (**lane 2**), or negative control UMR-106-01 cells transfected with pcDNA3 (**lane 3**). 18S rRNA was amplified and served as the loading control.

of the cells were also stained positively with alkaline phosphatase, which is expressed by terminally differentiated chondrocytes (Fig. 1B).

To identify the β-adrenergic receptor subtypes expressed by the chondrocytes, RT-PCR using primers specific for each receptor subtype was performed. As shown in Figure 1E, only the β_2-AR transcript was detected. To determine if receptor protein was produced from the β_2-AR transcript expressed in these chondrocytes immunohistochemistry using an antibody specific for β_2-AR was performed. Positive staining for the β_2-AR was detected over approximately 20% of the cells in culture (Fig. 1D, orange arrows).

β_2-AR Activation of Adenylyl Cyclase in Growth Plate Chondrocytes

β-AR are coupled via $G_s\alpha$ to activation of adenylyl cyclase and subsequently to the PKA pathway. To determine the functionality of β_2-AR expressed on GPC, adenylyl cyclase activity was assessed (Fig. 2A). ISO, a non-selective β-AR agonist, increased the adenylyl cyclase activity by 100% compared to the unstimulated cells. Activation of adenylyl cyclase by ISO was blocked by PRO, a non-specific β-AR antagonist. Further pharmacological characterization of the β-adrenergic receptor subtype expressed demonstrated that Sal, a selective β_2-AR agonist, stimulated the cyclase activity to a level similar to that of ISO, while BRL-37344 (BRL), a selective β_3-AR agonist, had no effect on adenylyl cyclase activity. Metroprolol (Met), a selective β_1-AR antagonist, was not able to block the ISO stimulation of AC activity. Forskolin (FSK), a cell permeable adenylyl cyclase activator, was used as a positive control in the assay. These data confirm that β_2-AR is the only functional β-AR subtype expressed on GPC.

The cyclic AMP response element (CRE)-binding protein (CREB) is one of the transcription factors that are activated by PKA down-stream of adenylyl cyclase activation. Activated PKA activates CREB in the cell nucleus by phosphorylating serine residue 133 (ser133). Phosphorylated CREB subsequently induces gene transcription by recruiting other transcriptional co-activators

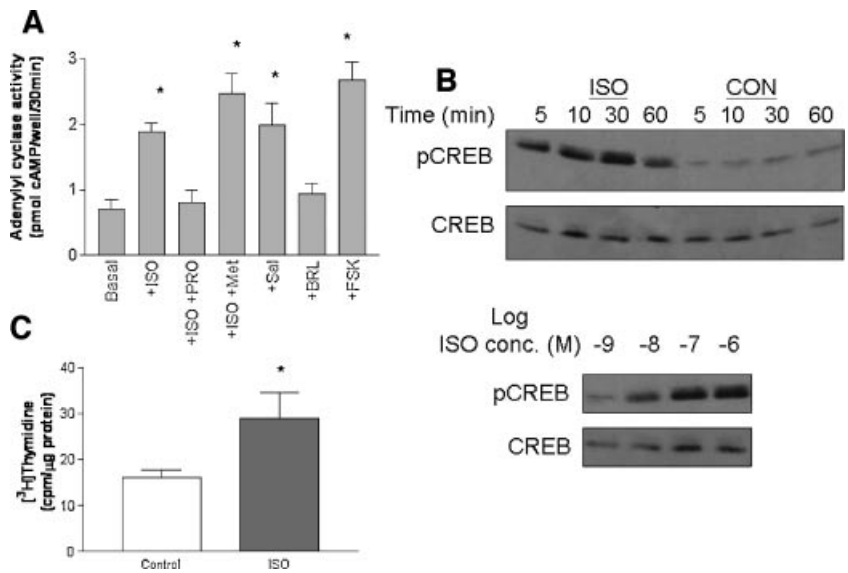

Fig. 2. β_2-AR activation of adenylyl cyclase, CREB phosphorylation and [^{3}H]-thymidine incorporation. **A**: Primary chondrocytes were treated with various agonists: isoproterenol (ISO), Salbutamol (Sal), BRL-37344 (BRL) or antagonists: PRO, Metroprolol (Met) all at 1 μM concentrations for 30 min and the adenylyl cyclase activity was assayed as outlined in Materials and Methods Section. Ten micromolar forskolin (+FSK) was used as a positive control. Values represent mean ± SE, and * represent significant different from the basal level ($P < 0.05$). **B**: Primary chondrocytes were treated with 1 μM ISO for the indicated time periods, or for 30 min with the indicated ISO concentrations. Total cell lysates (30 μg for p-CREB, and 15 μg for total CREB) were analyzed by SDS–PAGE and western blotting using specific antibodies against phosphor-CREB (p-CREB) or total CREB protein (CREB). A representative blot from three independent experiments is shown. **C**: Primary chondrocytes were treated with 1 μM ISO for 24 h with addition of [^{3}H]-thymidine for the final 4 h of stimulation. The amount of [^{3}H]-thymidine incorporated into cellular DNA was assessed as outlined in Materials and Methods Section. Values represent mean ± SE, and * represent significant different from the basal level ($P < 0.05$).

to CRE [reviewed in Johannessen et al., 2004]. In order to examine the effect of β_2-AR on CREB regulation in the chondrocytes, phosphorylation of CREB was determined by Western blotting using an antibody specific for ser133 phosphorylated CREB. Phosphorylation of CREB stimulated by ISO was both concentration (1 nM–1 μM) and time-dependent. It was first observed 5 min after stimulation, and sustained for at least 1 h (Fig. 2B). ISO phosphorylation of CREB was completely abolished by PRO, and ISO had no effect on the expression level of CREB.

Stimulation of adenylyl cyclase in GPC has been shown to stimulate growth of these cells, therefore we tested the effect of stimulation with ISO for 24 h on the incorporation of [^{3}H] thymidine into the chondrocytes in culture. As shown in Figure 2C treatment with ISO significantly stimulated thymidine incorporation into the cells.

β_2-AR Regulation of Mitogen-Activated Protein Kinase (MAP Kinase) ERK1/2 and MAP Kinase Phosphatase 1(MKP1) in Growth Plate Chondrocytes

β_2-AR have been demonstrated to couple to activation of the mitogen-activated protein kinases ERK1/2 by several mechanisms [Maudsley et al., 2000; Shenoy et al., 2006]. We tested the effect of β_2-AR on the ERK1/2 phosphorylation in chondrocytes using an antibody specific for phosphorylated ERK1/2 in Western blot analysis. ISO increased ERK1/2 phosphorylation transiently with return to basal by 30 min, while ISO had no effect on the total ERK1/2 expression level. PRO was able to completely abolish the effect of ISO on ERK1/2 activation (Fig. 3). MAP kinase phosphatase 1 (MKP1) belongs to the dual-specificity phosphatase family, which dephosphorylates MAP kinases and subsequently inactivates them [Dickinson and Keyse, 2006]. Since ERK1/2 phosphorylation stimulated by ISO in chondrocytes was only transient, we next determined if stimulation of β_2-AR could increase MKP1 expression. ISO stimulation of the cells resulted in an increase in MKP1 protein expression within 30 min and this effect was also blocked completely by PRO (Fig. 3). These data show that ISO can activate the ERK1/2 signaling pathway, but the intensity or duration of this effect may be limited by stimulation of MKP1.

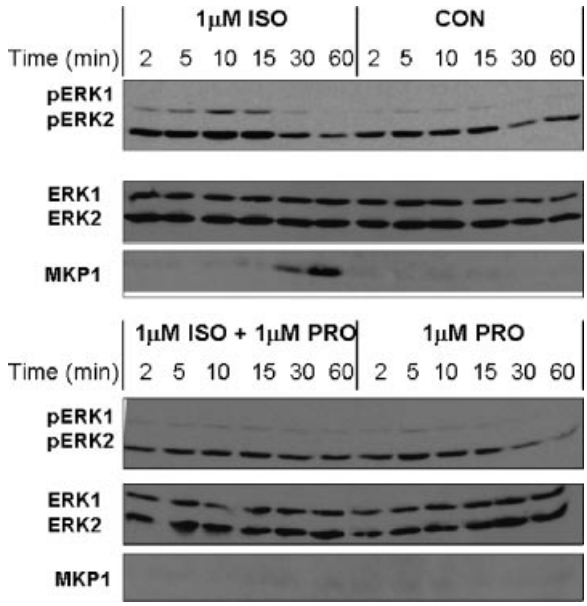

Fig. 3. β_2-AR activation of ERK1/2 and MKP1. Primary GPC were treated with 1 μM ISO, 1 μM PRO, or both ISO and PRO for the indicated time periods. Total cell lysates (30 μg for p-ERK1/2 and MKP1, 10 μg for total ERK1/2) were analyzed by SDS–PAGE and Western blotting using specific antibodies against phosphor-ERK1/2 (p-ERK1/2), total ERK1/2 or MKP1 protein. A representative blot from three independent experiments was shown.

Isoproterenol-Stimulation Inhibits Col X and Ihh mRNA

Both Col X and Ihh are important marker genes during endochondral bone development. Ihh is expressed primarily in pre-hypertrophic chondrocytes, and regulates endochondral bone development by orchestrating both chondrocyte proliferation and differentiation [Lai and Mitchell, 2005]. Col X is exclusively expressed in hypertrophic chondrocytes. Although the function of Col X in endochondral ossification still remains unknown, it is believed that its regulation and hypertrophic chondrocyte differentiation are related. We determined the effect of ISO on these two genes using real-time RT-PCR. ISO decreased both Ihh (Fig. 4A) and Col X (Fig. 4B) mRNA levels in 24 h, and these effects were completely blocked by PRO. Parathyroid hormone and FSK, a cell permeable adenylyl cyclase activator, also had a similar effect on the two mRNA levels.

Incubation of the cells with ISO for 24 h did not alter the excretion of proteoglycans as evidenced by staining with Alcian blue (Fig. 5A) or staining for alkaline phosphatase (Fig. 5B), suggesting that the cells

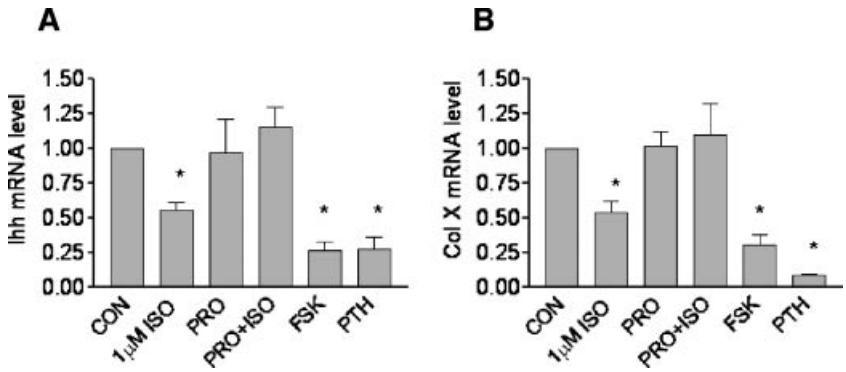

Fig. 4. β_2-AR down-regulation of Col X and Ihh mRNA. Primary GPC were treated with 1 μM ISO, 1 μM PRO, both ISO, and PRO or 10 μM FSK for 24 h. Total RNA was isolated and subjected to reverse transcription. cDNA was used in real-time PCR to determine the levels of (**A**) Indian hedgehog (Ihh) mRNA and (**B**) type X collagen (Col X). The mRNA levels are presented as the ratio of the mRNA in treated samples to the untreated sample (CON). Values represent mean ± SE, and * represents significant different from the untreated sample ($P < 0.05$).

maintained their chondrocytic phenotype with this treatment.

DISCUSSION

The primary cells used in this study maintained a chondrocytic phenotype in culture, secreting a proteoglycan-rich matrix that stained positively with Alcian blue. Further evidence of their chondrocytic phenotype came from demonstration that the cells express transcripts encoding Ihh and Col X which are marker genes for pre-hypertrophic and hypertrophic chondrocytes, respectively. Since at least some of the cells are undergoing cellular replication as evidenced by thymidine incorporation, the cells in culture appear to be a mixture of proliferating chondrocytes with post-mitotic pre-hypertrophic and hypertrophic chondrocytes.

We set out to determine if chondrocytes express β-AR. Using multiple methods including RT-PCR, immunohistochemistry, and functional assays with pharmacological agents, we

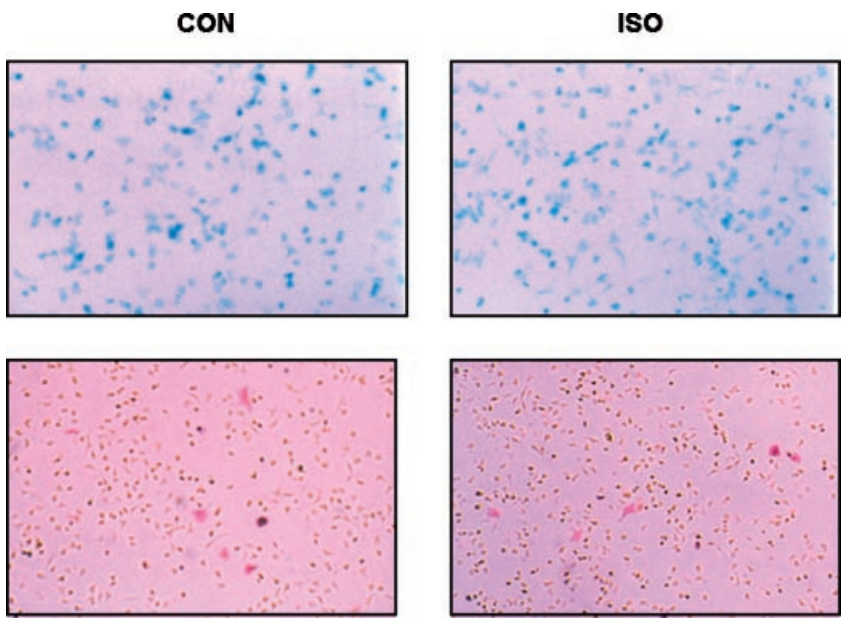

Fig. 5. Effect of isoproterenol on proteoglycan and alkaline phosphatase produced by primary chondrocytes in monolayer culture. Primary chondrocytes were treated with (ISO) or without (CON)1 μM ISO and then fixed and stained as outlined in Materials and Methods Section. Proteoglycans secreted by the cells are stained blue using Alcian blue (**top panels**). Alkaline phosphatase produced by the cells is shown as the red stain (**lower panels**).

demonstrated that the cells express only the β_2-subtype of the receptor, which is similar to the expression pattern reported in osteoblasts. β-AR are coupled to adenylyl cyclase activation in all tissues in which they are expressed including cardiovascular, lung and adipose tissues as well as in osteoblasts. In chondrocytes the β_2-AR are similarly coupled to activation of adenylyl cyclase, and this was further demonstrated by stimulation of CREB phosphorylation downstream of PKA activation. Numerous studies have demonstrated the functional role of the PKA/CREB pathway in mediating regulation of endochondral bone development by other G protein-coupled receptors. In chick chondrocytes, parathyroid hormone-related peptide (PTHrP), an important regulator of endochondral bone development, inhibited multiple differentiation related genes including Runx2, Col X and alkaline phosphatase by activating CREB [Ionescu et al., 2001; Li et al., 2004b]. PTHrP activation of CREB could also block BMP2 stimulation of Ihh and Col X [Ionescu et al., 2004]. Similarly, the PKA/CREB pathway partially mediates PGE_2 inhibition of Col X, matrix metalloproteinase 13 and alkaline phosphatase in chick GPC [Li et al., 2004a]. These studies all demonstrate the important role of CREB in regulating gene expression in chondrocytes, and our studies suggest that CREB stimulation by the β_2-AR expressed in chondrocytes may play a similar role to that of PTHrP and PGE_2 in inhibiting Col X expression.

In addition to the activation of adenylyl cyclase, ISO also stimulated ERK1/2 phosphorylation in our chondrocyte cultures. In chondrocytes, ERK1/2 has been shown to be an important regulator of differentiation at various stages of maturation. For example, activation of fibroblast growth factor receptor 3 (FGFR3) results in decreased growth and differentiation of chondrocytes [Naski et al., 1996; Wang et al., 1999], and this may be mediated by ERK1/2 stimulation [Krejci et al., 2004; Raucci et al., 2004]. In our study ISO activation of β_2-AR stimulated ERK1/2 but also stimulated growth of the chondrocytes as evidenced by [^{3}H]-thymidine incorporation into the cells. The important difference between the effects of ISO and FGF may be the co-activation of PKA and transient activation of ERK1/2 by ISO, whereas FGF is reported to stimulate ERK1/2 in a sustained fashion and this was necessary for growth arrest [Raucci et al., 2004]. The more transient effect of β_2-AR stimulation on ERK1/2 is likely due to the concomitant induction of MKP1 expression in our cells that subsequently dephosphorylates and thus inactivates ERK1/2. MKP1 is an immediate early gene product, induced by growth factors and PKA. Its rapid response to cell stimuli that increase MAP kinase activation has been suggested to serve as a negative feedback mechanism to fine tune MAP kinase effects [Sun et al., 1993; Keyse, 2000]. While there has been no study on the effect of MKP1 in chondrocytes our study showed that stimulation of the β_2-AR profoundly induced its synthesis suggesting that MKP1 may serve as a mechanism to limit the duration of ERK1/2 activation and maintain a positive effect of β_2-AR on chondrocyte growth.

Having demonstrated the expression of functional β_2-AR in chondrocytes we turned our attention to the effect of these receptors on gene expression in these cells. We focused on two genes that have previously been shown to be regulated by PKA/CREB: Col X and Ihh. Stimulation of the cells by ISO significantly inhibited the expression of mRNAs encoding both of these proteins. Similar effects were also seen when the cells were stimulated by PTH or FSK, both of which activate adenylyl cyclase in these cells. These results suggest that adrenergic stimulation of chondrocytes may have a similar effect as that seen when PTHrP stimulates chondrocytes.

The endogenous ligands of β-AR, norepinephrine and epinephrine, are produced by adrenal chromaffin cells and sympathetic neurons. Cartilage within the growth plate is avascular, however, vascularization at the chondro-osseous junction appears at E15.5 in mice and is a potential source for delivery of catecholamines produced by the developing chromaffin cells. As catecholamines are likely to be delivered to the growth plate in close proximity to the vascular zone, they would reach their target chondrocytes by diffusion. This route of delivery is analogous to that of PTH produced in the fetal parathyroid gland delivered to the chondrocytes through the vascular system. Given the similarities in the effects of PTH and ISO on gene transcription in our cell population, we anticipate that the effects of catecholamines on endochondral bone formation may be similar to that of fetal PTH, namely a decrease in hypertrophic cells expressing Col X and stimulation of angiogenesis [Miao et al., 2002].

In conclusion, we have demonstrated for the first time β_2-AR expression on GPC, and their functional activation of adenylyl cyclase, phosphorylation of CREB and ERK1/2 and stimulation of MKP1 expression. A role for the sympathetic regulation of chondrocyte differentiation is suggested by the inhibition of Col X and Ihh mRNAs. Future studies are required to examine the expression of β_2-AR in the growth plate in vivo as well as to identify the roles of β_2-AR in regulating endochondral bone development.

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