TPC1 and TPC2 Promote Osteoclastogenesis

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Abstract: Osteoclast differentiation is one of the key steps that regulate bone mass and involves RANKL-induced changes in the intracellular Ca\(^{2+}\) levels. Previously, we reported the function of a lysosomal Ca\(^{2+}\) channel, two-pore channel (TPC) subtype 2 (TPC2), using TPC2-knockout RAW264.7 cell line (RAW) during osteoclastogenesis. However, the effects of overexpressed TPC2 have not been examined because of the relatively low lipid-based efficiency transfection in RAW. In this study, TPC2 was transfected in RAW and RAW-derived mature osteoclast-like cells using an improved lipid-based transfection method. TPC2 was predominantly localized in the ruffled border-like structure of the osteoclasts. Moreover, overexpression of TPC2 promoted osteoclast differentiation. In addition, expression levels of TPC1 were measured, and TPC1-expressing vectors were transfected into RAW to investigate the role of TPC1 in osteoclastogenesis. Osteoclast differentiation was promoted in TPC1-transfected RAW. Notably, TPC2 expression was not influenced by TPC1 overexpression. Furthermore, TPC1 was knocked down by siRNA, resulting in reduced TRAP activity. TRAP is a biochemical indicator of osteoclastogenesis. Our findings suggest that TPC1 and TPC2 have independent essential roles in the differentiation of osteoclasts.

Key words: Osteoclast, TPC1, TPC2

Introduction

Osteoclasts are derived from hematopoietic precursors that undergo multiple differentiation steps shared with those in the monocyte-macrophage lineage1-3. The osteoclast precursors differentiate based on the receptor activator of NF-κB (RANK)/RANK ligand (RANKL) signaling pathway of committed preosteoclasts1. RANK cooperates with immunoreceptor tyrosine-based activation motif adaptors to activate phospholipase C gamma, which promotes the release of intracellular Ca\(^{2+}\) from the endoplasmic reticulum (ER)4. With regard to osteoclast molecules associated with the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_\text{i}\)) pathway, we have previously reported the function of two-pore channel (TPC) subtype 2 (TPC2), a lysosomal Ca\(^{2+}\) channel, in osteoclastogenesis5,6.

Two subtypes of TPC, subtype 1 (TPC1) and TPC2, have been cloned as translation products of different genes in mammals. TPC1 localizes predominantly in early- and late-endosomes, while TPC2 is a specific lysosomal calcium channel that is not localized in endosomes, ER, Golgi, and mitochondria7-9. TPC2 belongs to the family of Ca\(^{2+}\)-permeable channels that are activated by nicotinic acid adenine dinucleotide phosphate (NAADP), a potent Ca\(^{2+}\)-mobilizing messenger10. NAADP promotes Ca\(^{2+}\) release from the lysosome through TPC2, leading to further Ca\(^{2+}\) release from the ER through inositol triphosphate receptors or ryanodine receptors11. In relation to osteoclastic studies, we previously demonstrated that, in the RANK/RANKL pathway, TPC2 promotes osteoclast differentiation through changes in [Ca\(^{2+}\)]\(_\text{i}\), and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) localization6,12. However, overexpression of TPC2 has not been investigated because of the low transfection efficiency in pre-osteoclast-like cells. Moreover, although the effects of TPC2 on osteoclastogenesis have been examined, the role of TPC1 remains unclear in bone biology.

Here, we demonstrated that TPC2 localizes in a site-specific manner in mature osteoclast-like cells, and overexpressed TPC2 promotes osteoclast differentiation. Furthermore, when we overexpressed and knocked down TPC1, we found that it is a regulator of osteoclast differentiation.

Materials and Methods

Cell culture and animals

RAW 264.7 mouse osteoclast precursor-like cells (RAW; American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle medium (GE Healthcare Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. All cells were maintained at 37°C and 5% CO\(_2\) in a humidified atmosphere. All chemicals used were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise specified. For osteoclast differentiation, cells were seeded at 2.0 × 10\(^5\) cells/cm\(^2\) in the presence of 50 ng/ml soluble RANKL (Oriental Yeast, Tokyo, Japan).
A total of 2 (4-week-old) male C57BL/6J mice (Charles River Japan, Yokohama, Japan) were sacrificed by using anesthesia overdose. Mouse kidneys were immediately excised for extraction of total RNA. All studies involving animals were performed in accordance with the procedures approved by the Tokyo Medical and Dental University and Osaka Dental University Animal Care and Use Committees (42101, 18-01001).

Measurement of tartrate-resistant acid phosphate (TRAP) activity
TRAP activity was measured as described previously5,6). Briefly, cells were lysed in extraction buffer (150 mM NaCl, 50 mM Tris, 1% Nonidet P-40, pH 8.0) supplemented with protease inhibitors. A 20 μl aliquot of the lysate was then added to 200 μl of TRAP buffer (50 mM Na-acetate, 25 mM Na-tartrate, 0.4 mM MnCl₂, 0.4% N,N-dimethylformamide, 0.2 mg/ml fast red violet, 0.5 mg/ml naphthol AS-MX phosphate, pH 5.0). After incubation for 2 h at 37°C, the absorbance was measured at 540 nm using SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data were normalized to the total protein content determined using a bicinchoninic acid kit.

Gene overexpression and knockdown
Plasmids expressing the fusion protein TPC2-EGFP (Addgene, Watertown, MA, USA) were used for overexpression, as previously described10,11). Plasmid constructs expressing TPC2 and TPC1 were generated in the mammalian expression vector pCl-neo (Promega Japan, Tokyo, Japan). The pCl-neo without a DNA insert was used as a control. RAWs were transfected with expression plasmids using a lipid-based transfection reagent Lipofectamine 2000 (Thermo Fisher Scientific K.K., Tokyo, Japan), according to the manufacturer’s instructions. In addition to the lipofectamine-plasmid DNA complex, 10 μg/ml KALA peptide (Bachem, Torrance, CA, USA) was added to the cell culture medium to improve transfection efficiency10). TPC1- and control-siRNAs were purchased from Thermo Scientific K.K. for knockdown experiments. The siRNA was transfected into RAW cells using Lipofectamin RNAi-MAX (Thermo Fisher Scientific K.K.) according to the manufacturer’s protocols. Total RNA was extracted 24 h after transfection. Fluorescence images were obtained 72 h after transfection using fluorescence microscopy (All in One Microscopy, Keyence, Osaka, Japan).

Figure 1. Overexpression of TPC2 promotes osteoclast differentiation. (A–C) Representative images of TPC2-EGFP (A, C) and phase-contrast (B) at 72 h after transfection (Bar = 20 μm). (A–B) TPC2-EGFP predominantly localized in the ruffled border-like structure characterized by finger-like individual filopodia (white circle) in RAW-derived mature osteoclasts. (C) Enlarged images of TPC2-EGFP in RAW (white line). Dot-like fluorescence localized at the periphery of the cell but not at the center. (Bar = 20 μm). (D) Expression levels of TPC2 in RAW at 24 h after transfection. (E) TRAP activities in TPC2 or control plasmid vector-expressed RAW in the presence (+) or absence (−) of RANKL at 3 days after addition of RANKL. All values are represented as mean ± SEM (n = 5); *p <0.05. Control: transfection of control plasmid vector; TPC2: transfection of TPC2-expressing plasmid vector.
Figure 2. TPC1 regulates RANKL-induced osteoclastogenesis. (A, B) Expression levels of (A) TPC1 and (B) TPC2 in RAW and mice kidney. RANKL was added for 5 days. (C) Expression levels of TPC1 in TPC2 or control vector-expressed RAW in the presence or absence of RANKL. Control: transfection of control plasmid vector; TPC2: transfection of TPC2-expressing plasmid vector. (D) Expression levels of TPC1 in RAW at 24 h after transfection. (E) TRAP activities in TPC1-transfected or control vector-transfected RAW in the presence (+) or absence (−) of RANKL at 3 days after addition of RANKL. (F) Expression levels of TPC2 in TPC1 or control vector-expressed RAW in the presence (+) or absence (−) of RANKL. Control: transfection of control plasmid vector; TPC1: transfection of TPC1-expressing plasmid vector. (G) TPC1-knockdown by siRNA. TPC1 expression was reduced by siRNA in RAW at 24 h after transfection of control (Control) or TPC1-siRNA (TPC1). (H) TRAP activities in control (Control) or TPC1-siRNA (TPC1) transfected RAW at 3 days after addition of RANKL. All values are represented as mean ± SEM (n = 5); *p < 0.05.
Gene expression assays

Gene expression was measured as described previously. Total RNA was extracted from RAW and mouse kidneys using an RNeasy Kit (Qiagen KK, Tokyo, Japan) according to the manufacturer’s instructions. First-strand cDNA was produced from total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, San Francisco, CA, USA). Quantitative real-time PCR was performed in a Step One Real-Time PCR System (Applied Biosystems) using SYBR Green and specific forward and reverse primers. Transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels. The forward and reverse primer sequences, respectively, were as follows: TPC1, 5′-catgaactacagccgaaga-3′ and 5′-ectgcetctcaagataaaag-3′; TPC2, 5′-gaaccggactcagatgcaca-3′ and 5′-tctgcttgctctcaagat-3′; GAPDH, 5′-agaaggtggtgaagacgcgcat-3′ and 5′-cgaaaggtaaggtgagttg-3′.

Statistical analysis

Data were expressed as the mean and standard error of the mean (SEM) and analyzed using the Student’s t-test or analysis of variance. Tukey’s honestly significant difference test was applied as a post-hoc test. The level of statistical significance was set at p < 0.05. All analyses were performed using SPSS software (SPSS Japan, Tokyo, Japan).

Results

TPC2 promotes osteoclastogenesis in RAW.

TPC2 is a lysosomal membrane protein that predominantly localizes in an acidic lysosome. To confirm the localization of TPC2 in RAW and RAW-derived mature osteoclast-like cells, TPC2-EGFP was transfected with KALA peptide, which increases the transfection efficiency. We hypothesized that TPC2 localizes around the ruffled border of osteoclasts where the acidic lysosome gathers and fuses to the plasma membrane. Site-specific localization was observed using TPC2-EGFP (Fig. 1A, B). The TPC2-EGFP signal was predominantly localized at one end of RAW-derived mature osteoclast-like cells (Fig. 1A), which is a ruffled border-like structure characterized by a number of individual filopodia, but not on the opposite side of the cells. Furthermore, a dot-like EGFP signal was observed at the periphery of RAW cells (Fig. 1C). These results suggest that TPC2 localizes acidic lysosomes in RAW and RAW-derived mature osteoclasts, similar to other cells.

In our previous studies, deletion of TPC2 inhibited osteoclast differentiation under normal conditions, although the effects of overexpression of TPC2 have not been examined. Thus, to investigate the effects of overexpressed TPC2 on osteoclast differentiation, TPC2 was transfected in RAW. At 24 h after TPC2 gene transfection, increased expression levels of TPC2 were observed compared with that in the control (Fig. 1D). There was no significant difference in TRAP activity between control and TPC2 before induction of osteoclast differentiation by RANKL (Fig. 1E). However, TRAP activity in TPC2-overexpressed RAW cells was significantly higher than that in control plasmid vector-expressing RAW after the addition of RANKL. These results indicate that increased expression of TPC2 promotes osteoclastogenesis.

TPC1 regulates osteoclast differentiation in RAW.

There are two subtypes of two pore channels, TPC1 and TPC2, and both channels regulate intracellular Ca²⁺ levels. If TPC1 is expressed in osteoclasts, TPC1 will affect osteoclastogenesis, similar to TPC2. To investigate the effects of TPC1 on osteoclastogenesis, the expression levels of TPC1 were measured in RAW and RAW-derived osteoclast-like cells (Fig. 2A). The expression levels of TPC1 in RAW were similar to those in the kidney, where the expression levels of both TPC1 and TPC2 are high. Similar to our previous reports, the expression levels of TPC2 were increased by the addition of RANKL (Fig. 2B), while that of TPC1 showed no difference (Fig. 2A). To exclude the possibility that TPC2 overexpression affect TPC1 expression, gene expression analysis was performed. The expression levels of TPC1 did not differ between control- and TPC2-transfected RAW with or without RANKL (Fig. 2C). Next, to investigate the effects of TPC1 on osteoclast differentiation, TPC1 was transfected in RAW. The expression level of TPC1 increased in comparison to that in the control (Fig. 2D). Therefore, TRAP activity was measured in TPC1-transfected RAW. Before the addition of RANKL, TRAP activities did not differ between control and TPC1-transfected RAW (Fig. 2E). After the addition of RANKL, TRAP activity in TPC1-transfected RAW cells was significantly higher than that of control-transfected RAW (Fig. 2E). To confirm the relationships between TPC1 and TPC2, gene expression analysis was performed again. No difference was detected in expression levels of TPC2 between control-transfected and TPC1-transfected RAW with and without RANKL (Fig. 2F). Next, we knocked down TPC1 by siRNA. Transfection of TPC1-siRNA decreased TPC1 expression levels in comparison to control siRNA transfected RAW (Fig. 2G). Knockdown of TPC1 decreased TRAP activity (Fig. 2H) at three days after addition of RANKL. These results indicate that TPC1 promoted osteoclast differentiation similar to TPC2.

Discussion

Our study demonstrates that both TPC1 and TPC2 regulates osteoclastogenesis in RAW. TPC2-deletion inhibited osteoclastogenesis under normal conditions, while the overexpression of TPC2 was not investigated. Using by improved transfection methods, TPC2-transfected RAW exhibited the increased TRAP activities which is an index of osteoclastogenesis. Further we confirmed that the other TPC subtypes, TPC1 was expressed and played a key role in osteoclast differentiation independent of TPC2 expression. Our identification of both TPCs adds a novel contributor for osteoclastogenesis.

TPC2 is a lysosomal Ca²⁺ permeable ion channel that regulates osteoclast differentiation. We showed that knockout of TPC2 inhibited RANKL-induced osteoclastogenesis. However, the effects of overexpressed TPC2 have not been examined because of the low transfection efficiency in RAW. In this study, we overexpressed TPC2 using the improved lipid-based transfection method. Lysosomal TPC2-EGFP localized around the ruffled border-like structure indicating that TPC2 may play a role for acid release in mature osteoclasts. Notably, the TPC2-EGFP signal was hardly observed at the plasma membrane, although the osteoclastic lysosome fused to the membrane to release acid.

Further studies are required to address this phenomenon.

TPC1 is a member of the TPC family and localizes in early- and late-endosomal compartments but not in lysosomes. This channel controls intracellular Ca²⁺ levels and has important functions in various cells such as mast cells, kidneys, and cardiomyocytes; however, the role of TPC1 in osteoclasts has not been investigated. In this study, we found that TPC1 exists in osteoclast-like cells and regulates osteoclastogenesis. Unlike TPC2 expression, expression levels of TPC1 were the same during osteoclast differentiation, suggesting that TPC1 functions before the induction of osteoclast differentiation. The mechanisms underlying these effects remain unclear. Changes in intracellular Ca²⁺ lev-
els should be examined in future studies.

In summary, our results demonstrated the effects of overexpressed TPCs on osteoclast differentiation. RANKL-induced osteoclastogenesis was not only affected by TPC2 but also by TPC1. From the results of TPC1- overexpression, knockdown and TRAP activity measurements it was observed that TPC1 promoted osteoclastogenesis. Our findings suggest that the osteoclastic function of both TPCs may be a target for the development of novel therapeutic agents for skeletal disorders in relation to osteoclasts.

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Conflict of Interest
The authors declared no conflicts of interest.

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