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The role of calcium and nicotinic acid adenine dinucleotide phosphate (NAADP) in osteoclast formation and resorption

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Abstract

Osteoclasts are specialised bone-resorbing cells which form by fusion of circulating mononuclear phagocyte precursors. Bone resorption results in the release of large amounts of calcium into the extracellular fluid (ECF) but it is not certain whether changes in extracellular calcium concentration $[Ca^{2+}]_e$ influence osteoclast formation and resorption. In this study we sought to determine the effect of $[Ca^{2+}]_e$ and NAADP, a potent calcium mobilising messenger that induces calcium uptake, on human osteoclast formation and resorption. CD14⁺ human monocytes were cultured with M-CSF and RANKL in the presence of different concentrations of calcium and NAADP and the effect on osteoclast formation and resorption evaluated. We found that the number of TRAP⁺ multinucleated and the extent of lacunar resorption was reduced when there was an increase in extracellular calcium and NAADP. This was associated with a decrease in RANK mRNA expression by CD14⁺ cells. At high concentrations (20mM) of $[Ca^{2+}]_e$ mature osteoclast resorption activity remained unaltered relative to control cultures. Our findings indicate that osteoclast formation is inhibited by a rise in $[Ca^{2+}]_e$ and that RANK expression by mononuclear phagocyte osteoclast precursors is also $[Ca^{2+}]_e$ dependent. Changes in NAADP also influence osteoclast formation, suggesting a role for this molecule in calcium handling. Osteoclasts remained capable of lacunar resorption, even at high ECF $[Ca^{2+}]_e$, in keeping with their role in physiological and pathological bone resorption.

Keywords

Calcium; Osteoclast; NAADP; RANK

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Conflict of Interest Statement

X Cheng, E Hookway, T Kashima, U Oppermann, A Galione and NA Athanasou declare that they have no conflict of interest.

Introduction

Osteoclasts and osteoclast precursors are multinucleated cells that are specialised to carry out lacunar bone resorption [1]. Osteoclasts are formed by fusion of marrow-derived mononuclear phagocyte precursors which circulate in the monocyte fraction [2]. Osteoclast formation occurs in the presence of receptor activator for nuclear factor kappaB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) [3]. Osteoclasts are unique in being exposed to and having to function in a microenvironment in which there is a very high level of calcium concentration. Dissolution of the mineral phase of bone is accomplished by a specialised area of the osteoclast membrane, the ruffled border, which is surrounded by a tight sealing zone. A large amount of calcium within the resorption lacuna is endocytosed along the osteoclast ruffled border and then transcytosed to the baso-lateral membrane area away from the bone surface from where it is released in a constant steady-state manner [4]. Intracellular organelles which act as calcium stores include the endoplasmic reticulum, Golgi apparatus and acidic lysosome-related organelles [5, 6]. Macrophages and osteoclasts are very lysosome-rich cells and nicotinic acid adenine dinucleotide phosphate (NAADP) is a universal messenger that induces calcium release from intracellular acidic lysosome-like stores as well as calcium influx in some cases [7, 8]. An NAADP-mediated signalling pathway has been reported in many mammalian cell types but it has not been established whether it plays a role in osteoclast formation and resorption.

The calcium concentration at the resorption site may be as high as 20 mM and it is not clear what effect this extremely high level of calcium has on osteoclast formation and resorption [9]. In mouse marrow cell and osteoblast co-cultures, increased extracellular fluid calcium has been shown to stimulate osteoclast differentiation [10, 11]; in contrast, in hematopoietic blast cell culture, high extracellular calcium has been shown to inhibit osteoclast formation and activity [12]. It is not certain whether changes in extracellular calcium ($[Ca^{2+}]_e$) directly influence osteoclast formation and resorption and, to our knowledge, studies to investigate human osteoclast differentiation from CD14⁺ monocyte precursors at different levels of $[Ca^{2+}]_e$ have not been carried out.

In this study we have examined the effect of extracellular fluid calcium on human osteoclast formation and resorption. We have sought to determine whether changes in $[Ca^{2+}]_e$ alter osteoclastogenesis directly and whether $[Ca^{2+}]_e$ influences RANK expression on CD14⁺ mononuclear phagocyte osteoclast precursors. In addition, we have assessed the effect of NAADP on osteoclast formation and sought to determine whether lacunar resorption by mature osteoclasts is significantly altered at high levels of $[Ca^{2+}]_e$.

Materials and Methods

Effect of extracellular calcium and NAADP on human osteoclast formation

CD14⁺ monocytes were isolated from human buffy coat blood as previously described [13]. Cell sorting into CD14⁺ cell fractions was selected from peripheral blood mononuclear cells using an AutoMACS cell separator (Miltenyl Biotech, Surrey, UK). After incubating cells with MACS CD14 Micro Beads, the suspension was passed through the *MACS* Magnetic cell separator. CD14⁺ cells were added (1×10^5 cell/well) to 96-well tissue culture plates

containing glass coverslips and dentine slices, α -MEM culture medium (Lonza, Wokingham, UK) supplemented with 10% heat-inactivated FCS, 2mmol glutamine (Lonza), 100 iu/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Paisley, UK). After two hours incubation in a humidified incubator at 37°C and 5% carbon dioxide, dentine slices and coverslips were transferred to 16mm tissue culture wells containing 1ml of medium supplemented with M-CSF (25 ng/ml) and soluble RANKL (50 ng/ml), both obtained from R&D (Abingdon, UK). All cultures were incubated for 12-14 days during which time the culture medium was replenished every 3-4 days. Negative control cultures consisted of CD14+ cells incubated as above in the absence of one or both of the above factors.

To determine the effect of calcium on osteoclast formation, CD14+ monocytes were cultured in medium containing calcium (1.8-20 mM) or NAADP (0-500 μ M) for 12-14 days. Evidence of the extent of osteoclast differentiation was assessed as previously described [14] by histochemical analysis of the expression of the osteoclast-associated enzyme Tartrate-Resistant Acid Phosphatase (TRAP).

In addition, functional evidence of osteoclast formation was provided by demonstration of lacunar resorption pit formation on dentine slices as previously described [13]. The extent of lacunar resorption was measured by determining surface area resorption by image analysis.

Effect of extracellular calcium and NAADP on osteoclast resorption activity

Functional mature human osteoclasts were formed from peripheral blood by culturing CD14+ monocytes on hydrophobic dishes (Corning, UK) with M-CSF (25 ng/ml) and RANKL (50 ng/ml) for 19 days until osteoclasts were fully differentiated as previously described [15]. The hydrophobic surface prevents mononuclear osteoclast precursors and mature osteoclasts attaching to the dish surface; this allows collection of differentiated mature osteoclasts without causing damage to the osteoclast plasma membrane. Osteoclasts formed in this way were then seeded onto coverslips or dentine slices and cultured in medium containing calcium (0-20mM) for 1,3 or 5 days. Trypan blue uptake was used to identify osteoclasts with compromised membrane integrity. The number of TRAP+ cells and the number of Trypan blue-positive cells was counted using ImageJ. The effect of calcium (1.8-20 mM) and NAADP (0-500 μ M) on the resorbing activity of osteoclasts cultured on dentine slices was analysed using a lacunar resorption assay as described above.

Effects of extracellular calcium on RANK mRNA expression in CD14+ PBMC

CD14+ peripheral blood monocytes were isolated from fresh blood from three healthy human donors as described above. 1.5×10^5 cells/well were added to a 48 well plate in media containing varying concentrations of calcium (1.8mM-20mM). After one hour, 25ng/ml M-CSF was added and cells were cultured for 24 hours. RNA was extracted using the Direct-zol kit (Zymo Research) according to the manufacturer's instructions including on-column DNA digest, quantified using a Nanodrop1000 and up to 2 μ g reverse transcribed. qPCR was performed using 5 μ l Fast SYBR Green Master Mix 2 μ l of cDNA diluted 1:10 and primers at a concentration of 225nM in a final volume of 10 μ l. qPCR was performed on a Viia7 system (Applied Biosciences) using fast conditions (one cycle of 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds) with three replicates of each

condition. Relative expression of RANK was calculated using the CT method using beta-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-2-microglobulin (B2M) as reference genes and normalised to the sample cultured in media with 1.8mM Ca^{2+} and M-CSF.

The following primers were used: RANK forward 5'

CACCAAATGAACCCCATGTTTAC3', RANK reverse 5'

GGACTCCTTATCTCCACTTAGGC 3', ACTB forward 5'

GGATGCAGAAGGAGATCACTG 3' ACTB reverse 5'

CGATCCACACGGAGTACTTG 3', GAPDH forward 5'

GAAGGTGAAGGTCGGAGTC 3', GAPDH reverse 5'

GAAGATGGTGATGGGATTTC 3'

Statistical analysis

Statistical analysis was performed in GraphPad Prism version 5. Different treatment groups were compared with each other using a one-way ANOVA, not assuming a Gaussian distribution, with post-hoc testing using Dunn's multiple comparison test. Significance was designated as $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

Results

Effect of calcium and NAADP on osteoclast formation

A dose response inhibitory effect on osteoclast formation in increasing concentrations of extracellular calcium was noted in cultures of human monocytes incubated with RANKL and M-CSF. The number of TRAP⁺ multinucleated cells and the extent of lacunar resorption showed a step-wise decrease over the concentration range 1.8-20mM Ca^{2+} (Figure 1A, Figure 2). Cultures of monocytes incubated on dentine slices with RANKL and M-CSF showed a similar decrease in lacunar resorption reflecting the reduction in the number of multinucleated cells (Figure 1B). Minimal or no osteoclast formation was seen in RANKL and M-CSF-treated monocyte cultures incubated at 20mM Ca^{2+} calcium. The number of osteoclasts formed in monocyte cultures incubated at 5mM Ca^{2+} was not significantly different to that seen in control cultures.

The addition of NAADP also had an effect on the number of osteoclasts and the extent of resorption seen in monocyte cultures incubated with RANKL and M-CSF (Figure 3A, 3B). In general, at high NAADP concentration (100 and 500 μM), a decrease in osteoclast formation and resorption was noted. Osteoclast numbers and resorption were not significantly different to controls at 10mM but were decreased at higher concentrations (100 and 500 μM).

The effect of calcium and NAADP on osteoclast resorption activity

The ability of mature osteoclasts (formed from CD14⁺ cells under normocalcaemic conditions) to resorb bone, as measured by the formation of lacunar resorption pits, was not significantly altered at high calcium concentrations (Figure 4A). In addition, increasing concentrations of NAADP did not influence the function of mature osteoclasts to resorb bone (Figure 4B).

Effect of calcium on RANK expression by CD14⁺ cells

Increasing $[Ca^{2+}]_e$ reduced the expression of RANK in CD14⁺ cells stimulated with M-CSF in a dose-dependent manner, with a significant reduction seen at 10mM $[Ca^{2+}]_e$ and a highly significant reduction observed at $[Ca^{2+}]_e$ above 15mM (Figure 5).

Discussion

Osteoclasts are multinucleated cells that are specialised to carry out lacunar bone resorption. These cells are formed from CD14⁺ mononuclear phagocyte precursors that circulate in the monocyte fraction. In this study, using a cell culture system whereby human osteoclasts are formed from the CD14⁺ peripheral blood mononuclear cells (PBMCs), we found that high $[Ca^{2+}]_e$ reduces the formation of osteoclasts. RANK expression on CD14⁺ cells was also reduced, suggesting that this may represent a negative feedback mechanism on osteoclast formation. The effect of [NAADP] on osteoclast formation was similar to that of $[Ca^{2+}]_e$ suggesting that this molecule could play a role in the $[Ca^{2+}]_e$ effect on osteoclast differentiation. In contrast, we found that high $[Ca^{2+}]_e$ levels (20nM) or high [NAADP] (500mM), did not inhibit lacunar resorption by mature osteoclasts.

Calcium is the fifth most abundant inorganic element in the body. 99% of body calcium is in bone and teeth in the form of hydroxyapatite [15]. Approximately 1% of total body calcium is intracellular with 0.1% extracellular. A small component of bone calcium is rapidly exchangeable with the extracellular compartment and in this way contributes to the rapidly responsive regulation of extracellular calcium. A reduction in $[Ca^{2+}]_e$ stimulates the release of PTH which acts to enhance osteoclast formation and resorption, resulting in the liberation of calcium and phosphate from the skeleton. The osteoclast is unique in mobilising massive quantities of calcium from bone. This results in high levels of calcium at the site where osteoclasts are formed by fusion of mononuclear precursors. In this study we found that when the $[Ca^{2+}]_e$ concentration was increased, there was a decrease in human osteoclast formation from CD14⁺ monocytes which showed a decrease in RANK expression. The Ca^{2+} -NFATc1 signalling pathway is essential for osteoclast differentiation [16, 17] and there is evidence that osteoclasts express calcium-sensing ryanodine receptor (RyR). NFATc1 is activated by Ca^{2+} signalling and calcium oscillation that occurs during osteoclastogenesis induces NFATc1 through an autoamplification mechanism. RANKL induces oscillations in the intracellular concentration of calcium which plays a switch-on role in NFATc1 expression and mouse osteoclast differentiation. These oscillations are essential for osteoclast formation, particularly in the terminal differentiation of mononuclear phagocytes into osteoclasts.

Most studies on the effect of extracellular calcium on osteoclast formation have been carried out in mice. Some of these studies have shown that high extracellular calcium stimulates osteoclast-like cell formation and bone resorbing activity [10, 11]. Other studies looking at the effect of factors that influence osteoclast differentiation, such as zinc, gallium and calcium release-activated channels have shown inhibition of osteoclast differentiation in response to an increase in $[Ca^{2+}]_e$ and have noted that this is associated with changes in the Ca^{2+} -calcineurin-NFATc1 signalling pathway [18–22]. Our findings indicate that human osteoclastogenesis is also sensitive to $[Ca^{2+}]_e$ from CD14⁺ monocyte/macrophage osteoclast precursors and that this may in part be modulated through changes in RANK expression by human mononuclear phagocyte osteoclast precursors.

In contrast to the effect of extracellular calcium on osteoclast formation, we found lacunar resorption by mature osteoclasts, derived under normocalcaemic conditions, was little altered by the presence of high $[Ca^{2+}]_e$. Changes in $[Ca^{2+}]_e$ have previously been shown to influence the behaviour of osteoclastic giant cells in giant cell tumour of bone [23, 24]. The osteoclast acidifies the extracellular compartment by secreting protons across the ruffled border membrane via a vacuolar-like H^+ -ATPase proton pump [1, 23]. The protons are provided by the enzyme carbonic anhydrase type II, which generates H^+ and HCO_3^- ions from CO_2 . Intracellular pH is maintained by a chloride/bicarbonate exchanger on the anti-resorptive surface and electroneutrality is maintained by a chloride channel (CLC-7) located in the ruffled border membrane, which transports Cl^- into the resorption lacuna [1]. Ambient calcium concentration levels may be as high as 8–20 mM following bone mineral dissolution by osteoclast activity. Such extracellular calcium concentration changes alter bone resorbing activity of isolated rat osteoclasts and this is accompanied by rapid and sustained changes in cytosolic calcium concentration and cell retraction with inhibition of enzyme release and bone resorption. High calcium at sites of active osteoclast resorption have been shown in animal models to cause cell retraction, disappearance of the sealing zone and apoptosis, all of which would result in a decrease in bone resorption [7, 9]. These findings suggest the existence of long and short term feedback mechanisms on osteoclast activity, controlled by an increase in $[Ca^{2+}]_e$. However, our findings indicate that osteoclast resorption activity by mature osteoclasts was not altered by extracellular concentrations of calcium. Functional compartments for calcium mobilization in osteoclasts may enable the osteoclast to operate whilst in contact with a high $[Ca^{2+}]_e$ environment, but the precise molecular pathways and intracellular mechanisms used by osteoclasts to deal with a high calcium load have not been determined.

Our findings accord with the morphological findings in hyper-resorptive conditions, such as hyperparathyroidism, where there is a very high $[Ca^{2+}]_e$, numerous osteoclasts are seen on the bone surface. Functional compartments for calcium mobilization in osteoclasts may enable the osteoclast to operate whilst in contact with a high $[Ca^{2+}]_e$ environment, but the precise molecular pathways and intracellular mechanisms used by osteoclasts to deal with a high calcium load have not been determined.

Macrophages and osteoclasts are both lysosome-rich cells. NAADP is a universal messenger that induces calcium release from intracellular stores as well as calcium influx in some cases [7, 8]. The NAADP-mediated signalling pathway has been reported in many mammalian cell

types. NAADP is the most potent calcium mobilising messenger that has been discovered so far, with a low nanomolar concentration for activating its receptor. NAADP has been demonstrated to induce calcium release from acidic lysosome-like stores via a pathway distinct from other calcium – releasing messengers, inositol triphosphate (IP3) and cyclic ADP Ribose (cADPR). The production of NAADP is catalysed by ADP-ribosyl cyclases via a base-exchange reaction that converts the nicotinamide group of NADP to the nicotinic acid at acidic pH (pH~4.5). CD38, a homolog of ADP-ribosyl cyclase, which catalyses the synthesis of NAADP, is present on the plasma membrane [17] of both monocytes and osteoclasts [22, 23]. Moreover, the low pH in the hemivacuole provides an acidic environment that favours NAADP synthesis. It has been shown that two pore channels (TPCs), a family of cation channels, are located on lysosome-like acidic stores and that NAADP is likely to induce calcium release through TPC2 [25]. Our findings indicate that changes in the concentration of NAADP influence osteoclast formation from CD14+ mononuclear phagocytes, suggesting that it may also play a role in modulating calcium transport in these cells. It has recently been shown that two-pore channel 2 (TPC2) is expressed in osteoclast precursor cells and that breaking down TPC2 in these cells suppresses RANKL-induced key events including TRAP expression. Moreover knockdowns of TPC2 reduce the levels of RANKL-induced dynamic waving of calcium in osteoclast precursors. It is postulated that the target of TPC2 is nuclear localisation of NFATC1. TPC2 suppression also inhibited osteoclast pit formation in culture.

To complete bulk calcium transport, the high calcium solution at the osteoclast attachment site must be moved to and diluted in the extracellular space. Some calcium may be released when the osteoclast detaches but the bulk of the transport of calcium occurs through the osteoclast by vacuolar transcytosis. There is good evidence for the presence of calcium binding proteins and an epithelial calcium channel TRPV5 that support a calcium transport mechanism. The osteoclast has several other transport proteins including a Ca^{2+} -ATPase, which is expressed highly in osteoclasts. The massive calcium movement that occurs in bone resorption undoubtedly requires that the osteoclast has a mechanism for protecting cytoplasmic and organelle calcium content even if the bulk transport is vesicular. Osteoclasts are very lysosome-rich cells and are thus likely to contain NAADP receptors, although NAADP cannot cross the plasma membrane because it is highly negatively charged, it has been shown that NAADP can be taken up by some cell types [26]. It is possible that any NAADP produced in the hemivacuole would be taken up into osteoclasts, where it would play a role in calcium signalling and influence osteoclast differentiation and function.

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Fig 1. CD14⁺ monocytes incubated with RANKL and M-CSF in [Ca²⁺]_e 1.8-20mM showing: A) Osteoclast formation as measured by the number of TRAP⁺ multinucleated cells (MNCs) formed in 14 day cultures and B) The extent of lacunar resorption on dentine slices. Results were normalised to the number under the control conditions and expressed as the mean percentage ± standard error. Results represent the mean of 3 independent experiments

Fig 2. CD14+ monocytes cultures incubated with RANKL and M-CSF in (a) 1.8mM [Ca²⁺]_e, showing numerous TRAP+ MNCs and (b) 20mM [Ca²⁺]_e, showing an absence of TRAP+ MNCs. Scale bar represents 100µm

Fig 3. CD14⁺ monocytes incubated with RANKL and M-CSF in [NAADP] 0-500 μ M showing: (a) Osteoclast formation as measured by the number of TRAP⁺ multinucleated cells (MNCs) formed in 14 day cultures; and (b) The extent of lacunar resorption on dentine slices. Results were normalised to the number under the control conditions and expressed as the mean percentage $\pm$ standard error. Results represent the mean of 3 independent experiments

Fig 4.

The extent of mature osteoclast lacunar resorption at different concentrations of calcium (a) and NAADP (b). Results were normalised to the number in the control conditional and expressed as the mean percentage $\pm$ standard error

Fig 5.

Expression of RANK 24h after addition of M-CSF to CD14⁺ monocytes in varying concentrations of calcium as assessed by qPCR. Results were normalised to the control condition and expressed as the mean $\pm$ standard error. Results represent the mean of 3 independent experiments