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TNFα Increases the Expression of β2 Adrenergic Receptors in Osteoblasts

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Tumor necrosis factor alpha (TNFα) is a multifunctional cytokine that is elevated in inflammatory diseases such as atherosclerosis, diabetes and rheumatoid arthritis. Recent evidence has suggested that β2 adrenergic receptor (β2AR) activation in osteoblasts suppresses osteogenic activity. In the present study, we explored whether TNFα modulates βAR expression in osteoblastic cells and whether this regulation is associated with the inhibition of osteoblast differentiation by TNFα. In the experiments, we used C2C12 cells, MC3T3-E1 cells and primary cultured mouse bone marrow stromal cells. Among the three subtypes of βAR, β2 and β3AR were found in our analysis to be upregulated by TNFα. Moreover, isoproterenol-induced cAMP production was observed to be significantly enhanced in TNFα-primed C2C12 cells, indicating that TNFα enhances β2AR signaling in osteoblasts. TNFα was further found in C2C12 cells to suppress bone morphogenetic protein 2-induced alkaline phosphatase (ALP) activity and the expression of osteogenic marker genes including Runx2, ALP and osteocalcin. Propranolol, a β2AR antagonist, attenuated this TNFα suppression of osteogenic differentiation. TNFα increased the expression of receptor activator of NF-κB ligand (RANKL), an essential osteoclastogenic factor, in C2C12 cells which was again blocked by propranolol. In summary, our data show that TNFα increases β2AR expression in osteoblasts and that a blockade of β2AR attenuates the suppression of osteogenic differentiation and stimulation of RANKL expression by TNFα. These findings imply that a crosstalk between TNFα and β2AR signaling pathways might occur in osteoblasts to modulate their function.

Key words: TNFα, β2 adrenergic receptor, osteoblasts

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Introduction

Mammalian bone is highly innervated with sympathetic nerve fibers. Several neurotransmitters, neuropeptides, and their receptors are detectable in the bone and bone marrow microenvironment, where they locally control the development and differentiation of osteoblasts and osteoclasts (Togari and Arai, 2008). β2 Adrenergic receptors (AR) are the major adrenergic receptor subtype expressed in osteoblasts, via which the sympathetic nervous system regulates bone formation and resorption (Feve *et al*., 1991; El Hadri *et al*., 1996; Fu *et al*., 2005). It has been demonstrated that β AR activation suppresses osteoblastic activity and the rate of bone formation (Fu *et al*., 2005) while enhancing osteoclastic differentiation (Elefteriou et al., 2005).

Tumor necrosis factor alpha (TNFα) is a multifunctional cytokine that regulates various cellular and biological processes such as cell proliferation, differentiation, apoptosis and immune function (Cope *et al*., 1997). Increased production of TNFα has been implicated in a number of human diseases involving inflammation, such as rheumatoid arthritis, diabetes and vascular disease (Maini, 1996; Navarro-Gonzalez *et al*., 2009). During the rheumatoid arthritis pathological processes, TNFα is largely produced from inflamed synovium and causes activation and maturation of osteoclasts deviated from bone remodelling cycle, resulting in both local and systemic bone resorption. In addition to direct stimulatory effect on osteoclast precursor cells, TNF also

enhances osteoclastogenesis through the up-regulation of receptor activator of nuclear factor kappa-B ligand (RANKL) which is an essential cytokine for osteoclast differentiation (Lacey *et al*., 1998; Zhang *et al*., 2001; Kim *et al*., 2002). TNFα also suppresses the osteoblast differentiation and bone formation partly through NF-κB activation (McMahon and Ueki, 2008; Lee *et al*., 2010).

It has been reported that β2 AR expression is increased by TNFα in murine 3T3-F442A adipocytes (Hadri *et al*., 1997). No one study, to our knowledge, has studied the possibility of TNFα regulation on β AR expression and its downstream signaling in association with TNFα inhibition of osteoblastogenesis and TNFα stimulation of osteoclastogenesis. In the present study, we investigated whether TNFα regulates β AR expression in osteoblasts. We also examined whether the blockade of β AR could reverse TNFα inhibition of osteoblast differentiation and/or TNFα stimulation of RANKL expression.

Materials and Methods

Reagents and antibodies

Alpha modified Eagle's medium (αMEM) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Hyclone (Logan, UT, USA) and fetal bovine serum (FBS) was from BioWhittaker (Walkersville, MD, USA). Bioactive recombinant human bone morphogenetic protein 2 (BMP2) and TNFα protein were obtained from R&D Systems (Minneapolis, MN, USA). easy-BLUE™ for total RNA extraction and *Star*Taq™ polymerase for polymerase chain reaction (PCR) amplification were purchased from iNtRON Biotechnology (Sungnam, Korea). AccuPower RT PreMix for the first-strand cDNA synthesis was purchased from Bioneer (Daejeon, Korea). SYBR premix EX Taq was obtained from TaKaRa (Otsu, Japan). PCR primers were synthesized by Cosmogenetech (Seoul, Korea). Lipofect-AMINE™ reagent was purchased from Invitrogen (Carlsbad, CA, USA). Anti-β2 AR antibodies were purchased from Abcam (Cambridge, UK) and goat anti-rabbit HRP-conjugated antibody was from Santa Cruz (Santa Cruz, CA, USA).

Cell culture

Osteoblastic MC3T3-E1 cells and primary cultured mouse bone marrow stromal cells (bMSCs) were maintained in α-MEM supplemented with 10% FBS. C2C12, a murine mesenchymal precursor cell line, was maintained in DMEM supplemented with 10% FBS. To promote osteogenic differentiation, MC3T3-E1 cells and bMSCs were cultured in osteogenic media (consisting of regular growth media plus 50 μM ascorbic acid and 10 mM β-glycerophosphate), with media replaced every other day. To promote osteogenic differentiation of C2C12, the cells were cultured in DMEM supplemented with 5% FBS and 50 ng/ml of BMP2, with media replaced every other day.

Preparation of mouse bMSCs

Bone marrow cells were prepared from 5 month-old C57Black/6J mice as follows. Four mice were used for the preparation of bone marrow aspirates. The femora and tibiae were dissected free of soft tissues and kept in cold phosphate-buffered saline on ice. The bones were cut open at both ends and flushed with growth media using a 22-gauge syringe followed by filtration through a 70-μm nylon mesh filter. Bone marrow aspirates were dispersed with a 25-gauge syringe to produce a single cell suspension.

Measurement of intracellular cAMP accumulation

C2C12 cells were pretreated or not with 10 ng/ml TNFα for 2 hr and then stimulated with vehicle or isoproterenol (1 μM) for 15 min. The amount of cAMP in cell lysates was determined by the cAMP ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Reverse transcription-PCR (RT-PCR)

To evaluate mRNA expression, semi-quantitative RT-PCR or quantitative real time PCR was performed. Total RNAs were isolated using easy-BLUE™ RNA Extraction Reagents. cDNA was synthesized from total RNA by using AccuPower RT PreMix and was subsequently used for PCR amplification. Semi-quantitative RT-PCR products were electrophoresed on 1.2% agarose gel and visualized under UV light by ethidium bromide staining. Mouse genes and their primer sequences for PCR were as follows: β2 AR forward (f) 5'-GCC TGC TGA CCA AGA ATA AGG CC-3', β2 AR reverse (r) 5'-CCC ATC CTG CTC CAC CT-3'; β3 AR (f) 5'-ATG GCT CCG TGG CCT CAC-3', β3 AR (r) 5'-CCC AAC GGC CAG TGG CCA GTC AGC G-3'; GAPDH (f) 5'-TCA CCA TCT TCC AGG AGC G-3' and GAPDH (r) 5'-CTG CTT ACC ACC TTC TTG A-3'. Florescence-based real time PCR was carried out using SYBR premix EX Taq in an AB 7500 Fast Real-Time system (Applied Biosystems; Foster City, CA, USA). The following primers were used for PCR: β2 AR (f) 5'-GGA CAA CCT CAT CCC TAA-3', β2 AR (r) 5'-AGA GTA GCC GTT CCC ATA-3'; alkaline phosphatase (ALP) (f) 5-CCA ACT CTT TTG TGC CAG AGA-3, ALP (r) 5-GGC TAC ATT GGT GTT GAG CTT TT-3; osteocalcin (OCN) (f) 5-CTG ACA AAG CCT TCA TGT CCA A-3, OCN (r) 5-GCG CCG GAG TCT GTT CAC TA-3; Runx2 (f) 5-TTC TCC AAC CCA CGA ATG CAC-3, Runx2 (r) 5-CAG GTA CGT GTG GTA GTG AGT-3; GAPDH (f) 5-TCA ATG ACA ACT TTG TCA AGC-3 and GAPDH (r) 5-CCA GGG TTT CTT ACT CCT TGG-3. For quantification, GAPDH was used as the reference for normalization of each sample.

Western blot analysis

After appropriate treatments, cells were washed with

phosphate-buffered saline and scraped into lysis buffer consisting of 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μM leupeptin and 1 μM pepstatin, and sonicated briefly. Proteins were subjected to SDS-PAGE and subsequently electro-transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween20 and incubated with the indicated primary antibody followed by incubation with HRP-conjugated secondary antibody. Immune complexes were visualized with Supex reagent (Dyne-Bio, Sungnam, Korea) and luminescence was detected in LAS1000 (Fuji PhotoFilm, Tokyo, Japan).

Statistical analysis

The data are presented as means ± SD. The statistical significance of the results was assessed by the Student's *t*-test. A p value < 0.05 was considered statistically significant.

Results

TNFα increases β2 AR expression in osteoblast lineage cells

Since β2 AR is the major AR subtype expressed in osteoblasts, we first evaluated the effect of TNFα on β2 AR gene expression. C2C12 cells are mesenchymal precursor cells which can differentiate into myoblasts and osteoblasts. MC3T3-E1 cells are pre-osteoblasts. Primary bMSCs are a mixed cell population that contains cells, such as mesenchymal stem cells and pre-osteoblasts. These cells were incubated in osteogenic medium and exposed to 10 ng/ml of TNFα for 2 and 24 hr. TNFα led to an increment of β2 AR mRNA expression in all of the cells examined in a time-dependent manner, as quantified by real time PCR (Fig. 1A). To determine whether the transcriptional stimulation of β2 AR expression in response to TNFα was translated into an increase in β2 AR protein expression, we performed western blot analyses. As shown in Fig. 1B, β2 AR protein expression in C2C12 cells, MC3T3-E1 cells and bMSCs increased upon TNFα treatment, similarly to that observed at the mRNA level. These results indicate that TNFα up-regulated β2 AR expression levels in osteoblast lineage cells. In addition, modulation of β AR expression by TNFα was not limited to the β2 AR subtype. Exposure of C2C12 cells to TNFα slightly increased β3 AR mRNA expression (Fig. 1C). However, β1 AR expression was not detected in C2C12 cells regardless of TNFα treatment (data not shown).

To determine whether TNFα really enhances β AR signaling in osteoblasts, we measured cAMP production that is induced by isoproterenol, a β AR agonist. Isoproterenol significantly increased cellular cAMP level in C2C12 cells. Priming of C2C12 cells with TNFα for 2 hr further enhanced isoproterenol-induced cAMP production (Fig. 2). These results indicate that TNFα up-regulates β AR down-stream signaling in osteoblasts.

Propranolol partially alleviates TNFα inhibition of osteogenic differentiation

In vitro and *in vivo* studies have been shown that propranolol, a β AR antagonist, promotes osteogenesis and bone gain, inhibits bone resorption and protects bone from sympathetic nervous system stimulation-induced loss (Takeda *et al*., 2002; Bonnet *et al*., 2008; Sato *et al*., 2010). Therefore,

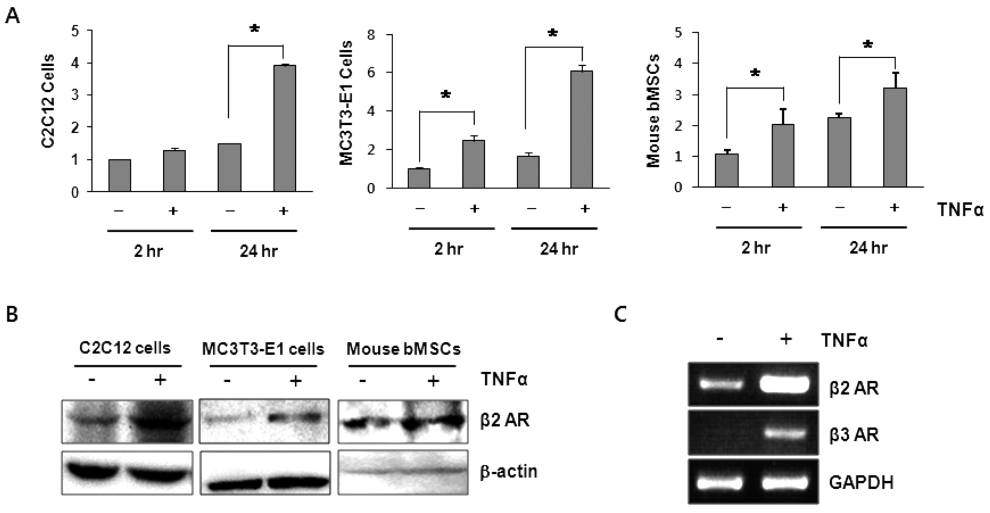

Fig. 1. TNFα increases the expression levels of β2 adrenergic receptor (β2 AR) in osteoblasts. C2C12, MC3T3-E1 and primary cultured mouse bone marrow stromal cells (bMSCs) were incubated in osteogenic media in the presence or absence of TNFα (10 ng/ml) for 2 hr (A) or 24 hr (A-C). β2 AR expression was analyzed by quantitative RT-PCR (A), western blot (B) and semiquantitative RT-PCR (C). β3 AR expression level was also examined in C2C12 cells (C). (A) Relative transcripts level of β2 AR was normalized to GAPDH. The data represent the mean ± SD of duplicates. * p < 0.05, statistically significant.

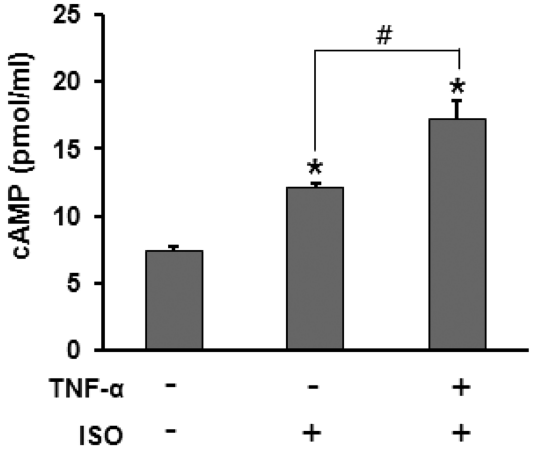

Fig. 2. TNFα priming enhances isoproterenol-induced cAMP production. C2C12 cells were pretreated with TNFα (10 ng/ml) for 2 hr and then stimulated with vehicle or isoproterenol (1 μM) for 15 min. Then, cAMP level was measured with cell lysates using ELISA kit. The data represent mean ± SD of three independent experiments. *$p < 0.05$, compared to vehicle only; #$p < 0.05$ for indicated comparison.

we hypothesized that TNFα might suppress osteoblast differentiation partially through the up-regulation of β2 AR signaling in osteoblasts and that the deleterious effect of TNFα on the osteoblasts might be attenuated by blockade of β2 AR signaling. We induced osteoblastic differentiation of C2C12 cells by BMP2 treatment for 48 hr in the presence or absence of TNFα, isoproterenol and propranolol. Then, osteoblastic differentiation was evaluated by cytochemical staining of ALP and quantitative RT-PCR analyses of ALP, OCN and Runx2. ALP staining showed that TNFα suppressed BMP2-induced ALP activity in C2C12 cells (Fig. 3A). Addition of isoproterenol further decreased TNFα-suppressed ALP activity whereas propranolol slightly attenuated TNFα inhibition of ALP activity (Fig. 3A). BMP2 increased mRNA expression of osteoblast differentiation marker genes, such as Runx2, ALP and OCN (Fig. 3B). TNFα suppressed BMP2 enhancement of the expression of these genes whereas propranolol significantly attenuated the suppressive effect of TNFα (Fig. 3B). However, addition of isoproterenol did not further enhance the suppressive effect of TNFα on osteogenic marker gene expression in C2C12 cells. These results suggest that β AR signaling participates in TNFα suppression of osteogenic differentiation in osteoblasts.

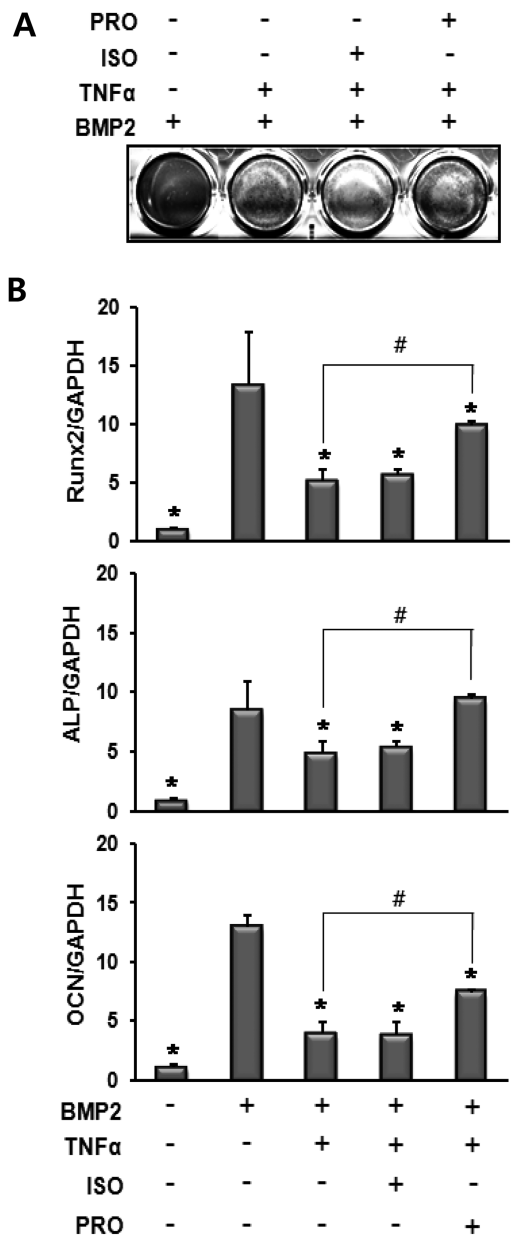

Fig. 3. Propranolol partially alleviates TNFα suppression of osteogenic differentiation. C2C12 cells were cultured in osteogenic medium supplemented with indicated reagents for 48 hr. At the end of culture period, ALP staining (A) and real time PCR (B) were performed to evaluate osteogenic differentiation. The data represent the mean ± SD of duplicates. *$p < 0.05$, compared to BMP2 only; #$p < 0.05$ for indicated comparison. PRO, 10 μM propranolol; ISO, 1 μM isoproterenol; TNFα, 10 ng/ml; BMP2, 50 ng/ml bone morphogenetic protein 2.

Propranolol blocks TNFα stimulation of RANKL expression in C2C12 cells

RANKL is highly expressed in immature osteoblasts and its expression is further stimulated by TNFα (Romas *et al*., 2002). Given that RANKL is essential for the regulation of osteoclastogenesis, we next examined the effect of β AR blockade on TNFα-induced RANKL expression in C2C12 cells. TNFα induced 3.5-fold increase in RANKL mRNA level (Fig. 4). Propranolol completely blocked TNFα induction of RANKL expression whereas addition of isoproterenol did not exert any significant effect (Fig. 4). Isopranolol or propranolol alone did not change the expression level of RANKL. These results suggest that β AR signaling is involved in TNFα induction of RANKL expression.

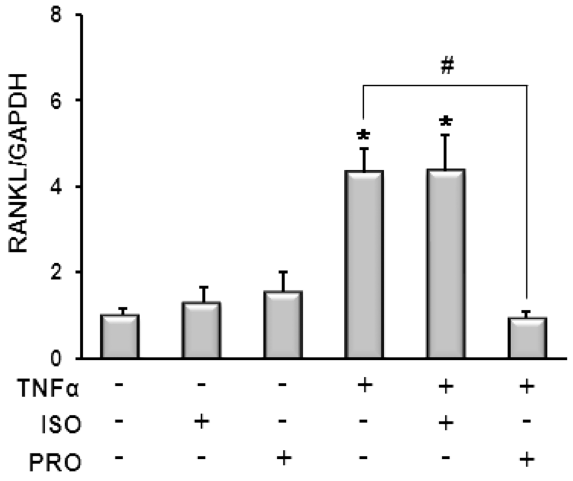

Fig. 4. Propranolol blocks TNFα stimulation of RANKL expression. C2C12 cells were cultured in osteogenic medium supplemented with the indicated reagents for 48 hr and real time PCR was performed. The data represent the mean ± SD of three independent experiments. *$p < 0.05$, compared to vehicle only; #$p < 0.05$ for indicated comparison.

Discussion

In the present study, we demonstrated that TNFα up-regulates β2 AR expression and their downstream signaling activity in osteoblasts. Since β2 AR signaling is considered as a negative regulator of osteoblast differentiation, it is questionable whether TNFα-induced up-regulation of this receptor subclass partially mediates TNFα suppression of osteogenic differentiation. Supporting this hypothesis, our results demonstrated that blockade of β AR signaling by propranolol attenuates TNFα suppression of osteogenic differentiation. Furthermore, propranolol completely pre- vents TNFα induction of RANKL expression in C2C12 cells.

TNF-α led to an increment of β2 AR mRNA and protein expression in C2C12 cells, MC3T3-E1 cells and in primary cultured bMSCs. In accordance with increase in β2 AR expression, TNFα priming enhanced isoproterenol-induced cAMP production. These results indicate that TNFα induction of β2 AR expression is functional in osteoblasts. This is in agreement with the previous finding that TNFα increased β2 AR expression in 3T3-F442A adipocytes (Hadri *et al*., 1997). In that study, TNFα increased both β2 AR gene transcription and β2 AR mRNA half-life while decreasing β3 AR mRNA expression in 3T3-F442A adipocytes. In current study, however, β3AR mRNA level increased with TNFα treatment in C2C12 cells. β2 AR is the major beta adrenergic receptor subtype expressed in murine osteoblasts (Feve *et al*., 1991; Collins *et al*., 1994) whereas β3 AR is considered as a marker of adipose conversion of murine adipocytic cells (Feve *et al*., 1991; El Hadri *et al*., 1996). Because the regulation of β3 AR expression by glucocorticoids, cAMP, or phorbol esters has been shown to depend on species (Granneman and Lahners, 1994), we cannot yet extrapolate the present results to human osteoblasts. But a previous study has also shown that TNFα slightly induced β2 AR expression in human lung cells (Stern and Kunos, 1988), suggesting the possibility that TNFα might up-regulate β2 AR expression in human osteoblasts. From a different perspective, increased β2 and β3 AR expression in C2C12cells was observed in osteogenic condition in current study, suggesting that there might be an additional β AR expression-regulation mechanism influenced by osteogenic process.

Although TNFα priming increased isoproterenol-induced cAMP production, addition of isoproterenol did not enhance TNFα suppression of osteogenic differentiation or TNFα stimulation of RANKL production. Furthermore, in the absence of TNFα, isoproterenol alone did not exhibit significant inhibitory effect on osteoblast differentiation in our culture system even when we used higher concentration of isoproterenol than 1 μM (data not shown). However, β AR blockade with propranolol consistently attenuated TNFα suppression of osteogenic differentiation and TNFα stimulation of RANKL production. Because we used *in vitro* culture system that is deficient of sympathetic innervations, we do not have any idea explaining how β AR blockade diminishes TNFα action in osteoblasts. Our group has previously reported that RANKL, a ligand for RANK which belongs to the same receptor family as TNF receptor, transactivates epidermal growth factor receptors in a ligand-independent manner in osteoclasts (Yi *et al*., 2008). In addition, another report demonstrated that TNFα transactivates epidermal growth factor receptors in human tracheal smooth muscle cells (Lee *et al*., 2007). These reports suggest the possibility that TNFα transactivates β AR and subsequently activated downstream signaling of β AR partially mediates TNFα action in osteoblasts. To clarify the role of β2 AR signaling in TNFα action in osteoblasts, further investigation with β2 AR silencing approach is necessary.

In conclusion, our findings demonstrate that TNFα induces β2 AR expression in osteoblast lineage cells and that blockade of β AR attenuates TNFα action in osteoblasts.

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