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Risedronate increases osteoblastic differentiation and function through connexin43

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ABSTRACT

Bisphosphonates are potent antiresorptive drugs which have antifracture efficacy by reducing bone turnover rate and increasing bone mineral density. In addition to inhibiting osteoclast function, bisphosphonates have been reported to also promote survival of osteocyte and osteoblast via an anti-apoptotic effect, mediated by opening of hemi-gap junction channels formed by connexin43 (Cx43). In this study, we investigated the effect of risedronate, one amino-bisphosphonate, on osteoblast differentiation and Cx43 expression using the mesenchymal cell line C2C12. Risedronate dose-dependently increased the activity of osterix (OSE)-luciferase containing Runx2 response element with highest activity at 50 μM. The activity of osteocalcin (OC)- and bone sialoprotein (BSP)-luciferase reporters, markers of osteoblast differentiation, were also increased by risedronate. When risedronate and BMP2 were used in combination, alkaline phosphatase (ALP) activity increased to a larger extent than when BMP2 was used alone. Risedronate as well as the pro-osteogenic transcription factors, Runx2, Osterix or Dlx5, increased transcriptional activity of the Cx43 promoter in a dose-dependent manner. In the presence of Runx2 or Dlx5, risedronate had an additive effect on Cx43 promoter activity. Accordingly, risedronate increased protein expression of Cx43, Runx2, Osterix, and Dlx5. These results suggest that risedronate promotes osteoblastic differentiation and positively regulates Cx43 gene transcription.

1. Introduction

Bone is a dynamic tissue that is remodeled constantly by osteoclasts and osteoblasts throughout life. An imbalance between bone formation and resorption is the underlying cause of osteoporosis, a disorder characterized by enhanced skeletal fragility as a result of reduced bone quantity and quality. Bisphosphonates, synthetic compounds with a chemical structure similar to pyrophosphate (an inhibitor of calcification), have been widely used for the treatment of postmenopausal osteoporosis, glucocorticoid-induced osteoporosis, Paget's disease of bone, and cancer-induced bone loss [1,2]. The main effect of bisphosphonates is inhibition of osteoclast bone resorption. Nitrogen-containing bisphosphonates inhibit farnesyl pyrophosphate synthase (FPP), a key step in the mevalonate pathway [3]. Inhibition of this enzyme interferes with isoprenylation of small GTPases, eventually leading to osteoclast inhibition and apoptosis [4]. Intriguingly, a series of studies from one group suggests that bisphosphonate may have an opposite, anti-apoptotic effect on osteoblasts and osteocytes, *in vitro* and *in vivo* [5–7]. The same group further proposes that this effect of bisphosphonates on bone forming cells is mediated by opening of hemi-gap junction channels formed by connexin43 (Cx43), and down-stream activation of Src and extracellular signal-regulated kinases (ERKs) [8–10]. They also show that Cx43 is essential for osteocyte survival and for communication with other cells [11]. However, the biologic relevance of such anti-apoptotic effect for bisphosphonate pharmacologic action is unclear, since in mice conditionally deficient of Cx43 alendronate or risedronate prevent glucocorticoid and ovariectomy-induced bone loss as well as in wild type animals [7,12], without affecting bone formation [12].

Cx43 is the major gap junction protein present in osteolineage cells, where it provides a mechanism for cell–cell communication via gap junctions [13–15]. As noted, Cx43 can also be assembled in unopposed gap junction hemichannels [8,9,16], though the biologic role of these hemichannels is increasingly being challenged [12,17]. In any case, mouse genetics studies have established that Cx43 is critically important for bone development and adult skeletal growth [18,19]. Cx43 modulates the differentiation and secretory activity of bone forming cells [12,19–21]. Cx43 also modulates parathyroid hormone stimulatory action on matrix production by MC3T3 cells [22]; and Cx43 gene expression is stimulated by

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prostaglandin E2 and parathyroid hormone, critical factors for systemic and local control in bone remodeling [23,24]. Further, the anabolic response to PTH is attenuated in mice with an osteoblast-specific deletion of Cx43 [19].

We studied the effect of the amino-bisphosphonate, risedronate on Cx43 and key transcriptional factors during osteoblastic differentiation. We find that risedronate increases the transcriptional activity and expression of Cx43 and genes critical for osteogenic differentiation. Thus, we suggest that risedronate promotes osteoblastic differentiation and function in association with upregulation of Cx43.

2. Materials and methods

2.1. Cell culture

Culture media and antibiotics were purchased from Invitrogen. HEK293 cells and C2C12 cells were maintained at 37 °C in a 5% CO_2 atmosphere in DMEM supplemented with 10% fetal bovine serum and antibiotics–antimycotics.

2.2. Plasmids and antibodies

Hemagglutinin (HA)-tagged Osterix, -Dlx5, -Runx2 were constructed in a CMV promoter-derived mammalian expression vector (pCS4+). 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 generously provided by N. Kim (Chonnam National University). Cx43 promoter-reporter construct was provided by Stephen Lye (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). Antibodies against Connexin43 (Santa Cruz, USA), hemagglutinin (HA) (antibody 12CA5; Roche) and tubulin (Sigma–Aldrich, St. Louis, MO, USA) were used.

2.3. DNA transfection and reporter assay

Transient transfections were performed using the calcium phosphate-mediated and Lipofectamine plus methods. For luciferase assays, HEK293 cells were plated on 24-well plates the day before transfection. For luciferase assays, cells were transfected with the indicated plasmids along with pCMV-β-Gal and lysed 36 h after transfection. Luciferase activities were measured using Luciferase Reporter Assay Kit (Promega) and normalized to the corresponding β-gal activities for transfection efficiency.

2.4. Immunoblotting

Twenty four hours after transfection, HEK293 cells were lysed in ice-cold lysis buffer [25 mM Hepes (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 250 M Hepes (pH μg/ml leupeptin, and 10 μg/ml aprotinin]. Lysates were cleared by centrifugation and the supernatants were subjected to immunoblotting. Proteins were resolved by SDS–PAGE and transferred to PVDF membranes. Proteins were visualized using appropriate primary antibodies, horseradish peroxidase-coupled secondary antibodies and chemiluminescent Western blotting reagent (Amersham Bioscience).

2.5. Alkaline phosphatase (ALP) staining

C2C12 cells fixed in 4% paraformaldehyde for 10 min at room temperature, were washed several times with PBS and stained with NBT/BCIP solution (Sigma) for 15 min at room temperature. The ALP-positive cells were stained blue/purple.

2.6. Statistical analysis

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 $p < 0.05$ indicating significance.

3. Results

3.1. Risedronate stimulates the activity of osteogenic transcriptional factors

We first analyzed the effect of risedronate on the transcriptional activity of OSE-luciferase, which contains Runx2 binding sites, and of OC and BSP-luciferase, known markers of osteoblast differentiation. Risedronate increased the activity of OSE, OC and BSP-luciferase in a dose-dependent manner. In the three promoter assays, each promoter activity reached the maximum level at 50 μM risedronate (Fig. 1A–C).

3.2. Risedronate enhances osteoblast differentiation in vitro

To evaluate the effect of risedronate on osteoblast differentiation, we performed alkaline phosphatase (ALP) staining. ALP is an osteoblast-specific marker that is induced in early stages of osteoblast differentiation. C2C12 cells were plated into 24-well plates and treated with or without BMP2 (5 ng/ml). Risedronate dose-dependently increased ALP activity in C2C12 cells, and it further induced enhanced ALP staining in the presence of BMP-2 (Fig. 2), consistent with the notion that risedronate has a positive action on osteoblast differentiation.

3.3. Risedronate and osteogenic transcription factors enhance Cx43 gene transcription and protein expression

We then examined whether Runx2, Osterix and Dlx5, and risedronate regulate Cx43 gene transcription. As shown in Fig. 3, connexin43 promoter activity was upregulated by co-transfection of increasing concentrations of a Runx2-expressing construct in C2C12 cells (Fig. 3A). A similar effect was obtained with an Osterix construct, although Cx43-luc induction was significant at the highest concentration of the expression plasmid (Fig. 3B). A dose-dependent stimulatory effect on Cx43-luc was also observed with Dlx5 (Fig. 3C). Importantly, risedronate also increased Cx43 promoter activity (Fig. 3D). In cells transfected with Runx2 (0.5 μg plasmid), risedronate increased Cx43 promoter activity dose-dependently, up to 25 μM risedronate (Fig. 3E). A similar interaction was also observed between Dlx5 and risedronate. In the presence of 0.5 μg of Dlx5, Cx43 promoter activities increases in a bell-shape curve with the highest level at 10 μM risedronate (Fig. 3F). Thus, Cx43 transcription is stimulated by osteogenic transcription factors, and risedronate enhances this effect. Consistent with the promoter-reporter results, Western blot analysis confirmed that risedronate increase the abundance of Cx43 protein, dose-dependently (Fig. 4A). Expression of Osterix, Dlx5 and Runx2 was modestly, it at all affected by risedronate (Fig. 4B).

4. Discussion

Risedronate is a nitrogen-containing bisphosphonate that has been approved by FDA to prevent and treat osteoporosis, and to

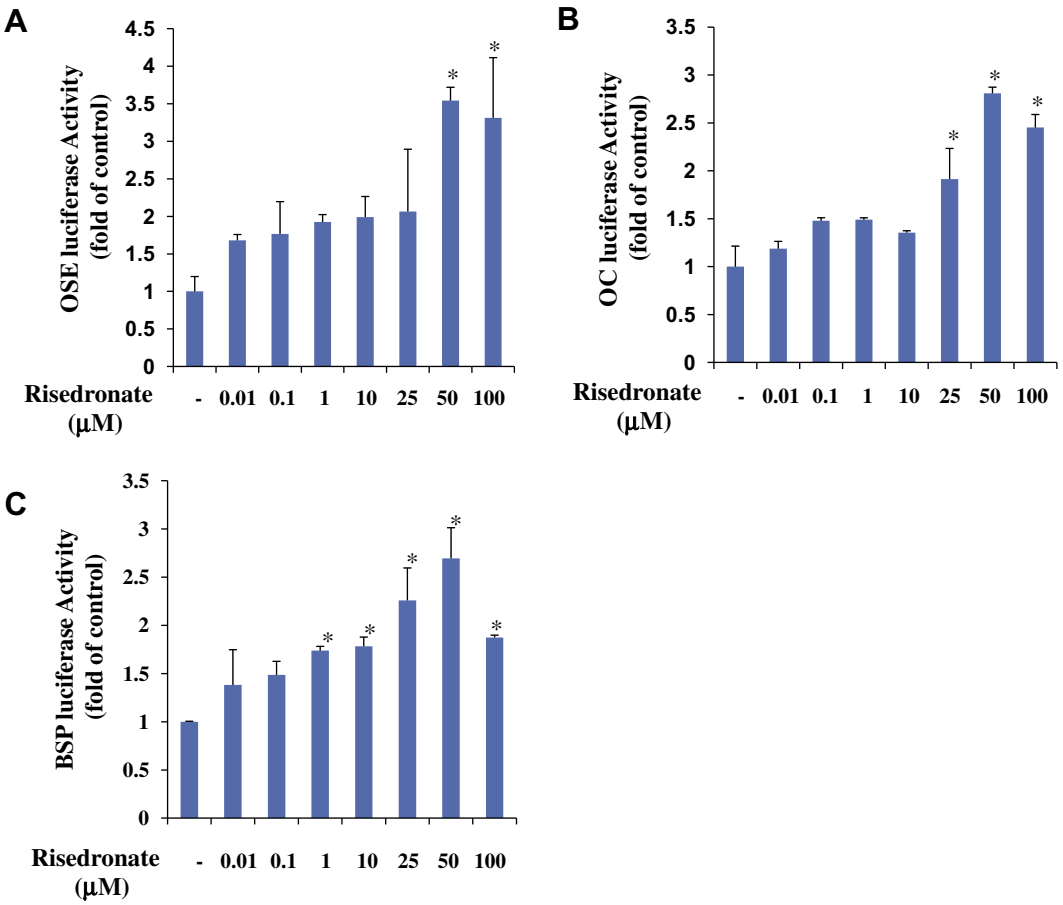

Fig. 1. Risedronate enhances the activity of osteogenic transcriptional factors. C2C12 cells were transfected with OC-, BSP-, or OSE-Luc plasmid, and a luciferase reporter assay was performed 48 h later. Each bar represents three independent experiments. *$p < 0.05$ versus control.

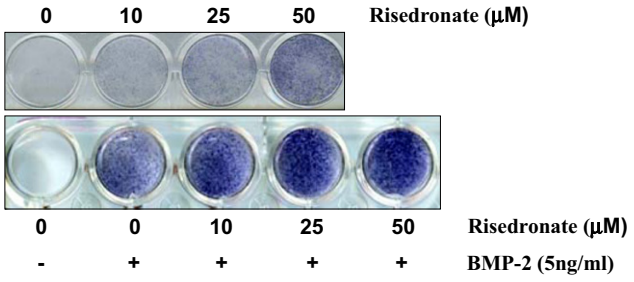

Fig. 2. Risedronate stimulates osteoblast differentiation in C2C12 cells. Cells were incubated until sub-confluent and then cultured in the presence of Risedronate with/without BMP2 (5 ng/ml). On day four, the cells were subjected to ALP staining.

reduce the risk of fracture. Bisphosphonates prevent bone loss primarily by promoting osteoclast apoptosis, while the contribution of inhibition of osteoblast apoptosis is still controversial. Some studies have demonstrated that nitrogen-containing bisphosphonate effects on osteoclasts are related to interference with cell differentiation and survival [25,26], and inhibition of farnesyl pyrophosphate synthase, which is responsible for the isoprenylation of small GTPases in the mevalonate pathway. Small GTPases are important for morphology, cytoskeletal arrangement, vesicular trafficking and membrane ruffling of osteoclasts, which control bone resorption [3]. On the other hand, bisphosphonates have been shown to increase proliferation, differentiation and mineralization in an osteoblastic lineage and an *in vivo* model [27,28]. Some studies, though not all, have shown an anti-apoptotic effect of bisphosphonates in osteoblasts and osteocytes, an effect possibly mediated by Cx43 hemichannel opening, leading to activation of Src/ERK kinase [7,9,29].

This study demonstrates that risedronate enhances *in vitro* osteoblastic differentiation and the osteogenic effect of three major transcription factors, Runx2, Osterix and Dlx5. Consistently, risedronate also stimulates Runx2 transcriptional activity, as demonstrated by a positive effect on OSE-luc, as well as osteocalcin and BSP promoter activity. Coupled with the enhancement of ALP activity in cell cultures, these studies demonstrate that risedronate has direct stimulatory effects on the C2C12 cells. The fact that risedronate did not appreciably alter Runx2 (and Osterix or Dlx5) protein expression is not surprising, as Runx2 is primarily regulated by phosphorylation in committed osteoblasts. More to the point, our study demonstrates that risedronate upregulates Cx43 transcription and protein expression. Based on the established function of Cx43 in osteoblast differentiation and function [22,30], this result further underlines a positive role of risedronate on bone forming cells. Cx43 expression and function is upregulated by other pro-osteogenic factors, such as prostaglandin E2, parathyroid hormone and bone morphogenetic protein-2 [24,31]; and is also modulated by PI3K/AKT [32]. Furthermore, Runx2, Osterix and Dlx5, critical transcription factors for osteoblastic differentiation, also stimulate Cx43 transcription.

In this study, we tested only risedronate; and the results cannot be necessarily extrapolated to other aminobisphosphonates. Furthermore, despite favorable effects of bisphosphonate on osteoblasts and osteocytes via Cx43-mediated anti-apoptosis [5,7,9,11], bisphosphonate treatment does not affect bone formation in Cx43

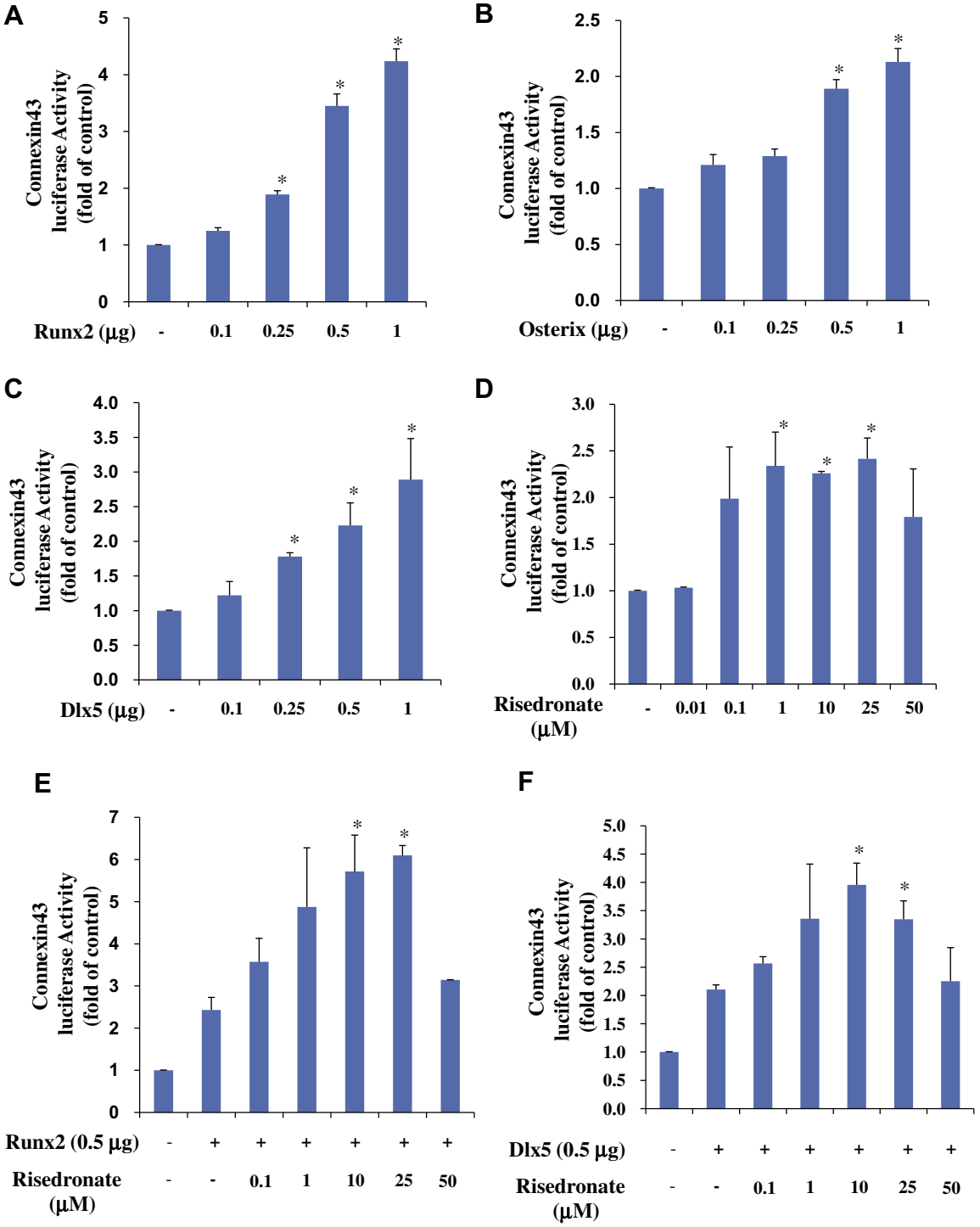

Fig. 3. Risedronate and osteogenic transcription factors regulate the expression of connexin43 at the transcriptional level. (A–C) C2C12 cells were transfected with an expression plasmid for HA-tagged Runx2 or Osterix or Dlx5 and with connexin43-Luc plasmid, and a luciferase reporter assay was performed 48 h later. Each bar represents three independent experiments. $^{*}p < 0.05$ versus control. (D) C2C12 cells were transfected with an expression plasmid with connexin43-Luc plasmid and then treated with Risedronate. Luciferase reporter assay was performed 48 h later. Each bar represents three independent experiments. $^{*}p < 0.05$ versus control. (E–F) C2C12 cells were transfected with an expression plasmid for HA-tagged Runx2 or Osterix or Dlx5 and with connexin43-Luc plasmid and then treated with Risedronate. Luciferase reporter assay was performed 48 h later. Risedronate stimulated the transcriptional activity of Runx2, Osterix, and Dlx5 at the connexin43- promoters. Each bar shows three independent experiments. $^{*}p < 0.05$ versus each transcription factor alone.

deficient mice, nor does Cx43 deficiency alter the pharmacologic action of alendronate or risedronate on bone mass and strength in ovariectomized mice [12], or after glucocorticoid treatment [7]. However, since most of the effect of bisphosphonates is mediated by their inhibitory activity on bone resorption, a contribution of a direct effect on bone forming cells cannot be ruled out.

Our data are compatible with the hypothesis that risedronate upregulates Cx43 expression as a manifestation of its stimulatory activity on osteoblast differentiation. It would be important to determine the molecular mechanism by which risedronate regulates the Cx43 gene transcription, and the contribution of Runx2 and the other osteogenic transcription factors to this effect.

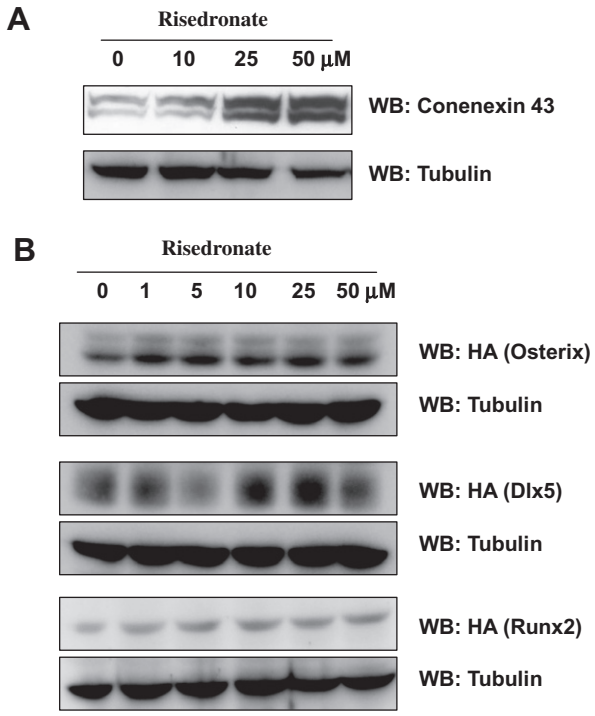

Fig. 4. Risedronate enhances the protein expression of Connexin43 and osteogenic transcription factors. (A) Cells were incubated until sub-confluent and then cultured in the presence of Risedronate. (B) C2C12 cells were transfected with an expression plasmid for HA-tagged Runx2 or Osterix or Dlx5 and then treated with Risedronate at various concentrations. The expression level of Runx2 or Osterix or Dlx5 (upper panel) was determined by Western blot analysis using anti-HA antibody. Tubulin (lower panel) was used as a loading control. Each blot shown is representative of three independent experiments giving similar results.

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