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Inhibition of SAPK/JNK leads to enhanced IL-1-induced IL-6 synthesis in osteoblasts

Akira Kondo [a,b], Takanobu Otsuka [a], Rie Matsushima-Nishiwaki [b], Gen Kuroyanagi [a,b], Jun Mizutani [a], Ikuo Wada [a], Osamu Kozawa [b], Haruhiko Tokuda [b,c,*]

[a] Department of Orthopedic Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan
[b] Department of Pharmacology, Gifu University, Graduate School of Medicine, Gifu 501-1194, Japan
[c] Department of Clinical Laboratory, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan

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ABSTRACT

Stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK)[1] which belongs to the MAP kinase superfamily regulates many cellular events. We previously reported that interleukin 1 (IL-1) stimulates the synthesis of interleukin 6 (IL-6) through activation of ERK and p38 MAP kinase in osteoblast-like MC3T3-E1 cells, and that AMP-activated protein kinase (AMPK) negatively regulates the IL-1-induced IL-6 synthesis through IκB/NF-κB pathway. In the present study, we investigated the role of SAPK/JNK in the IL-1-stimulated IL-6 synthesis in these cells. IL-1 induced the phosphorylation of SAPK/JNK. SP600125, an inhibitor of SAPK/JNK, increased the release and the mRNA expression levels of IL-6 induced by IL-1. IL-1-stimulated IL-6 release was significantly up-regulated in SAPK/JNK-knocked down cells. SP600125 remarkably suppressed the IL-1-induced phosphorylation of both IκB and NF-κB, whereas SP600125 failed to affect the IL-1-induced phosphorylation of AMPK, STAT3 or Src. Compound C, an AMPK inhibitor, attenuated the IL-1-induced phosphorylation of SAPK/JNK. SP600125 enhanced IL-1-stimulated IL-6 release also in normal human osteoblasts. These results strongly suggest that SAPK/JNK negatively regulates IL-1-stimulated IL-6 synthesis and acts at the point between AMPK and IκB/NF-κB in osteoblasts.

Introduction

Bone metabolism is a highly coordinated process of bone resorption by osteoclasts and bone formation by osteoblasts [1]. Bone resorption is mediated by the increased local production of inflammatory cytokines such as tumor necrosis factor-α and interleukin 1 (IL-1), so they are considered as bone resorptive agents. In osteoblasts, it has been reported that bone resorptive agents such as IL-1 stimulate the synthesis of interleukin 6 (IL-6), which is a pleiotropic cytokine that has important physiological effects on a wide range of cell functions such as promoting B cell differentiation, T cell activation and inducing acute phase proteins [2–7]. As for bone metabolism, IL-6 has been shown to stimulate bone resorption and induce osteoclast differentiation [2,5,7,8]. Regarding these findings, IL-6 secreted from osteoblasts plays a crucial role as a downstream effector of bone resorptive agents. We previously reported that IL-1 stimulates IL-6 synthesis through the activation of p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase, and that protein kinase C activated by IL-1 negatively regulates IL-6 synthesis at a point upstream of p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells [9]. In addition, we have recently reported that IL-1-activates AMP-activated protein kinase (AMPK), which negatively regulates IL-1-stimulated IL-6 synthesis through Inhibitor of κB (IκB)/nuclear factor-κB (NF-κB) pathway in these cells [10]. However, the details behind IL-6 synthesis in osteoblasts remain to be elucidated.

It is generally recognized that three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) are known as central elements used by mammalian cells to transduce the various messages of variety of extracellular stimulators [11,12]. As for the relationship between bone metabolism and SAPK/JNK, we previously reported that SAPK/JNK acts as a positive regulator in the synthesis of vascular endothelial growth factor (VEGF) stimulated by basic fibroblast growth factor, prostaglandin E1 and transforming growth factor-β in osteoblast-like MC3T3-E1 cells [13–15]. As for IL-6 synthesis, we have shown that SAPK/JNK is involved in platelet-derived growth factor-BB-stimulated IL-6 synthesis in MC3T3-E1 cells [16]. However, the exact role of SAPK/JNK in osteoblasts has not yet been fully clarified.

* Corresponding author at: National Center for Geriatrics and Gerontology, Department of Clinical Laboratory, Obu, Aichi 474-8511 Japan. Fax: +81 562 46 8396.
E-mail address: tokuda@ncgg.go.jp (H. Tokuda).

[1] *Abbreviations used*: Stress-activated protein kinase/c-Jun N-terminal kinase, SAPK/JNK; Interleukin 1, IL-1; Interleukin 6, IL-6; AMP-activated protein kinase, AMPK; Mitogen-activated protein, MAP; Inhibitor of κB, IκB; Nuclear factor-κB, NF-κB; Vascular endothelial growth factor, VEGF; Enzyme-linked immunosorbent assay, ELISA; Normal Human Osteoblasts, NHOst; Glyceraldehyde-3-phosphate dehydrogenase, GAPDH; α-minimum essential medium, α-MEM; Fetal calf serum, FCS; Sodium dodecyl sulfate, SDS; Polyacrylamide gel electrophoresis, PAGE.

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In the present study, we investigated the mechanism underlying IL-1-stimulated IL-6 synthesis on the point whether SAPK/JNK is involved in the IL-1-stimulated IL-6 synthesis as well as the involvement of AMPK-IκB/NF-κB pathway in osteoblast-like MC3T3-E1 cells. We herein show that SAPK/JNK negatively regulates IL-1-stimulated IL-6 synthesis and acts at the point between AMPK and IκB/NF-κB in osteoblasts.

Materials and methods

Reagents

IL-1 was obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). Mouse and human IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). SP600125 and compound C were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). Normal Human Osteoblasts (NHOst) were purchased from CAMBREX (Charles, IA, USA). Phospho-specific SAPK/JNK (Thr-183/Tyr-185) antibodies, SAPK/JNK antibodies, phospho-specific IκB (Ser-32) antibodies, IκB antibodies, phospho-specific NF-κB (Ser-468) antibodies, NF-κB antibodies, phospho-specific AMPKα (Thr-172) antibodies, AMPKα antibodies, phospho-specific STAT3 antibodies and phospho-specific Src antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An ECL Western blotting detection system was obtained from GE Healthcare UK Ltd. (Buckinghamshire, UK). Control short interfering RNA (siRNA; Silencer Negative Control no. 1 siRNA) was purchased from Ambion (Austin, TX) and siLentFect was purchased from Bio-Rad (Hercules, CA, USA). Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA). SAPK/JNK siRNA and an Omniscript Reverse Transcriptase kit were purchased from QIAGEN (Hilden, Germany). Fast-start DNA Master SYBR Green I was purchased from Roche Diagnostics (Mannheim, Germany). BCA Protein Assay Reagent kit was purchased from Thermo Scientific (Rockford, IL, USA). Other materials and chemicals were obtained from commercial sources. SP600125 or compound C was dissolved in dimethyl sulfoxide. The maximum concentration was 0.1%, which did not affect the IL-6 assay or the protein detection using Western blotting.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [17], were maintained as previously described [18]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm diameter dishes (5×10^4 cells/dish) or 90-mm diameter dishes (2×10^5 cells/dish) in α-MEM containing 10% FCS. After 5 days, the medium was exchanged to α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

NHOst were seeded into 35-mm diameter dishes (5×10^4 cells/dish) in α-MEM containing 10% FCS. After 17 days, the medium was exchanged to α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

siRNA transfection

To knock down SAPK/JNK in MC3T3-E1 cells, the cells were transfected with negative control siRNA or SAPK/JNK siRNA utilizing siLentFect according to the manufacturer's protocol. In brief, the cells (1×10^5 cells) were seeded into 35-mm diameter dishes in α-MEM containing 10% FCS and sub-cultured for 48 h. The cells were then incubated at 37 °C with 50 nM siRNA-siLentFect complexes. After 24 h, the medium was exchanged to α-MEM containing 0.3% FCS. The cells were subsequently used for experiments after 24 h.

IL-6 assay

The cultured cells were stimulated by 3 ng/ml of IL-1 in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with SP600125 for 60 min. The conditioned medium was collected at the end of the incubation, and the IL-6 concentration was measured by cell species-responsible IL-6 ELISA kit. The absorbance of enzyme-linked immunosorbent assay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Western blot analysis

The cultured cells were stimulated by 3 ng/ml of IL-1 in α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with SP600125 or compound C for 60 min. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli [19] in 10% polyacrylamide gel. Western blot analysis was performed as previously described [20] by using phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific IκB antibodies, IκB antibodies, GAPDH antibodies, phospho-specific NF-κB (Ser-468) antibodies, NF-κB antibodies, phosphor-specific AMPKα (Thr-172) antibodies, AMPKα antibodies, phospho-specific STAT3 antibodies and phospho-specific Src antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as secondary antibodies. Peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

Real-time RT-PCR

The cultured cells were pretreated with SP600125 for 60 min and stimulated by 3 ng/ml of IL-1 for 2 h. Total RNA was isolated and transcribed into cDNA using Trizol reagent and an Omniscript Reverse Transcriptase kit. Real-time RT–PCR was performed using a Light Cycler system (Roche Diagnostics) in capillaries and Fast-Start DNA Master SYBR Green I provided with the kit. Sense and antisense primers for mouse IL-6 or GAPDH mRNA were purchased from Takara Bio Inc. (Tokyo, Japan) (primer set ID: MA039013). The amplified products were determined by melting curve analysis and agarose electrophoresis. IL-6 mRNA levels were normalized with those of GAPDH mRNA.

Determination

The protein levels of the cells were measured by BCA Protein Assay Reagent kit according to the manufacture's protocol. The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and $p < 0.05$ was considered significant. All data are presented as the mean ± S.E.M. of triplicate determinations from three independent cell preparations.