Bisphosphonates Act Directly on the Osteoclast to Induce Caspase Cleavage of Mst1 Kinase during Apoptosis

A LINK BETWEEN INHIBITION OF THE MEVALONATE PATHWAY AND REGULATION OF AN APOPTOSIS-PROMOTING KINASE*

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Bisphosphonates (BPs) include potent inhibitors of bone resorption used to treat osteoporosis and other bone diseases. BPs directly or indirectly induce apoptosis in osteoclasts, the bone resorbing cells, and this may play a role in inhibition of bone resorption. Little is known about downstream mediators of apoptosis in osteoclasts, which are difficult to culture. Using purified osteoclasts, we examined the effects of alendronate, risedronate, pamidronate, etidronate, and clodronate on apoptosis and signaling kinases. All BPs induce caspase-dependent formation of pyknotic nuclei and cleavage of Mammalian Sterile 20-like (Mst) kinase 1 to form the active 34-kDa species associated with apoptosis. Withdrawal of serum and of macrophage colony stimulating factor, necessary for survival of purified osteoclasts, or treatment with staurosporine also induce apoptosis and caspase cleavage of Mst1. Consistent with their inhibition of the mevalonate pathway, apoptosis and cleavage of Mst1 kinase induced by alendronate, risedronate, and lavastatin, but not clodronate, are blocked by geranylgeraniol, a precursor of geranylgeranylnyl diphosphate. Together these findings suggest that BPs act directly on the osteoclast to induce apoptosis and that caspase cleavage of Mst1 kinase is part of the apoptotic pathway. For alendronate and risedronate, these events seem to be downstream of inhibition of geranylgeranylation.

Bisphosphonates (BPs) include potent inhibitors of bone resorption used for the treatment of osteoporosis, Paget’s disease, bone metastases, and other bone diseases. It is generally accepted that BPs inhibit bone resorption by acting directly or indirectly on osteoclasts, cells of hematopoietic origin. Until recently the molecular mechanism of action of BPs was not well understood. Recent pharmacological studies suggest that BPs inhibit bone resorption (10, 11). It is not known if the induction of apoptosis is the result of direct action of BPs on the osteoclast, and little is known about the biochemical pathways involved. Coxon et al. (12) have shown the induction of caspases in BP-treated J774 macrophages undergoing apoptosis. However, macrophages may not fully mimic osteoclast responses to bisphosphonates, since apoptosis in these cells is blocked by addition of either farnesyl diphosphate or geranylgeranyl diporphosphate (3) and only the latter is implicated as rate-limiting for N-BP effects on the osteoclast (4). Recent evidence suggests that Mammalian Sterile 20-like (Mst) kinase 1 is a substrate for caspase 3 in several hematopoietic cells and can induce apoptosis in mesenchymal cell lines (13–15). The caspase cleavage site of Mst1 (DEMD), situated between the amino-terminal kinase domain and carboxyl-terminal regulatory and dimerization domains, matches the consensus sequence for caspase 3. After Mst1 cleavage, the kinase domain, separated from its regulatory elements, has high catalytic activity.

In this study we investigated the effect of several BPs on apoptosis and activation of signaling kinases in purified osteoclasts. BPs induced osteoclast apoptosis and activation of the 34-kDa Mst1 kinase by up to 13-fold, while reducing the activity of Mst1 (59 kDa) and Mst2 (60 kDa) kinases by up to 50%. Slight 34-kDa Mst2 kinase activity was also observed, and two other responsive kinases of 50 and 36 kDa remain unidentified. Induction of apoptosis by withdrawal of serum and macrophage colony-stimulating factor (M-CSF) elicited kinase responses similar to those produced by BPs, while staurosporine activated both full-length Mst and 34-kDa Mst1 kinase activities without altering the 50-kDa kinase activity. The findings indicate that BPs act directly on osteoclasts to induce caspase cleavage of Mst1, as a signaling intermediate in the induction of apoptosis, and suggest that geranylgeranylation is the upstream target of ALN and RIS in inducing apoptosis.

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1 The abbreviations used are: BP, bisphosphonate; FFP, farnesyl diporphosphate; PAM, pamidronate; ALN, alendronate; Mst, Mammalian Sterile 20-like; RIS, risedronate; CL2, clodronate; GGOH, geranylgeraniol; N-BP, nitrogen-containing bisphosphonate; TRAP, tartrate-resistant acid phosphatase; Oc, osteoclast; PBS, phosphate-buffered saline; M-CSF, macrophage colony-stimulating factor; HBS, HEPES buffered solution; LOV, lovastatin; EHDP, etidronate.
EXPERIMENTAL PROCEDURES

Osteoclast (Oc) Formation—Co-cultures of murine osteoblasts and marrow cells were prepared as described by Wesolowski et al. (16) with the following modifications. Bone marrow cells were harvested from 6-week-old male Balb/C mice by flushing the marrow spaces of freshly isolated long bones (tibiae and femora) with α-minimal essential medium (Life Technologies, Inc.) containing penicillin/streptomycin (100 IU/ml and 100 μg/ml, respectively). Bone marrow cells were suspended in Oc medium: α-minimal essential medium supplemented with fetal calf serum (10% v/v; HyClone Laboratories, Logan, UT) and 10 ml 125-(OH)₂ vitamin D₃ (Biomol, Plymouth Meeting, PA). Bone marrow cells were then added to subconfluent monolayers of murine MB1.8 cells and cultured for 6–7 days at 37 °C in the presence of 5% CO₂. Co-cultures were first treated with type I collagenase (Wako Pure Chemical Industries, Ltd., Japan) at a concentration of 1 mg/ml in phosphate-buffered saline (PBS) for 1 h at 37 °C. Suspended osteoblasts were gently aspirated, leaving a mixture enriched in pre-osteoclasts and the remaining MB1.8 osteoblasts. All cells were released with EDTA (0.2 g/liter in PBS) for 20 min at 37 °C and then re-plated in Oc medium and cultured for an additional 3 days.

Oc Apoptosis Assay—Oc-forming cultures, generated as above in 24 well plates, were treated with type I collagenase (Sigma) in PBS to remove all osteoblasts, followed by EDTA to remove pre-osteoclasts. Ocs were then maintained in Oc medium supplemented with M-CSF (R&D Systems, Minneapolis, MN) at 5 ng/ml. Ocs were treated with indicated compounds in the Oc medium for 18 h and stained for tartrate-resistant acid phosphatase (TRAP) and with Hoechst nuclear (no. 33342; Sigma) stain as follows. Cells were fixed with 10% formaldehyde (48 °Case inhibitor mixture (Sigma): 4-(2-aminoethyl)-benzenesulfonyl fluoride and instead supplemented with a protease inhibitor mixture (Sigma) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM aprotinin, 1 mM leupeptin, 1 mM N-acetyl-L-cysteine, 1 mM EDTA, 10 μg/ml leupeptin, 1 mM pepstatin A, 1 mM antipain, 1 mM bestatin, 1 mM thiorphan, 100 μM Pepstatin A, and 20 μM peptatin A. Gels were then washed twice with Kinase Buffer (20 mM HEPES, pH 7.6, 5 mM MgCl₂, 10 mM β-glycerophosphate (Sigma), 1 mM NaCl, and 1 mM EGTA) and washed three times with PBS to remove all osteoblasts, followed by EDTA to remove osteoclasts. Ocs were then stained with Fast Red Violet LB (Sigma) dissolved in TRAP buffer (sodium tartrate 100 mM, sodium phosphate 50 mM, pH 5.0) for 10 min at 37 °C. After TRAP staining, nuclear staining was performed with 5 naphthol AS-MX phosphate (100 mg/ml, pH 5.0) for 10 min at 37 °C. TRAP staining was performed as described under “Experimental Procedures,” and apoptosis in these cells was scored based on the presence of pyknotic nuclei. This method correlated with TUNEL staining in this system (data not shown). Base-line apoptosis in untreated Ocs maintained for 18 h was ~9%. Typically, nuclei were deployed in ringlike structures (Fig. 1A).

RESULTS

Alendronate and Risedronate Induction of Osteoclast Apoptosis Is Blocked by Geranylgeraniol and Caspase Inhibitors—N-BPs were shown to disrupt the cytoskeleton, abolish the ruffled border, and induce apoptosis in osteoclasts and macrophages (3, 10, 19). We found that GGOH prevents alendronate inhibition of osteoclastic bone resorption (4), suggesting that geranylgeranyl diphosphate, an isoprenylation precursor derived from the mevalonate pathway or from GGOH, is rate-limiting for osteoclast activity. Geranylgeranylated G-proteins regulate cytoskeletal organization, vesicular trafficking, and apoptosis (20, 21). Focusing on the latter, we examined the effects of GGOH and of the caspase inhibitor Z-VAD-FMK on alendronate-, risedronate-, and EHDP-induced apoptosis in purified Ocs. Ocs were identified by TRAP staining as described under “Experimental Procedures,” and apoptosis in these cells was scored based on the presence of pyknotic nuclei. This method correlated with TUNEL staining in this system (data not shown). Base-line apoptosis in untreated Ocs maintained for 18 h was ~9%. Typically, nuclei were deployed in ringlike structures (Fig. 1A).

Treatment with ALN (30 μM) for 18 h increased the number of cells with nuclear condensation (apoptosis) by 3-fold (Fig. 1, B and E). RIS (30 μM) produced similar effects (Fig. 1E). Both GGOH (Fig. 1, C and E) and Z-VAD-FMK (Fig. 1, D and E) prevented ALN- or RIS-induced apoptosis, reducing the number of cells with condensed nuclei to control levels. However, while GGOH prevented both nuclear condensation and disappearance of the ringlike structure in N-BP-treated Ocs, Z-VAD-FMK-treated cells showed normal nuclear morphology without the ringlike arrangement. Instead, these nuclei were clustered in one or more regions of the osteoclast. Apoptosis induced by CL2 and EHDP was also suppressed by Z-VAD-FMK (Fig. 1E) but was not affected by GGOH. Apoptosis induced by the withdrawal of M-CSF was also standard procedures (Fig. 1G).

Bisphosphonates Induce Kinase Signaling during BP-induced Apoptosis in Purified Osteoclasts—Several geranylgeranylated proteins, including members of the Ras family of small GTPases, activate signaling pathways and control apoptosis through their regulation of protein kinases. We assessed kinase activity in purified murine Oc-like cells obtained as described under “Experimental Procedures.” In-gel kinase assays of Oc lysates (Fig. 2) showed that treatment with ALN (panel A),
PAM (panel B), RIS (panel C), or CL2 (panel D) for 12–20 h increased the activity of 34- and 36-kDa kinases 5–13-fold when compared with untreated controls (0 h) or to osteoclasts treated for 1–2 h, respectively. After 16 h, 50-, 59-, and 60-kDa kinases showed decreases in activity of up to 50% (most notable in Figs. 2D and 3). ALN and RIS triggered these kinase responses at 10–100 μM. At 30 μM, RIS (Fig. 2C) elicited a slightly higher activation of the 34- and 36-kDa kinases than ALN (Fig. 2A). PAM had no effect at 10 or 30 μM, was slightly effective at 60 μM (data not shown), while 100 μM PAM (Fig. 2B) elicited about the same response as 30 μM ALN (Fig. 2A) or RIS (Fig. 2C). However, at 100 μM PAM-treated cultures also showed the accumulation of debris in the medium, which was not observed with ALN or RIS. Treatment with EHDP (300 μM; Fig. 2E) for 2–24 h activated the 34-kDa kinase less than 2-fold and there was no effect on kinases migrating at 36, 50, 59, or 60 kDa. In many experiments, EHDP elicited no response at all (data not shown). Tiludronate up to 300 μM did not significantly alter kinase activities (data not shown).

The Oc cultures used in these experiments were 95% pure by cell count, suggesting that the BPs act directly on the osteoclast. Since Ocs in these populations are large and multinucleated, they may contribute >99% of the protein and kinase.
Bisphosphonates Induce Caspase Cleavage of Mst Kinase

Several studies have recently described an apoptosis response, associated with caspase cleavage of the 59-kDa Mst1 and Mst2 kinases to form catalytically active species migrating at 34–36 kDa (13–15). Caspase cleavage of Mst1 occurs at the DEMD sequence (residues 323–326), while Mst2 is cleaved at DELD (residues 319–322). The catalytically active 34-kDa kinase domain is readily immunoprecipitated using antibodies directed only to the Mst amino terminus (13). Anti-Mst1 amino-terminal antibody (Fig. 3, lanes 3 (control) and 4 (ALN)) immunoprecipitated the 34-kDa kinase activated by the BPs in the osteoclast, while anti-Mst2 was much less effective (lanes 5 and 6). This suggested that the 34-kDa kinase activated by BPs was predominantly the amino-terminal fragment of Mst1. Antibodies directed to the non-catalytic carboxyl-terminal domain of Mst1 and Mst2 immunoprecipitated only the full-length Mst1 and Mst2 isoforms from both untreated (lane 7) and ALN-treated (lane 8) osteoclast lysates.

To determine whether the 34-kDa Mst1 kinase was generated as a result of caspase activities, purified Ocs were treated with ALN (Fig. 4, lanes 4–6), RIS (lanes 7–9), or CL2 (lanes 10–12) in the absence or presence of Z-VAD-FMK (lanes 2, 5, 8, and 11) or Z-DEVD-FMK (lanes 6, 9, and 12). Kinase activity of 34-kDa Mst1 was reduced by either caspase inhibitors block Mst1 kinase cleavage induced by bisphosphonates. Ocs were prepared and analyzed for in-gel kinase activity as described in Fig. 2. Osteoclasts were not treated with bisphosphonate (lanes 1–3) or treated with 30 μM ALN (lanes 4–6), 30 μM RIS (lanes 7–9), and 100 μM CL2 (lanes 10–12) for 20 h in the absence or presence of Z-VAD-FMK (lanes 2, 5, 8, and 11) or Z-DEVD-FMK (lanes 6, 9, and 12). Radioactive bands were visualized by phosphorimaging. Molecular mass markers (in kDa) are indicated to the left. Kinase identities are shown on the right.

Identification of Mst1 Kinase as the Target of Bisphosphonate Action—To identify the kinases that respond to BPs in the osteoclast, we used antibodies against kinases with molecular masses in the observed range and assayed immunoprecipitates using in-gel kinase assay. The kinases activated by ALN (Fig. 3) and other BPs (data not shown) were only immunoprecipitated with antibodies against Mst1 or Mst2, also known as kinase responsive to stress (Krs) 2 and 1, respectively. Antibodies directed to the amino terminus of either Mst1 (Fig. 3, lanes 3 (control) and 4 (ALN)) or Mst2 (lanes 5 (control) and 6 (ALN)) immunoprecipitated both kinases in the 59-kDa (Mst1)/60-kDa (Mst2) doublet in Oc lysates of control and ALN-treated cells. Mst1:Mst1 dimerization has been described and involves a carboxyl-terminal domain that is conserved in Mst2 (22). Consistent with the formation of Mst1:Mst2 heterodimers, either type of antibody precipitated both Mst1 and Mst2. Anti-Mst1 immunoprecipitated Mst1 to a greater extent than Mst2, and vice versa. This is explained by assuming random association, where anti-Mst1 precipitates Mst1:Mst1, Mst1:Mst2, and Mst2:Mst1, but not Mst2:Mst2 (yielding a 2:1 ratio), while anti-Mst2 will give a similar ratio favoring Mst2. Although bands were not separated sufficiently for quantitation, the data are consistent with this ratio. Immunodepletion with a mixture of both anti-Mst1 and Mst2 antibodies removed >95% of all kinase activity migrating at 59/60 kDa, suggesting that this activity doublet was comprised primarily of these kinases (data not shown).
Bisphosphonates Induce Caspase Cleavage of Mst Kinase

A

B

C

FIG. 5. Geranylgeraniol prevents induction of Mst1 kinase cleavage caused by inhibition of the mevalonate pathway. Ocs were prepared and analyzed by in-gel kinase assay as described in Fig. 2. Osteoclasts were maintained in the presence of M-CSF (5 ng/ml) and treated as follows. A, osteoclasts were purified and cultured for 24 h before lysis. 10 μM LOV was added to each culture 0 h (lane 1), 1 h (lane 2), 12 h (lane 3), 16 h (lane 4), 20 h (lane 5), and 24 h (lane 6) prior to lysis preparation. B, osteoclasts were not treated with inhibitors (lanes 1–3) or treated with 30 μM ALN (lanes 4–6), 30 μM RIS (lanes 7–9), or 10 μM LOV (lanes 10–12) for 19 h in the absence (lanes 1, 4, 7, and 10) or presence of 10 μM farnesol (lanes 2, 5, 8, and 11) or 10 μM GGOH (lanes 3, 6, 9, and 12). C, osteoclasts were not treated with inhibitors (lanes 1 and 2) or treated with 30 μM ALN (lanes 3 and 4), 30 μM RIS (lanes 5 and 6), 100 μM CL2 (lanes 7 and 8), 10 μM LOV (lanes 9 and 10), or 100 μM Na3VO4 (lanes 11 and 12) for 20 h in the absence or presence of 10 μM GGOH (lanes 2, 4, 6, 8, 10, and 12). Radioactive bands were visualized by phosphorimaging. Molecular mass markers (in kDa) are indicated to the left. Kinase identities are shown on the right.

caspase inhibitor, relative to cells treated with ALN, RIS, or CL2, alone (lanes 4, 7, and 10, respectively). These data suggest that BP-induced activation of the 34-kDa species resulted from caspase cleavage of full-length Mst1. In the absence of BP treatment, both caspase inhibitors reduced baseline 34-kDa Mst1 activities (lanes 2 and 3) relative to control (lane 1). The findings indicate that the activity migrating at 36-kDa kinase was also caspase-dependent, although the identity of this kinase remains to be determined. Analyses of p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase using GST-ATF2 as a kinase substrate for in-gel kinase assays and using phosphospecific antibodies to probe immunoblots showed that these kinases were not activated in osteoclasts treated with ALN (data not shown).

GGOH Blocks Mst1 Cleavage Induced by ALN, RIS, or Lovastatin, but Not by CL2—Recent studies suggest that ALN and other N-BPs act on the Oc by inhibiting protein geranylgeranlylation, related to their inhibition of the mevalonate pathway (4, 5). To examine if inhibition of the mevalonate pathway is sufficient to induce caspase cleavage of Mst1 kinase, osteoclast cultures were treated with the hydroxymethylglutaryl-CoA reductase inhibitor, lovastatin (LOV) for 2–24 h. In-gel kinase assays (Fig. 5A) showed that, like N-BPs (Fig. 2), LOV induced 34-kDa Mst1 and 36-kDa kinases and decreased Mst1, Mst2, and 50-kDa kinase activities. The profile of these responses was similar to that of ALN, PAM, and RIS (Fig. 2, A–C, respectively) indicating that inhibition of the mevalonate pathway can induce Mst1 kinase cleavage in these cells.

Recent studies showed that induction of apoptosis in J774 macrophages by N-BPs or statins was blocked by the addition of either geranylgeranyl or farnesyl precursors (3, 23). This is consistent with FPP synthase as the target for N-BP action but does not separate between geranylgeranylation and farnesylation as rate-limiting targets in N-BP action on the Oc. To distinguish between these two potential N-BP targets (4), we treated purified osteoclasts with ALN (Fig. 5B, lanes 4–6), RIS (lanes 7–9), and LOV (lanes 10–12) in the absence or presence of farnesol (lanes 2, 5, 8, and 11) or GGOH (lanes 3, 6, 9, and 12). Increase in 34-kDa Mst1 activity with either BP or LOV was blocked by GGOH but not by farnesol, although a partial reduction in 34-kDa kinase activity was seen in the presence of farnesol in some experiments (data not shown and Ref. 4). Other assays showed that mevalonic acid lactone, a metabolite situated between N-BP and LOV sites of action, was effective in blocking LOV, but not ALN or RIS, induction of Mst1 kinase cleavage (data not shown and Ref. 4).

To evaluate the proposed differences between the mechanism of action of N-BPs and of BPs lacking nitrogen, purified osteoclasts were treated with ALN (Fig. 5C, lanes 3 and 4), RIS (lanes 5 and 6), and CL2 (lanes 7 and 8) in the absence (lanes 3, 5, and 7) or presence of GGOH (lanes 4, 6, and 8). EHDP was not examined, since it elicited little or, occasionally, no response from 34-kDa Mst1 kinase. LOV (lanes 9 and 10) and Na3VO4 (lanes 11 and 12) were used to inhibit the mevalonate pathway and protein-tyrosine phosphatases, respectively. As previously shown in Fig. 5B, ALN-, RIS-, and LOV-induced Mst1 kinase cleavage was blocked by GGOH with kinase activities similar to those observed in untreated controls (Fig. 5C, lane 1). Similar analyses with PAM showed a significant, but incomplete, reduction in 34-kDa Mst1 activity when GGOH was added during treatment (data not shown). On the other hand, activation of 34-kDa Mst1 by CL2, which does not inhibit the mevalonate pathway, was not blocked by GGOH (lane 8). Na3VO4 (lanes 9 and 10) elicited only a modest response with activity levels comparable to those observed with EHDP (Fig. 2E). Other concentrations of vanadate resulted in similar responses (data not shown).

Mst1 Kinase Is Cleaved during Osteoclast Apoptosis Induced by Withdrawal of Serum and M-CSF or by Treatment with Stauroporine—To determine whether Mst1 kinase cleavage was unique to the BP and LOV responses or was more broadly associated with Oc apoptosis, we examined kinase activities during osteoclast apoptosis induced by treatment with staurosporine or withdrawal of serum and M-CSF. Treatment of B-cells with staurosporine, a potent inducer of apoptosis, leads to both activation of full-length Mst1 and Mst2 and caspase cleavage (data not shown and Ref. 4).

To contrast to treatment with BPs or LOV, staurosporine elicited little reduction in activity of the 50-kDa kinase, although activity of the 36-kDa kinase was increased with kinetics comparable to that of the 34-kDa Mst1 kinase. Withdrawal of M-CSF was shown to induce osteoclast apoptosis within 18 h of treatment (Fig. 1E). Withdrawal of serum
lanes 1–6 (lanes 1–6) or presence of 50 μM Z-VAD-FMK (lane 7) or 10 μM GGOH (lane 8). Radioactive bands were visualized by phosphorimaging. Molecular mass markers (in kDa) are indicated to the left. Kinase identities are shown on the right.

and M-CSF (Fig. 6B) activated 34-kDa Mst1 within 2 h (lane 4), and, by 4 h (lane 5), kinase activity was maximal. Activity of the 50-kDa kinase increased and then diminished over this time course. Inclusion of Z-VAD-FMK during the apoptosis-inducing 8 h incubation in the absence of serum and M-CSF (lane 7) blocked activation of the 34-kDa Mst1 cleavage product and diminution of the 50-kDa kinase activity, while GGOH (lane 8) had no effect. These findings suggest that Mst1 cleavage and 34-kDa Mst1 activation is a common pathway in osteoclast apoptosis, whether activated by BPs or by other apoptotic stimuli, while the 50-kDa kinase showed stimulus-specific responses.

DISCUSSION

BPs bind to bone and are taken up by osteoclasts (2). The intracellular action of BPs leads to loss of cytoskeletal structure and disappearance of the ruffled border, and ultimately, apoptosis (10, 11, 19). Oc apoptosis was proposed to be the mechanism by which bone resorption is inhibited by BPs. However, it was not known whether BPs induce Oc apoptosis through direct or indirect action, and there has been little insight into the signaling pathways controlling this event. We show here that apoptosis is induced by BPs in purified Ocs, consistent with direct action of BPs on Ocs. Furthermore, we identified a specific kinase involved in the signal transduction pathway leading to osteoclast apoptosis.

For the N-BPs, such as ALN and RIS, apoptosis and inhibition of bone resorption seem to be initiated by the inhibition of the mevalonate pathway enzymes, isopentenyl diphasophate synthase and/or FPP synthase (5). This causes a block in biosynthesis of cholesterol, FPP, and geranylgeranyl diphasophate. The prevention of N-BP effects by the addition of GGOH indicates that geranylgeranylation is rate-limiting in this process. Geranylgeranylation is the attachment of a 20-carbon (geranylgeranyl) lipid to certain proteins, including key regulatory G-proteins such as Rac, Rho, Cdc42, and various members of the Rab family (20, 21, 24). Geranylgeranylation is necessary to anchor these proteins on the plasma membrane or on intracellular membranes. Absence of geranylgeranylation and intracellular targeting of G-proteins results in a block or change in signaling events that ultimately lead to the induction of caspasess, possibly caspase 3, and to apoptosis.

Mst1 acts as a caspase 3 substrate and is cleaved into a highly active 34-kDa species that maintains the catalytic domain but lacks most of the carboxyl-terminal sequences (13). Included in these sequences is a domain necessary for the formation of Mst1:Mst1 dimers (22). We show here that Mst1 and Mst2 also form heterodimers that contain both isoforms, and this association is disrupted by caspase cleavage. Another domain located near the carboxyl terminus acts as a negative regulatory element, whose deletion by mutagenesis results in increased catalytic activity by up to 9-fold even when dimerization is maintained (22). Expression of Mst1 or the 34-kDa Mst1 kinase domain were shown to be sufficient to activate caspases and induce apoptosis in COS cells (13). The induction of Mst1 cleavage in the osteoclast by BPs and by the other apoptosis-inducing treatments suggests that this kinase is part of a signaling pathway leading to Oc programmed cell death.

Comparison of the potency of the tested BPs shows that the induction of Mst1 kinase cleavage by ALN and RIS in the Oc was comparable at matched concentrations, reflecting their similar efficacy in vivo (25). The mechanism for the occasional upper gastrointestinal effects produced by these agents, found to be similar for ALN and RIS in dogs and rats (26, 27), could also be due to inhibition of the mevalonate pathway. PAM induces Mst1 kinase cleavage and Oc apoptosis at 10-fold higher doses, consistent with the lower anti-resorptive potency of this N-BP. BPs lacking a nitrogen also induce caspase cleavage of Mst1; however, they require higher concentrations and their action is not through the mevalonate pathway. CL2 and, to a lesser extent, EHDP and tiludronate, are converted into toxic ATP analogs in the cell (6). We observe that both CL2 and EHDP induce apoptosis in the osteoclast, while CL2 induction of Mst1 cleavage is robust, EHDP effects are modest. Additional mechanisms may be involved in the action of these BPs. EHDP may act by inhibiting protein-tyrosine phosphatases, an effect produced by to all BPs in vitro, and tiludronate was reported to directly inhibit in vitro the osteoclast vacuolar ATPase (28).

Together, these data support the following model for BP

![Model for Bisphosphonate Intracellular Effects](http://www.jbc.org/)

**Fig. 6.** Staurosporine and withdrawal of both serum and M-CSF induce caspase cleavage of Mst1 kinase in osteoclasts. Ocs were prepared and analyzed by in-gel kinase assay as described in Fig. 2. Cells were treated with 100 nM staurosporine (A) or by withdrawal of both serum and M-CSF (B) 0 h (lane 1), 0.5 h (lane 2), 1 h (lane 3), 2 h (lane 4), 4 h (lane 5), and 8 h (lanes 6–8) prior to lysis in the absence (lanes 1–6) or presence of 50 μM Z-VAD-FMK (lane 7) or 10 μM GGOH (lane 8). Radioactive bands were visualized by phosphorimaging. Molecular mass markers (in kDa) are indicated to the left. Kinase identities are shown on the right.

**Fig. 7.** Schematic representation of intracellular effects of BPs in osteoclasts.
action (Fig. 7). All BPs concentrate on bone. The BPs examined in this study can act on the Oc directly to induce apoptosis. All apoptosis-inducing treatments trigger caspase cleavage of Mst1 kinase into an active 34-kDa species. Since the 34-kDa Mst1 kinase domain is itself capable of inducing the activation of caspses that cleave the full-length kinase (13), this can create an apoptosis auto-activation loop. For ALN and RIS, inhibition of bone resorption, induction of Mst1 kinase cleavage and induction of apoptosis are prevented by GGOH, suggesting that the mevalonate pathway intermediate, geranylgeranyl diphosphate, is rate-limiting for their effects. On the other hand, CL2 does not inhibit the mevalonate pathway, is not inhibited by GGOH, and induces Mst1 kinase cleavage and osteoclast apoptosis via a different mechanism (5, 6). EHDP, the least potent BP, induces apoptosis with weaker activation of Mst1 kinase cleavage, and may act via yet another mechanism. In conclusion, these findings further support the hypothesis that the mevalonate pathway is the intracellular target for the N-BPs, ALN and RIS, and identify Mst1 as a key intermediate in Oc apoptosis.

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