Biochemical and Biophysical Research Communications 409 (2011) 82–89

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Xanthohumol from the hop plant stimulates osteoblast differentiation by RUNX2 activation

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ARTICLE INFO

Article history:
Received 22 April 2011
Available online 1 May 2011

Keywords:
Xanthohumol
Osteoblast differentiation
RUNX2

ABSTRACT

Xanthohumol (XN), the principal prenylated flavonoid from the hop plant, an additive that contributes bitterness and flavor to beer, is known to be a potent phytoestrogen. Although XN has been identified as a chemopreventive agent and as an anti-infective agent, its effects on bone are unknown. In the present study, the effects of XN on osteoblast differentiation and function were determined by analyzing the activity of alkaline phosphatase (ALP), an osteoblast marker, and the regulation of RUNX2, a master gene of osteoblast differentiation, in a mesenchymal stem cell line. XN upregulated ALP activity and the expression of osteogenic marker genes. Additionally, XN increased the expression and transcriptional activity of RUNX2. To determine which signaling pathways are involved in the osteogenic effects of XN, we tested the effect of inhibitors of kinases known to regulate RUNX2. Enhancement of the transcriptional activity and expression of RUNX2 were inhibited by treatment with a p38 and an ERK inhibitor. These findings suggest that XN stimulates osteoblast differentiation by activation of RUNX2 via mechanisms related to the p38 MAPK and ERK signaling pathway. Regulation of RUNX2 activation by XN may be an important therapeutic target for osteoporosis.

1. Introduction

Osteoporosis occurs around the world. The disorder is defined by low bone mass and micro-architectural deterioration of bone tissue [1,2]. In particular, elderly postmenopausal women experience high rates of osteoporotic fractures, caused by an imbalance between bone formation by osteoblasts and bone resorption by osteoclasts, mediated by hormonal changes. To prevent or treat osteoporosis, various anti-resorptive therapies such as bisphosphonates [3], hormone replacement therapy (HRT), calcitonin, vitamin D, and calcium, and various anabolic agents, such as fluoride, anabolic steroids, and parathyroid hormone (Teriparatide) [4] have been used to date. However, conventional drug therapy has both pros and cons. For example, although chronic estrogen therapy has been used for treatment of osteoporosis and has positive effects on climacteric symptoms and the cardiovascular system, long-term use of hormonal therapy increases the possibility of breast and endometrial cancers [5,6]. There is a continuing need for agents that have a high recovery efficiency of bone mass without side effects. Natural products called phytoestrogens, which stimulate osteoblast differentiation and new bone formation, have received attention for the treatment of osteoporosis. Several plant-derived compounds have been identified from soy, hops, grapes, and wine and have positive effects on bone with no or fewer undesirable side effects.

It is known that isoflavones from soy retard bone loss in peri- and postmenopausal women [7–9]. Genistein, a soy-derived isoflavone, has been shown to inhibit bone resorption and to stimulate bone formation *in vivo* [10] and in an *in vitro* study using MC3T3-E1 cells [11]. A recent study suggested that syringetin from grapes and wine induced human osteoblast differentiation through the BMP2/ERK 1/2 pathway [12]. In addition to these well-known components from soy and grape, various other natural compounds have been shown to influence osteoblast differentiation.

Xanthohumol (XN), a structurally simple prenylated chalcone, is the main prenylflavonoid that is extracted from the hop plant, *Humulus lupulus* L., which is used as a beer additive for bitterness, flavor, and a preservative (antiseptic) effect. Among the abundant prenylated hop flavanones, 8-prenylnaringenin, an isomerization product of desmethylxanthohumol, is the most potent phytoestrogen identified to date; it is more active than the well-known phytoestrogens genistein and daidzein from soy [13]. The other hop prenylflavonoids, including XN and xanthogalenol, have very weak or no estrogenic effect in the yeast screen [14]. Interestingly, isoxanthohumol, the 5-O-methyl derivative of 8-prenylnaringenin, has no estrogenic activity. XN has little if any estrogenic effect,

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doi:10.1016/j.bbrc.2011.04.113

but rather has a potent cancer chemopreventive effect [15]. In *in vivo* and *in vitro* studies, XN inhibited tumor growth and angiogenesis and induced apoptosis of tumor cells [16–18]. Although in *in vitro* studies XN has weak or no estrogenic activity, this component has a strong effect on bone resorption, with IC_{50} values of about 1 μM [19]. Additionally, hops-derived compounds, including 8-prenylnaringenin, 6-prenylnaringenin, XN, and isoxanthohumol, regulate osteoblastic phenotypic gene expression and proliferation in MCF-7 breast cancer cells [20]. However, studies of the involvement of XN in bone differentiation have been limited, although this phytoestrogen has several biological activities.

In the present study, we first describe the osteogenic activity of XN and its underlying molecular mechanism. We show that XN enhanced the expression of RUNX2, which is the principal osteogenic master gene for bone formation, at the transcriptional and translational levels via the p38 MAPK and ERK signal transduction pathways. Thus, we suggest that XN increases osteogenic differentiation.

2. Materials and methods

2.1. Materials

Xanthohumol was obtained from Sigma Aldrich Co. (St. Louis, MO, USA), recombinant human BMP2 from R&D Systems (Minneapolis, MN, USA), the ALP activity assay kit and luciferase reporter assay kit from Promega (Sunnyvale, CA, USA), the TRIzol reagent and reverse transcriptase from Invitrogen (Carlsbad, CA, USA), p38 MAPK (SB203580), ERK (U0126), PKA (H89), and Akt inhibitor from Calbiochem (La Jolla, CA, USA), and ECL reagent and polyvinylidene difluoride (PVDF) membranes from Amersham Pharmacia Biotech (Uppsala, Sweden). Oligonucleotide PCR primers were custom-synthesized by Bioneer Co. (Korea). All chemicals were of the highest commercially available grade.

2.2. Plasmids and antibodies

HA-tagged RUNX2, -Osterix, and -Dlx5 were constructed in a CMV promoter-derived mammalian expression vector (pCS4-3HA). The plasmid pGL3-12-OSE-luc was constructed by inserting 12 tandem repeats of the RUNX binding site (AACCACA) into the multiple cloning site of pGL3-luciferase (Promega, Madison, WI, USA). OC promoter-Luc (1.3 kb of the osteocalcin promoter) and ALP-Luc were provided by N. Kim (Chonnam National University). BSP-Luc was used in this study. Antibodies against hemagglutinin (HA) (antibody 12CA5; Roche), Erk/phospho-Erk, p38/phospho-p38 and tubulin (Sigma Aldrich) were used.

2.3. Cell culture

All culture media and antibiotics were from Gibco-BRL. MC3T3-E1 and C2C12 cells, mouse preosteoblast cell and mouse mesenchymal stem cells, were maintained in DMEM with 10% or 20% fetal bovine serum, antibiotics, and antimycotics at 37 °C in an atmosphere of 5% CO_2.

2.4. DNA transfections and reporter assay

Transient transfections were performed using the calcium phosphate or Lipofectamine PLUS method, with pCMV β-gal as an internal control for transfection efficiency. For the luciferase assay, C2C12 cells were seeded on 24-well plates and incubated for 24 h before transfection with the reporter constructs. The cell lysates were analyzed for luciferase activity using a luciferase reporter assay kit (Promega).

2.5. ALP staining and von Kossa and Alizarin red S staining

Differentiated osteoblast cells were stained for ALP activity using the BCIP/NBT color development substrate (Promega). In MT3T3-E1 cells, mineralization was visualized by von Kossa staining. Cells in each well were fixed with 2% paraformaldehyde and then hydrated with water. Silver nitrate solution (5%) was placed into each well and incubated for 1 h. Wells were rinsed three times with distilled water, followed by reduction with thiosulfate solution for 5 min. The cells were counterstained for 5 min with nuclear fast red stain and rinsed with tap water. For bone nodule formation, MC3T3-E1 cells were cultured in osteogenic medium containing 400 μM ascorbic acid and 5 mM β-glycerophosphate to provide inorganic phosphate. The cells were used at days 14–21 for Alizarin red S staining. The cells were washed twice with PBS, and then fixed with 4% paraformaldehyde in PBS, pH 7.4. The fixed MC3T3-E1 cells were stained with 0.1% Alizarin red S solution for 60 min, and then photographs were taken with a Nikon camera.

2.6. RNA preparation and semi-quantitative RT-PCR

Total cellular RNA was prepared using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Random primers and reverse transcriptase (Invitrogen) were used to synthesize cDNA from 1 μg of total RNA. The following conditions were used for amplification by PCR: initial denaturation at 94 °C for 1 min, followed by 28–30 cycles of denaturation at 94 °C for 30 s, annealing at a temperature optimized for each primer pair for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The PCR primer sequences were as follows: ALP, F 5′-GAT CAT TCC CAC GTT TTC AC-3′ and R 5′-TGC GGG CTT GTG GGA CCT GC-3′; OC, F 5′-CTC CTG AGT CTG ACA AAG CCT T-3′ and R 5′-GCT GTG ACA TCC ATT ACT TGC-3′; Collα1, F 5′-TCT CCA CTC TTC TAG GTT CCT-3′ and R 5′-TTG GGT CAT TTC CAC ATG C-3′; BSP, F 5′-ACA CTT ACC GAG CTT ATG AGG-3′ and R 5′-TTG CGC AGT TAG CAA TAG CAC-3′; RUNX2, F 5′-AGC AAC AGC AAC AAC AGC AG-3′ and R 5′-GTA ATC TGA CTC TGT CCT TG-3′; Dlx5, F 5′-ACA GGA GTG TTT GAC-3′ and R 5′-CTA ATA AAG CGT CCC GGA-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), F 5′-ACC ACA GTC CAT GCC ATC AC-3′ and R 5′-TCC ACC ACC CTG TTG CTG TA-3′. GAPDH was used as an internal control.

2.7. Western blot analysis

Cells were pretreated with or without an inhibitor of p38 MAPK (SB203580), ERK (U0126), or PKA (H89) and then incubated with XN for 12 h. Cell lysates were prepared and resolved by SDS–PAGE, followed by electroblotting onto a PVDF membrane. The membrane was incubated with the appropriate primary antibody, followed by incubation with secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Bio-Rad Laboratories, Inc., Hercules, CA), according to the manufacturer's instructions.

2.8. Statistical analyses

All experiments were performed with triplicate independent samples and were repeated at least twice, giving qualitatively identical results. Results are expressed as mean ± standard error of the mean. Data were analyzed using Student's *t*-test, with p values <0.05 taken to indicate statistical significance.

3. Results

3.1. Xanthohumol stimulates osteogenic differentiation in osteoblastic cell lines

We studied the effect of XN on differentiation in MC3T3-E1 cells, an osteoblastic cell line derived from newborn mouse calvariae. MC3T3-E1 cells were cultured with different concentrations of XN for several days. ALP activity, an early-stage osteogenic differentiation marker, increased significantly in 7 days of culture of MC3T3-E1 cells, with dose-dependency for XN (Fig. 1A). To detect the effects of XN on mineralization, Alizarin red S and von Kossa staining were performed in MC3T3-E1 cells, which were cultured with different concentrations of XN for 14 or 18 days. Matrix mineralization, which is induced by bone morphogenic protein 2 (BMP2), a known inducer of bone formation, was significantly increased, according to the concentration of XN (Fig. 1B and C). To detect the effect of marker gene expression for osteogenic differentiation by XN, C2C12 mesenchymal stem cells were treated with XN and/or BMP2, and then total RNA was extracted from the cell lysates. As shown in Fig. 2A, the BMP2-induced mRNA level of osteogenic-specific markers was increased by XN. Not only ALP and collagen type Iα, early-stage osteogenic differentiation markers, but also BSP and OC, late-stage osteogenic differentiation markers, were positively affected by XN. Additionally, effects on gene expression of RUNX2 and Dlx5, major transcriptional factors for osteogenic differentiation, by XN were detected (Fig. 2B). These results suggest that XN may induce osteogenic differentiation throughout processes from the early to late phase.

To investigate the effects of XN on the transcriptional activity of osteogenic marker gene promoters, we used a luciferase reporter assay to detect the activity of OC-, ALP-, BSP-, and osteoblast-specific element (OSE)-luciferase genes in C2C12 cells. XN increased the transcriptional activities of these osteogenic markers in a dose-dependent manner (Figs. 2C and D and S1 A and B). Time dependency of XN was detected in OC-luciferase genes in C2C12 cells. XN increased the transcriptional activity of OC according to time (Fig. 2E). In particular, the OSE-luciferase gene construct contained 12 repeats of the RUNX2 binding site, suggesting that XN may promote osteogenic differentiation by specifically regulating the activity of RUNX2 in mesenchymal stem cells.

3.2. Xanthohumol enhances the expression and the transcriptional activity of RUNX2

As shown in Fig. 2F, XN enhanced the transcription of OSE-luciferase. As OSE-luciferase contains RUNX2 binding sites, it is important to examine the effect of XN on RUNX2, a major transcription

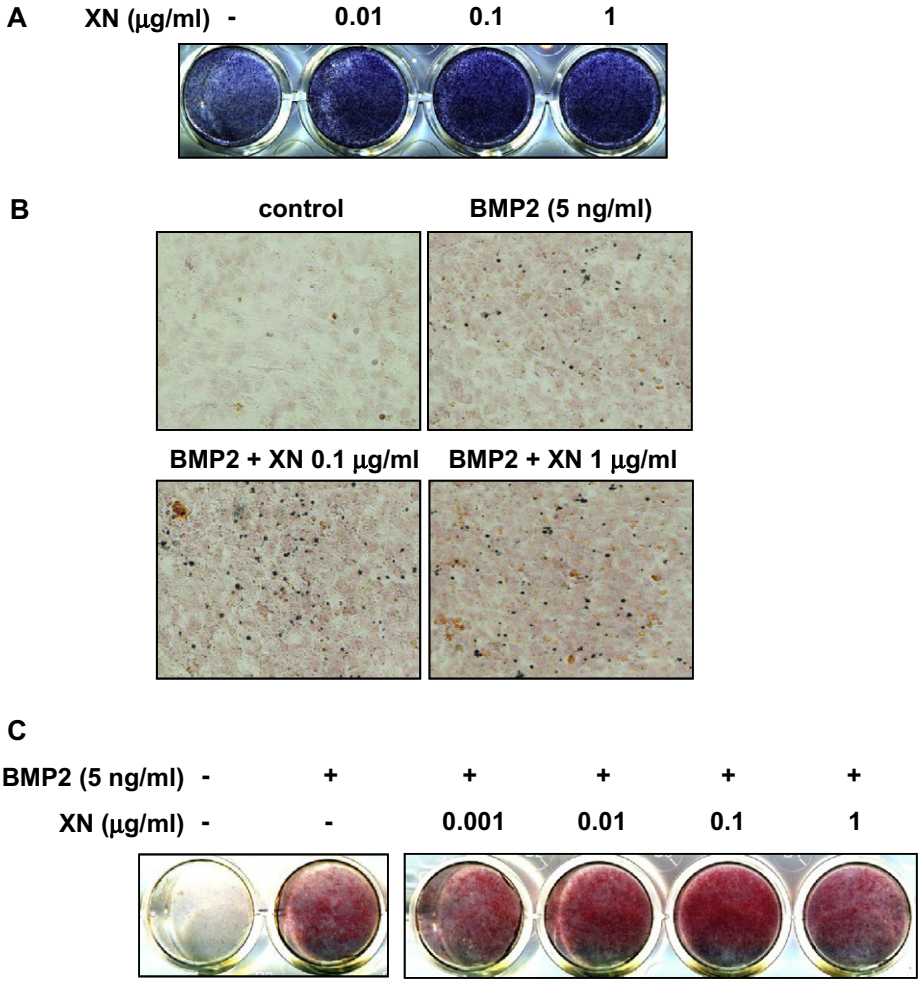

Fig. 1. Xanthohumol stimulates osteogenic differentiation in MC3T3-E1 cells. (A) Cells were incubated until sub-confluent and then cultured in the absence or the presence of XN (0.01, 0.1 and 1 μg/ml). On day 7, the cells were subjected to ALP staining. (B) Cells were treated with 0.1 or 1 μg/mL of XN and/or BMP2. On day 18, the cells were subjected to von Kossa staining. (C) Cells were treated with 1, 10, or 100 ng/ml or 1 μg/ml of XN and/or BMP2. On day 14, the cells were subjected to Alizarin red S staining. Each figure is representative of three independent experiments.

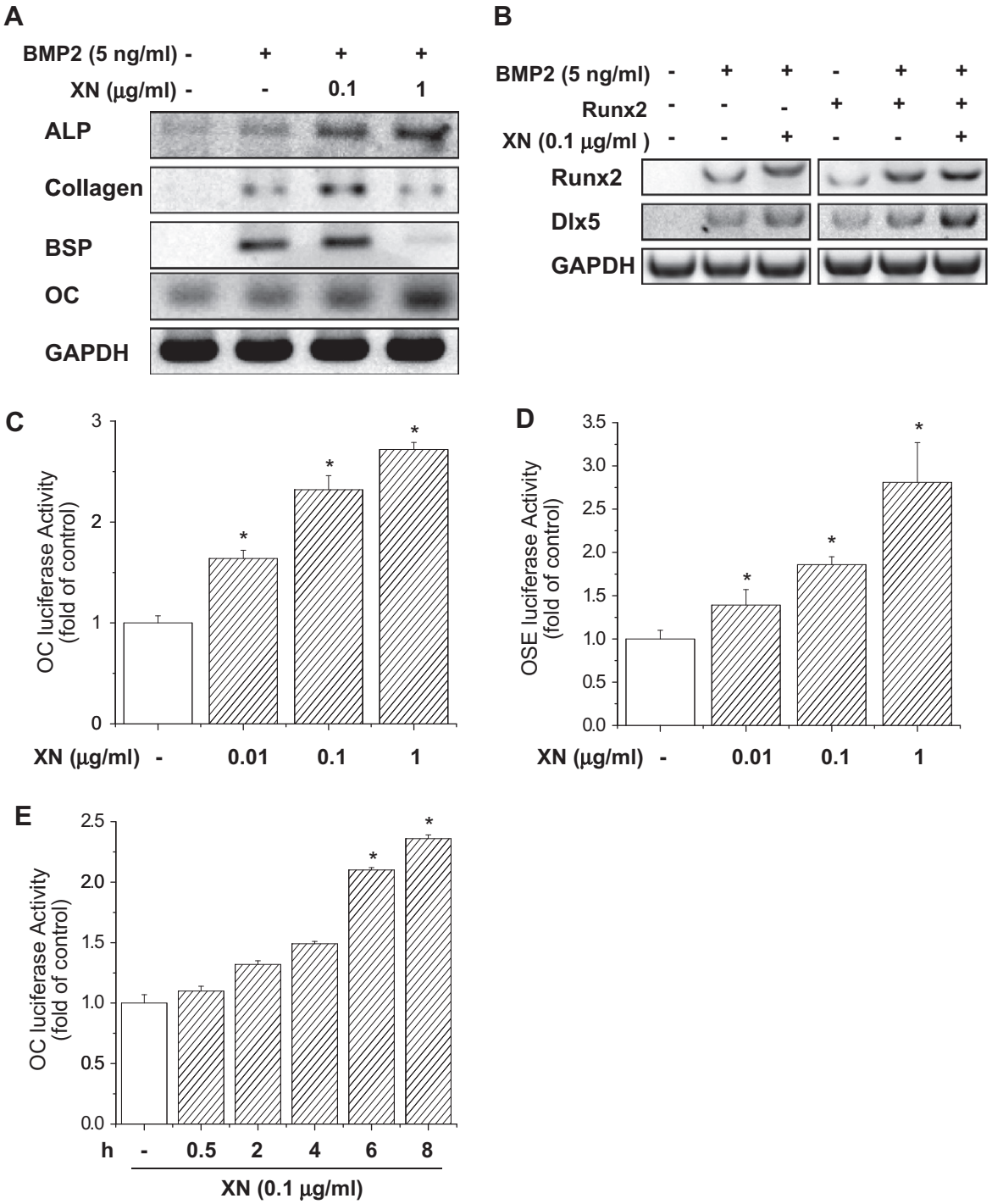

Fig. 2. Xanthohumol increases the expression of osteogenic differentiation factors and RUNX2. (A) C2C12 cells were treated with xanthohumol (0.1 or 1 μg/ml) and/or BMP2. Total RNA was extracted from the cell lysates, and cDNA was prepared from 1 μg of the total RNA. RT-PCR was performed using the cDNA and primers for ALP, collagen type 1α, BSP, OC, and GAPDH. (B) C2C12 cells were treated with XN (0.1 μg/ml) and/or BMP2 in the absence or presence of RUNX2. RT-PCR was performed using the cDNA and primers RUNX2, Dlx5, and GAPDH. (C and D) XN stimulates OC and OSE promoter-driven transcription. C2C12 cells were transfected with the OC- or OSE-Luc plasmid, and a luciferase reporter assay was performed 48 h later. (E) XN stimulates OC promoter-driven transcription in a time-dependent manner. In Fig. 2(C and D), each bar represents three independent experiments. $^{*}p < 0.05$, versus control.

factor in osteogenic differentiation. To examine whether XN affected the expression of not only RUNX2 but also Dlx5, major transcription factors in osteogenic differentiation, C2C12 cells were transfected with HA-tagged RUNX2, -Dlx5, or -Osx plasmid in the presence or absence of XN for 12 h. As shown in Fig. 3A, XN enhances the expression of only RUNX2, not Dlx5 or Osx, which is regulated and characterized by RUNX2. Next, we examined the effects of XN on the transcriptional activity of the promoters of the osteogenic marker genes OC, ALP, BSP, and OSE, which are well-known target genes of RUNX2 [21]. C2C12 cells were transfected with an expression plasmid for HA-tagged RUNX2 and with the OC-, ALP-, BSP-, or OSE-Luc plasmid, and XN-stimulated luciferase activity was assayed 48 h later. The reporter assays showed that XN enhanced the transcriptional activity of RUNX2 at the OC, ALP, BSP, and OSE promoters (Fig. 3B–E). These results suggest that XN stimulates osteogenic differentiation through enhancing the

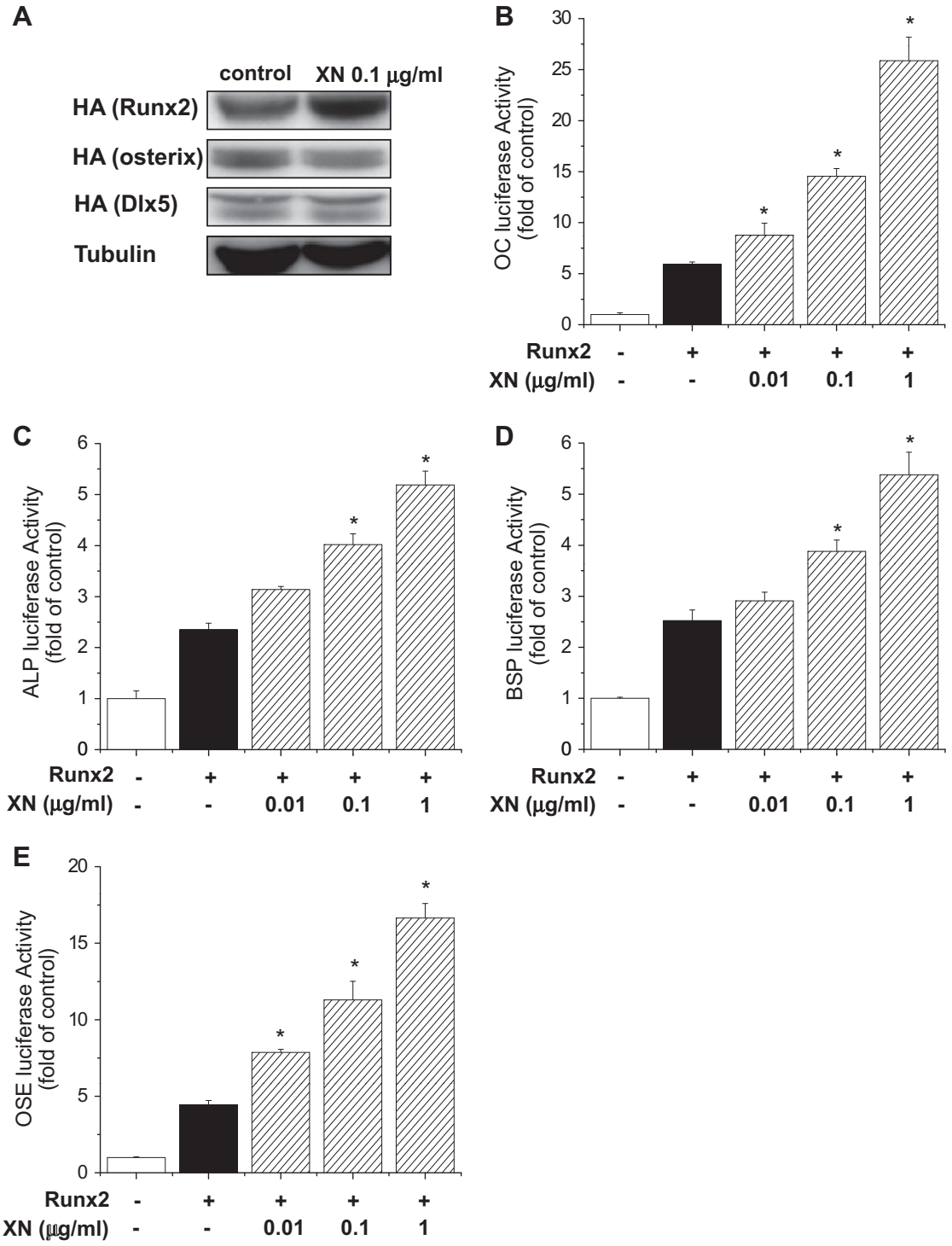

Fig. 3. Xanthohumol enhances the expression and transcriptional activity of only RUNX2 among several transcription factors. (A) C2C12 cells were transfected with an expression plasmid for HA-tagged RUNX2, Dlx5, or Osx and then treated with xanthohumol at various concentrations. The expression level of RUNX2, Dlx5, or Osx was determined by Western blot analysis using anti-HA antibody. Each blot shown is representative of three independent experiments giving similar results. (B–E) C2C12 cells were transfected with an expression plasmid for HA-tagged RUNX2 and with OC-, ALP-, BSP-, or OSE-Luc plasmid, and a luciferase reporter assay was performed 48 h later. Each bar shows three independent experiments. $^{*}p < 0.05$, versus RUNX2.

expression and the transcriptional activity of RUNX2, an important transcription factor for bone.

3.3. Xanthohumol stimulates osteogenic differentiation via activation of RUNX2 by the p38 MAPK and ERK signaling pathway

Phosphorylation is an important regulatory mechanism of the activity of RUNX2, a key transcription factor in osteogenic differentiation. RUNX2 is regulated by various signaling pathways including mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), PKC, and PKA [22–24]. To determine which signal transduction pathway is involved in the activation of RUNX2 by XN, the expression of various kinase proteins that seem to phosphorylate RUNX2 was detected in the presence or absence of XN. As shown in Fig. 4A, the expression of phospho-ERK and -p38 MAPK was increased by XN according to dose, but that of total

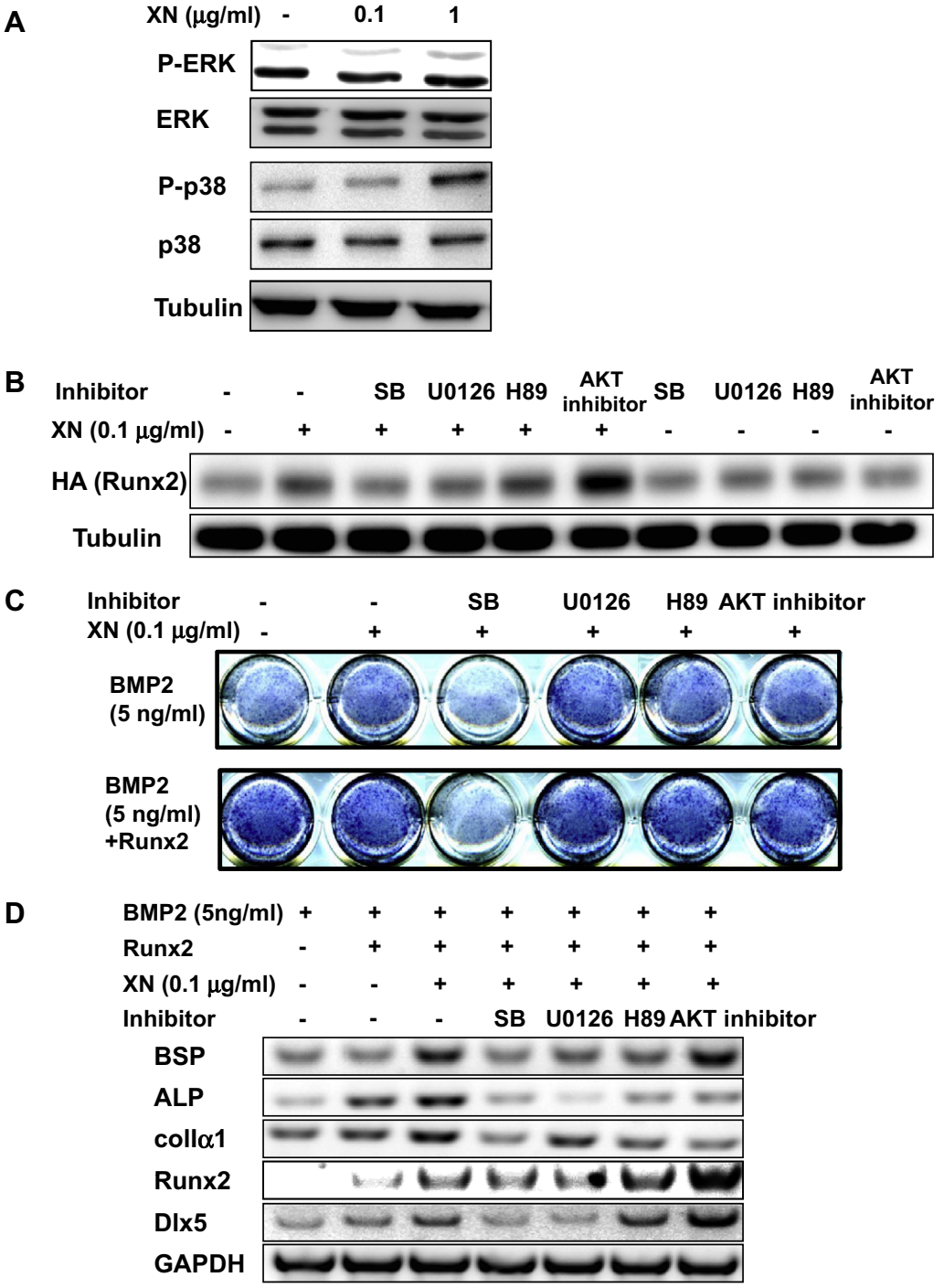

Fig. 4. Xanthohumol stimulates osteogenic differentiation via the activation of RUNX2 by the p38 MAPK and ERK signaling pathway. (A) XN increases the expression of ERK and p38 of the phosphorylation form. C2C12 cells were treated with XN at various concentrations. The expression level of the phospho-form or total protein on ERK or p38 was determined by Western blot analysis using each antibody. (B) C2C12 cells were transfected with an expression vector for HA-tagged RUNX2, pretreated with inhibitors, and then treated with XN. The expression level of RUNX2 was determined by Western blot analysis using anti-HA antibody. Each blot shown is representative of three independent experiments with similar results. (C) Non-transfected or RUNX2-transfected C2C12 cells were incubated until sub-confluent and then cultured with 100 ng/mL of XN and BMP in the absence or presence of inhibitors, followed by ALP staining on day 7. This figure is representative of three independent experiments. (D) C2C12 cells were transfected with an expression plasmid for HA-tagged RUNX2 and then treated with XN in the absence or presence of kinase inhibitors (SB203580, U0126, H89, AKT inhibitor). RT-PCR was performed using primers for RUNX2, Dlx5, ALP, Collα1, and BSP with the cDNA produced from 1 μg of total RNA. GAPDH was used as a control.

ERK and p38 was unchanged (Fig. 4A). This suggests that the activation of ERK and p38 MAPK by XN may phosphorylate RUNX2. We next examined whether the XN-enhanced expression of RUNX2 was affected by inhibitors of these kinases. As shown in Fig. 4B, SB203580, a p38 inhibitor, and U0126, an ERK inhibitor, inhibited the expression of RUNX2, but H89, a PKA inhibitor and AKT inhibitor, did not. In addition to this expression of RUNX2, XN-enhanced RUNX2-activated transcription of OC and OSE (S2 A and B), -ALP activity (Fig. 4C), and the transcription of osteogenic markers (Fig. 4D) were inhibited by SB203580 and U0126. These results

suggest that XN stimulated osteogenic differentiation through activated RUNX2 via the p38 MAPK and ERK signal transduction pathway.

4. Discussion

In this study, we identified the osteogenic differentiation ability of XN. Treatment of C2C12 or MC3T3-E1 cells with XN resulted in increased expression of RUNX2, an indispensable transcription factor in bone development, and osteogenic factors including ALP, collagen, BSP, and OC. Moreover, we determined that XN stimulated osteoblast differentiation by activation of RUNX2 via mechanisms related to the p38 MAPK and ERK signaling pathway.

RUNX2 has a key role in intramembranous or endochondral bone formation. A loss-of-function mutation of RUNX2 in humans induces cleidocranial dysplasia (CCD) [25]. Nullizygous mice do not form mineralized bone and lack mature osteoblast and osteogenic differentiation markers [26,27]. The function of RUNX2 is regulated at several levels, such as transcription, translation, post-translational modification, and protein–protein interactions, by multiple signal transduction pathways. In particular, phosphorylation of RUNX2 is an important mechanism that regulates its activity. It is known that a various kinases phosphorylate RUNX2. MAPK/ERK pathways, which are induced by insulin growth factor-1 (IGF-1), mechanical stress, or basic fibroblast growth factor-2 (bFGF-2), increase RUNX2 phosphorylation [23,28]. These inducers stimulate bone formation through phosphorylation of RUNX2 via the MAPK/ERK signaling pathway. Also, bFGF-2 stimulates the phosphorylation of RUNX2 through protein kinase c delta (PKC-ζ) [24], and parathyroid hormone (PTH) induces the expression of collagenase-3, which is activated through phosphorylated RUNX2 by protein kinase A (PKA) [22]. Moreover, in a previous study, we determined that saponin from the roots of *Platycodon grandiflorum* stimulated osteoblast differentiation via p38 MAPK- and ERK-dependent RUNX2 activation [29]. Thus, the regulation of RUNX2 activity by alteration of its phosphorylation status is important in bone formation. In this study, various kinase inhibitors were used to determine which mechanism was involved in RUNX2 activity for bone formation. p38 MAPK (SB203580 as inhibitor) and ERK (U0126 as inhibitor), but not PKC (H89 as inhibitor) or AKT, decreased the expression and the transcriptional activity of RUNX2, which was increased by XN, as well as ALP activity and the transcriptional expression of osteogenic differentiation markers. Additionally, treatment with XN increased the expression of phospho-ERK and -p38, but not AKT and JNK. These results suggest that the target protein and target kinase of XN are RUNX2 and p38/ERK, respectively.

In conclusion, we suggest that XN induces osteoblast differentiation by activating RUNX2 via the p38 MAPK and ERK signaling pathway. Usually, the usage of bone resorption inhibitors is very low because the degree of bone resorption in osteoporosis may exceed that of recovery of bone mass. Thus, therapy that involves enhancing bone formation has been emphasized rather than therapy that inhibits bone resorption. Therefore, it is necessary to further study whether XN inhibits bone resorption simultaneously as well as stimulating bone formation, as in this study, to assess its potential use as a therapeutic agent.

Acknowledgments

This study was supported by grants from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A090923).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.04.113.

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