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Tumor Necrosis Factor α up-regulates the Expression of beta2 Adrenergic Receptor via NF-κB-dependent Pathway in Osteoblasts

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Tumor necrosis factor alpha (TNFα) is a multifunctional inflammatory cytokine that regulates various cellular and biological processes. Increased levels of TNFα have been implicated in a number of human diseases including diabetes and arthritis. Sympathetic nervous system stimulation via the beta2-adrenergic receptor (β2AR) in osteoblasts suppresses osteogenic activity. We previously reported that TNFα up-regulates β2AR expression in murine osteoblastic cells and that this modulation is associated with TNFα inhibition of osteoblast differentiation. In our present study, we explored whether TNFα induces β2AR expression in human osteoblasts and then identified the downstream signaling pathway. Our results indicated that β2AR expression was increased in Saos-2 and C2C12 cells by TNFα treatment, and that this increase was blocked by the inhibition of NF-κB activation. Chromatin immunoprecipitation and luciferase reporter assay results indicated that NF-κB directly binds to its cognate elements on the β2AR promoter and thereby stimulates β2AR expression. These findings suggest that the activation of TNFα signaling in osteoblastic cells leads to an upregulation of β2AR and also that TNFα induces β2AR expression in an NF-κB-dependent manner.

Key words: TNFα, β2 adrenergic receptor, NF-κB, osteoblast

Introduction

Mammalian bone is highly innervated with sympathetic, sensory and glutaminergic nerve fibers. Various neurotransmitters, neuropeptides and their receptors are detectable in the bone and bone marrow microenvironment, where they locally regulate the differentiation and activity of bone and marrow cells [1]. The effect of sympathetic stimulation on bone is mainly mediated by the β2 adrenergic receptors (β2AR) which are the major adrenergic receptor subtype expressed in osteoblasts [2-4]. β2AR activation suppresses osteoblastic activity and bone formation rate [4] but enhances osteoclastic differentiation [5]. Administration of β adrenergic receptor antagonist demonstrated an increase in bone mass [6]. β2AR knockout mice have a high bone mass phenotype but no other endocrine abnormalities which affect bone metabolism [5].

Tumor necrosis factor alpha (TNFα) is a multifunctional inflammatory cytokine that regulates various cellular and biological processes [7]. Increased level of TNFα has been implicated in a number of human diseases including diabetes, rheumatoid arthritis and vascular disease [8,9]. During the

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inflammatory pathological processes, TNFα is largely produced from inflamed tissues and induces osteoclastic cell differentiation and activation, leading to local and systemic bone resorption. Besides the direct stimulatory effect on osteoclast precursor cells, TNFα also increases osteoclastogenesis through the up-regulation of receptor activator of nuclear factor kappa-B ligand (RANKL), a crucial cytokine for osteoclast differentiation, in osteoblasts and stromal cells [10-12]. TNFα is also known to suppress osteoblast differentiation and bone formation partly through the NF-κB signaling pathway [13,14].

We previously demonstrated that TNFα increases the expression of β2AR in murine osteoblastic cells [15]. Propranolol, a β adrenergic receptor antagonist, partially attenuated the TNFα-induced decline in osteoblast differentiation. In the present study, we extended the previous study by examining 1) whether TNFα increases β2AR expression in human osteoblasts and 2) which of the TNFα downstream signaling pathways is involved in the up-regulation of β2AR expression in osteoblasts.

Materials and Methods

Reagents and antibodies

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Hyclone (Logan, UT, USA) and fetal bovine serum (FBS) was from BioWhittaker (Walkersville, MD, USA). Bioactive recombinant human TNFα was obtained from R&D Systems (Minneapolis, MN, USA). Easy-BLUE™ for total RNA extraction and StarTaq™ 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). Anti-β2AR antibody was purchased from Abcam (Cambridge, UK) and anti-p65 NF-κB antibody and goat anti-rabbit HRP-conjugated antibody were from Santa Cruz (Santa Cruz, CA, USA). A reporter construct containing human beta 2 adrenergic receptor promoter sequences (-1037 to -1 bp; Adrb2-luc, HPRM23871) and Secrete-Pair™ Gaussia luciferase assay kit were purchased from GeneCopoeia (Rockville, MD, USA).

Cell culture

Saos-2, a human osteosarcoma cell line and C2C12, a murine mesenchymal precursor cell line, was maintained in DMEM supplemented with 10% FBS.

Reporter assay

Saos-2 and C2C12 cells were transfected with the indicated vectors by electroporation using a Microporator (Invitrogen; Carlsbad, CA, USA) and Neon tips (Invitrogen) in accordance with the manufacturer's instructions. In each transfection, 0.2 μg of reporter plasmid (β2AR-luc) or expression vector (p65 NF-κB or pcDNA3.1) were used as indicated. After 18 h, the cells and media were harvested and luciferase activity was measured using the Secrete-Pair™ Gaussia luciferase assay kit according to the manufacturer's instructions. The relative luminescence unit (% RLU) was calculated after normalizing the transfection efficiency by cell number.

Reverse transcription-PCR (RT-PCR)

To evaluate β2AR mRNA expression levels, quantitative real time PCR was performed. Total RNA was isolated using easy-BLUE™ RNA Extraction Reagents. cDNA was synthesized from total RNA using AccuPower RT PreMix and subsequently used for PCR amplification. 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 real time PCR: mouse β2AR (f) 5'-GGA CAA CCT CAT CCC TAA-3', (r) 5'-AGA GTA GCC GTT CCC ATA-3'; mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (f) 5'-TCA ATG ACA ACT TTG TCA AGC-3', (r) 5'-CCA GGG TTT CTT ACT CCT TGG-3'; human β2AR (f) 5'-GCC TGC TGA CCA AGA ATA AGG CC-3', (r) 5'-CCC ATC CTG CTC CAC CT-3' and human GAPDH (f) 5'-TCC CTG AGC TGA ACG GGA AG-3', (r) 5'-GGA GGA GTG GGT GTC GCT GT-3'. For quantification, GAPDH was used as the reference for normalization of each sample.

Western blot analysis

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 (DyneBio, Sungnam, Korea) and luminescence was detected in LAS1000 (Fuji PhotoFilm, Tokyo, Japan).

Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed as described previously [16]. C2C12 cells were cross-linked with 1% formaldehyde, lysed and sonicated to get DNA fragments of 200-800 bp. After preclearing with blocked protein G agarose, immunoprecipitation was carried out with anti-NF-κB antibody or equivalent concentrations of mouse IgG as a negative control. DNA was eluted from immune complexes, purified and subjected to PCR amplification of the region containing NF-κB binding element (-1951 to -1941 bp) in the mouse β2AR promoter using the following primers: (f) 5'-GCA CAG CAG CCC TAG ATT TC-3', (r) 5'-CCC GTT ATG TGC ACC AGA CT-3'.

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 β2AR expression in Saos-2 cells

We first evaluated the effect of TNFα on β2AR gene expression in human osteoblastic cells. Saos-2 is an osteogenic cell line derived from the osteosarcoma. These cells were incubated in growth medium and exposed to 10 ng/ml of TNFα for 2, 6 and 24 h. TNFα led to an increment of β2AR mRNA expression in a time-dependent manner, as quantified by real time PCR (Fig. 1A). To determine whether the transcriptional stimulation of β2AR expression in response to TNFα was translated into an increase in β2AR protein expression, we performed Western blot analysis. As shown in Fig. 1B, β2AR protein expression in Saos-2 cells

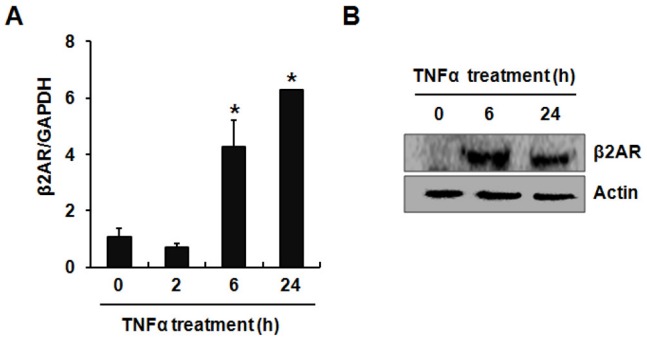

Fig. 1. TNFα increases the expression levels of β2 adrenergic receptor (β2AR) in human osteoblasts. Saos-2 cells were incubated in growth media in the presence or absence of TNFα (10 ng/ml) for 2, 6 or 24 h. β2AR expression was analyzed by real time PCR (A) and Western blotting (B). Relative transcripts level of β2AR was normalized to GAPDH. The data represent the mean + SEM of duplicates. *p<0.05, compared to 0 h.

increased upon TNFα treatment, similarly to that observed at the mRNA level (Fig. 1A). These results indicate that TNFα up-regulates β2AR expression in human osteoblast lineage cells, as previously demonstrated in mouse osteoblastic cells [15]. Modulation of β2AR expression by TNFα was not limited to Saos-2 cells. Exposure of primary human osteoblastic cells to TNFα increased β2AR mRNA expression (data not shown).

TNFα enhances β2AR expression in an NF-κB-dependent manner

Next, we determined the signaling pathway that was involved in TNFα induction of β2AR expression by treating cells with signal-specific inhibitors. Saos-2 cells and C2C12 cells were treated with TNFα (50 ng/ml) for the lengths of time in the presence or absence of an ERK inhibitor (U0126), a JNK inhibitor (SP600125), a p38 MAPK inhibitor (SB-203580) or an NF-κB inhibitor (BAY-11-7082). Real time-PCR analysis demonstrated that TNFα-induced β2AR expression was blocked by the inhibition of NF-κB activation, but not by inhibiting JNK, ERK or p38 MAPK activation (Fig. 2).

NF-κB binds to the putative NF-κB binding site on β2AR promoter and stimulates β2AR transcription

To investigate whether NF-κB directly regulates β2AR expression, we performed an *in silico* analysis to search for the NF-κB binding element (GGGRNNYYCC) in the mouse β2AR promoter using the Transcription Element Search System and found that one putative NF-κB binding element resides within -1951 to -1941 bp of the 2 kb β2AR promoter

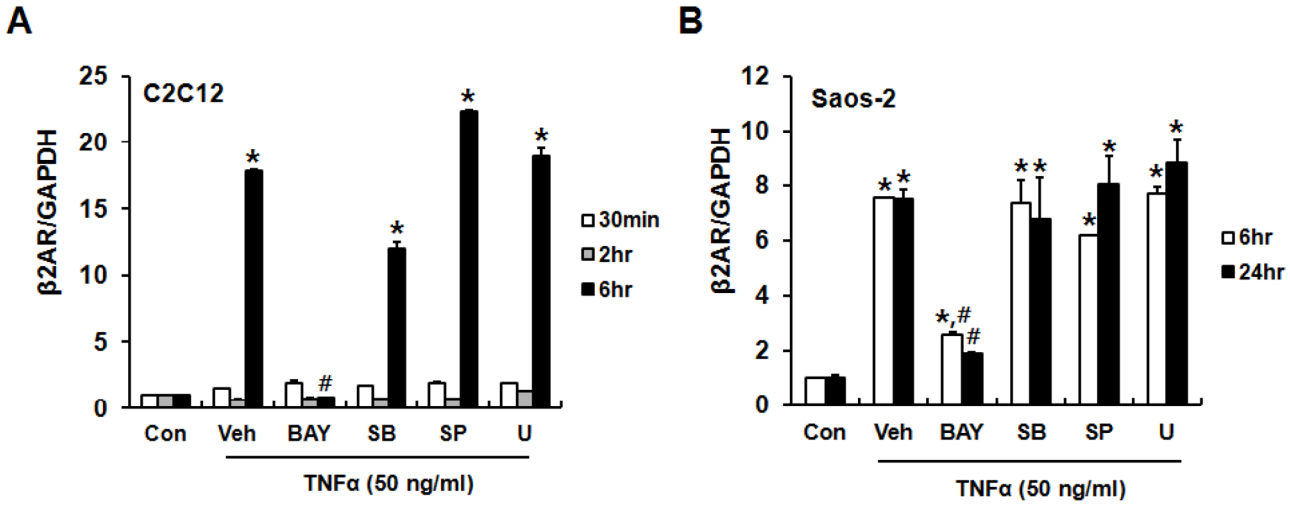

Fig. 2. TNFα enhances β2AR expression in an NF-κB-dependent manner. C2C12 (A) and Saos-2 (B) cells were incubated with TNFα (50 ng/ml) for indicated periods of time in the presence or absence of 10 μM of an ERK inhibitor (U0126, U), a JNK inhibitor (SP600125, SP), a p38 MAPK inhibitor (SB203580, SB) or an NF-κB inhibitor (BAY-11-7082, BAY). β2AR mRNA levels were then analyzed by real time-PCR. The relative β2AR mRNA level was normalized to GAPDH. The data represent the mean + SEM of triplicates. *p<0.05 vs Con of the respective time point, #p<0.05 vs TNFα + Veh of the respective time point.

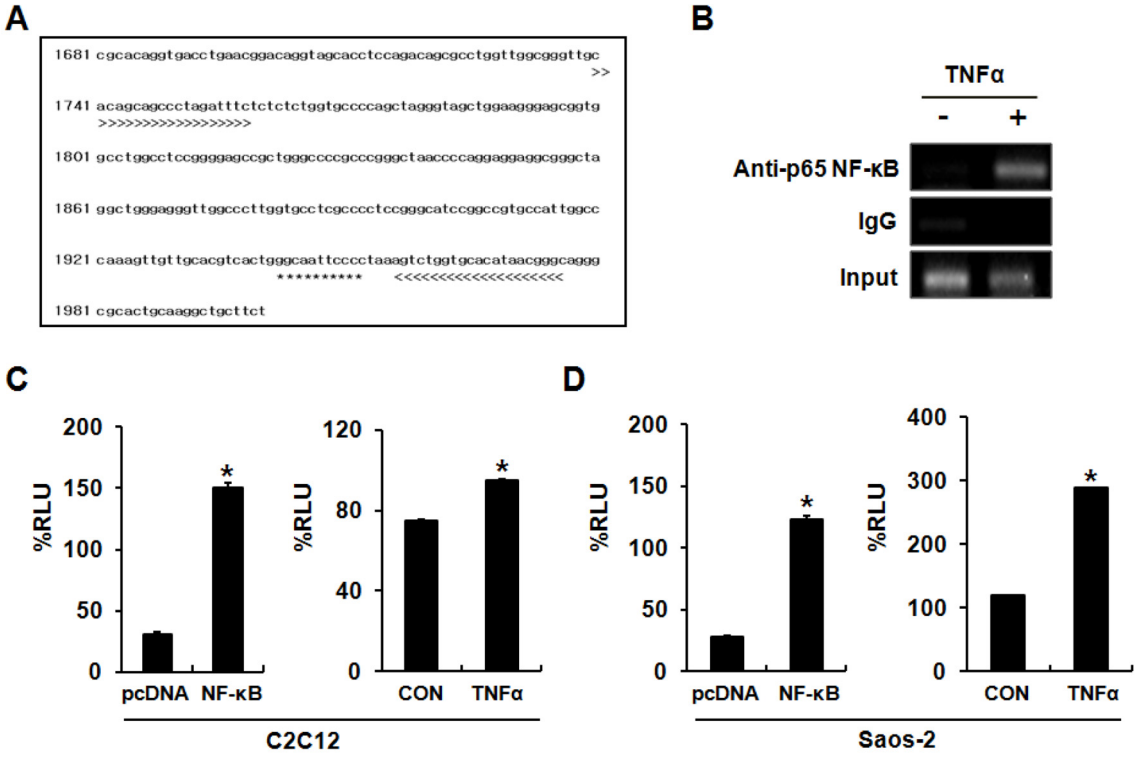

Fig. 3. NF-κB directly binds to the β2AR promoter and stimulates β2AR transcription. (A) Nucleotide sequence of the mouse β2AR promoter region that contains the NF-κB binding element (GGGRNNYYCC: indicated with asterisks). Arrow heads indicates the binding sites for the primers used in chromatin immunoprecipitation analysis. (B) C2C12 cells were incubated in growth media in the presence or absence of TNFα (10 ng/ml) for 6 h. Cellular DNA fragments were immunoprecipitated with anti-p65 NF-κB antibody or normal IgG. PCR amplification revealed that NF-κB binds to the DNA region encompassing the NF-κB binding element (-1951 to -1941 bp) on the mouse β2AR promoter. C2C12 cells (C) and Saos-2 cells (D) were transiently transfected with the indicated plasmids and incubated for 24 h (left panels) or were treated with TNFα (50 ng/ml) for 6 h (right panels). Then luciferase activity was measured and normalized by cell number. Data are represented as mean + SEM (n=6). *p<0.05 vs pcDNA or Con.

region (Fig. 3A). To examine whether NF-κB binds to these putative NF-κB binding motifs *in vivo*, we performed a ChIP analysis. A murine pluripotent mesenchymal precursor cell line C2C12 cells were treated with TNFα for 6 h, and DNA fragments were immunoprecipitated with anti-p65 NF-κB antibody or control IgG. PCR amplification of the β2AR promoter region containing putative NF-κB binding element revealed that NF-κB binds to the DNA region encompassing the putative binding element in the mouse β2AR gene promoter (Fig. 3B). PCR amplification of DNA fragments immunoprecipitated with control IgG did not produce any amplified DNA bands, suggesting that the PCR reactions are specific.

We then performed a luciferase reporter assay to examine whether p65 NF-κB overexpression or TNFα treatment transactivates the β2AR promoter. Overexpression of p65 NF-κB in C2C12 cells (Fig. 3C, left panel) and Saos-2 cells (Fig. 3D, left panel) significantly increased the reporter activity of β2AR-luc, which contains an approximately 1 kb of human β2AR promoter sequence. Treatment of cells with 50 ng/ml TNFα for 6 h significantly enhanced β2AR-luc reporter activity in C2C12 cells (Fig. 3C, right panel) and Saos-2 Cells (Fig. 3D, right panel). Taken together, these results suggest that NF-κB binds directly to the β2AR promoter, thus inducing the transcription of the β2AR gene.

Discussion

We previously demonstrated that TNFα up-regulates β2AR expression and blockade of βAR activation attenuates TNFα suppression of osteogenic differentiation in mouse osteoblasts. In the present study, we demonstrated that TNFα induces β2AR expression in human osteoblasts as well and TNFα stimulates β2AR transcription through the NF-κB-dependent pathway.

Noting the considerable similarity between mouse and human β2AR promoter sequences within 1 kb, we attempted to verify if TNFα leads to an increment of β2AR mRNA and protein expression in human osteoblastic cells. As expected, TNFα induced β2AR mRNA expression in Saos-2 cells in a time-dependent manner. Transcriptional stimulation of β2AR expression in response to TNFα was translated into an increase in β2AR protein expression, as verified by Western blot analysis.

The data in the present study supports the hypothesis that β2AR is a novel target of TNFα-activated NF-κB. We first showed that NF-κB, the major downstream signaling molecule of TNFα, binds to the putative NF-κB binding element on the β2AR promoter. Then we demonstrated that TNFα or NF-κB stimulates β2AR promoter activity. These results suggest that NF-κB directly binds to and transactivates the β2AR promoter, thus increasing β2AR expression.

There is some literature on the regulation of β2AR expression or responsiveness by inflammatory cytokines. Selective regulation of β2AR gene expression by interleukin-1 in human lung tumor cells was demonstrated [17]. β2AR responsiveness in airway smooth muscle is regulated through multiple PKA- and EP2 receptor-dependent mechanisms by interleukin-1 and TNFα [18]. However, no one study has proven yet whether TNFα signaling in osteoblasts regulates the transcription and expression of β2AR. The findings shown in this study demonstrate for the first time that activation of TNFα signaling in osteoblastic cells leads to upregulation of β2AR and that TNFα regulation of β2AR expression is induced in an NF-κB-dependent manner. Taken together, the data presented in this study suggest that inflammatory disease-related bone loss including arthritic bone loss or diabetic osteoporosis might, at least in part, be mediated or be exacerbated by increased sensitivity of bone cells to the sympathetic nervous system stimulation.

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Conflict of interest

All authors disclose that there are no conflicts of interest in this study.

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