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Effects of Bafilomycin A1: An Inhibitor of Vacuolar H (+)-ATPases on Endocytosis and Apoptosis in RAW Cells and RAW Cell-Derived Osteoclasts

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Abstract Bafilomycin A1, a specific inhibitor of V-ATPases, is a potent inhibitor of bone resorption, but the underlying mechanisms of its action remain unclear. In this study, we investigated the effect of Bafilomycin A1 on endocytosis and apoptosis in RAW cells and RAW cell-derived osteoclasts. Quantitative analysis by flow cytometry showed that Bafilomycin A1 increased total transferrin levels when RAW cells were exposed to labeled transferrin and decreased the total uptake of Dextran-rhodamine B, both in a dose- and time-dependent fashion, indicating that Bafilomycin influences receptor-mediated and fluid phase endocytosis in these cells. Furthermore, Bafilomycin A1 induced apoptosis of RAW cells in a dose dependent manner as evidenced by Annexin V flow cytometry. The action of Bafilomycin A1 on endocytotic events appeared to be more sensitive and occurred earlier than on its apoptosis inducing effects, suggesting that interrupting of endocytosis might be an early sign of Bafilomycin-mediated osteoclast inhibition. Semi-quantitative RT-PCR analysis showed that the gene transcripts of putative Bafilomycin A1 binding subunit, V-ATPase-subunit a3, were expressed in the preosteoclastic RAW cell line, and up-regulated during RANKL-induced osteoclastogenesis. Osteoclasts treated with Bafilomycin A1 exhibited apoptosis as well as altered cellular localization of Transferrin Alexa 647. Given that endocytosis and apoptosis are important processes during osteoclastic bone resorption, the potent effect of Bafilomycin A1 on endocytosis and apoptosis of osteoclasts and their precursor cells may in part account for Bafilomycin A1 inhibited bone resorption. J. Cell. Biochem. 88: 1256–1264, 2003.

Key words: bafilomycin A1; V-ATPases; endocytosis; apoptosis; RAW cells; osteoclasts

Osteoclasts, the multinucleated bone-resorbing cells that derived from precursor cells of the monocytic/macrophage lineage, are directly related to lytic bone diseases such as osteoporosis, osteoarthritis, and Paget's disease [Rodan and Martin, 2000; Teitelbaum, 2000]. Vacuolar H (+)-ATPase (V-ATPase) plays an important role in osteoclast differentiation and function [Laitala-Leinonen and Vaananen, 1999; Toyomura et al., 2000]. Osteoclast V-ATPases are expressed highly in the plasma membrane and in the vesicular membranes, including clathrin-coated vesicles, endosomes, the Golgi apparatus, and lysosomes [Marquez-Sterling et al., 1991; Martinez-Zaguilan et al., 1993]. In addition, suppression of gene expression of V-ATPases by antisense RNA and DNA inhibits bone resorption [Laitala and Vaananen, 1994; Laitala-Leinonen et al., 1996, 1999; Laitala-Leinonen and Vaananen, 1999]. Moreover, Bafilomycin A1, a highly potent and selective inhibitor of all the V-ATPases [Gagliardi et al., 1998a] has been shown to inhibit bone resorption [Sundquist et al., 1990; Sundquist and Marks, 1994] and to delay tooth eruption [Sundquist and Marks, 1994; Marks and Sundquist, 1995]. Bafilomycin A1 and its derivatives have therefore been proposed to be potential inhibitors of osteoclast V-ATPase with an accompanying bone antiresorptive activity [Gagliardi et al., 1998a,b, 1999]. However, the

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underlying cellular mechanisms of its action remain to be further elucidated.

Endocytosis and apoptosis are important cellular events for osteoclast function. Osteoclasts are able to engulf and degrade dying osteoblasts/bone-lining cells or immature osteocytes [Taniwaki and Katchburian, 1998] and phagocytose particles, including particles of polymeric and metallic biomaterials [Wang et al., 1997a,b]. It has been shown that matrix proteins, including degraded type I collagen, are endocytozed along the ruffled border within the resorption compartment and transcytozed through the osteoclast to the basolateral membrane [Nesbitt and Horton, 1997; Salo et al., 1997]. During osteoclastic bone resorption, the endocytic pathway is involved in the maintenance of a functional ruffled border and may contribute to the balance of the membrane traffic associated with transcytosis from the ruffled border to the basal plasma membrane [Palokangas et al., 1997]. All such studies emphasize that endocytosis is an important function in osteoclasts [Vaananen et al., 2000]. Multiple lines of evidence have also shown that osteoclast apoptosis can be induced by a variety of stimuli, such as estrogen [Kameda et al., 1997; Zecchi-Orlandini et al., 1999], bisphosphonate [Hughes et al., 1996; Rogers et al., 1996], nitric oxide [van't Hof and Ralston, 1997], TGF-β [Hughes et al., 1996], and tetracyclines [Bettany et al., 2000]. Induction of osteoclast apoptosis is, therefore, of particular importance in osteoclast development and survival.

To further examine the mechanism of Bafilomycin A1 in osteoclasts and their precursor cells, we examined the gene expression of the Bafilomycin A1 binding protein, the a3 subunit of V-ATPase, during osteoclastogenesis, as well as the effect of Bafilomycin A1 on endocytosis and apoptosis in RAW cells and RAW cell-derived osteoclasts [Hsu et al., 1999; Huang et al., 2000; Xu et al., 2000]. Our results have shown that Bafilomycin A1 affects both receptor-mediated and fluid phase endocytosis and induces apoptosis in preosteoclastic RAW cells as well as in RAW cell-derived osteoclasts. The action of Bafilomycin A1 on endocytotic events appears to be more sensitive and occurs earlier than on its apoptosis inducing effects. The effects of Bafilomycin A1 on both endocytosis and apoptosis might contribute to the inhibition of osteoclastic bone resorption. Our results also suggest that lower doses of Bafilomycin A1 might be used to inhibit bone resorption through interrupting endocytosis but without causing apoptosis.

EXPERIMENTAL PROCEDURES

Reagents

Dextran, rhodamine B conjugated, (10, 000 MW, neutral) and Transferrin from human serum, Alexa Fluor 546 and 647 conjugated were purchased from Molecular Probes (Eugene, OR). Bafilomycin A1 was purchased from Sigma (Sydney, Australia) and oligonucleotide primers were supplied by Genset Pacific Pty. Ltd. (Lismore, NSW, Australia). DNA polymerase was from Geneworks (Adelaide, Australia). GST-rRANKL 160–318 recombinant proteins were expressed and purified in our laboratory as previously described [Xu et al., 2000].

RAW Cells and Osteoclast Culture

$RAW_{264.7}$ cells were cultured in alpha-modified essential medium (α-MEM) (Biosciences Pty. Ltd.) supplemented with 10% FCS, 1% L-Glutamine, and 2% penicillin/streptomycin. For osteoclast culture, RAW cells were seeded in a 6-well plate to a density of 5×10^4 cells/well and cultured for 5–7 days in full α-MEM in the presence of 100 ng/ml of GST-rRANKL [Xu et al., 2000].

Confocal Microscopy Analysis

Cultured osteoclasts were treated with Bafilomycin A1 overnight. Transferrin Alexa Fluor 546 was added to a final concentration of 40 μg/ml and incubated for 30 min. The cells were fixed with 4% paraformaldehyde and nuclei were stained with Hoechst (Molecular probes). Fluorescent images were recorded using a Confocal Laser Scanning Microscope (MRC-1000 Biorad).

RNA Isolation and RT-PCR

Total RNA was isolated from RAW cells treated with GST-rRANKL according to the manufacturer's instructions (Ambion, Inc., Austin, TX). For RT-PCR, single-stranded cDNA was prepared from 2 μg of total RNA using reverse transcriptase with an oligo-dT primer. Two μl of each cDNA was subjected to PCR amplification using specific primers. For the amplification of V-ATPase a3 subunit, forward primer: 5′GGATCCGAATTCATCATGGGCTCTATGTTC3′; and reverse primer: 5′GGATCCTCTAGA

CTAGTCACTGTCCACAGT3′ were used and PCR-amplification was carried out with 32 cycles (94°C, 60 sec; 54°C, 60 sec; and 72°C, 60 sec). As an internal control, the single stranded cDNA was PCR-amplified for 25 cycles using 36 B4 forward primers: 5′TCATTGTGGGAGCAGACA3′; and reverse primer: 5′ TCCTCCGACTCTTCCTTT3′. For the amplification of mouse calcitonin receptor [Yamin et al., 1994], forward primer: 5′TGGTTGAGGTTGTGCCCA3′; and reverse primer: 5′CTCGTGGGTTTGCCTCATC3′ were used and PCR-amplification was carried out with 30 cycles (94°C, 40 sec; 62°C, 40 sec; and 72°C, 40 sec). For the amplification of cathepsin K, forward primer: 5′GGGAGAAAAACCTGAAGC3′; and reverse primer: 5′ATTCTGGGGACTCAGAGC3′ were used. For the amplification of TRAP, forward primer: 5′TGTGGCCATCTTTATGCT3′; and reverse primer: 5′GTCATTTCTTTGGGGCTT3′ were used. PCR-amplification was carried out with 30 cycles (94°C, 40 sec; 55°C, 40 sec; and 72°C, 40 sec).

Endocytic Assays: Transferrin and Dextran Uptake

For Transferrin uptake experiments, RAW cells were starved for 1 h with serum free medium and 20 μg/ml of Transferrin Alexa Fluor 647 was added. Transferrin uptake was measured by flow cytometry using a Becton Dickinson FACSCalibur (Registered trademark) (San Jose, CA) instrument. Alexa Fluor 647 was detected in FL4. Sample files containing at least 10,000 events were collected using CellQuest (Registered trademark) software (Version 3.1f, Becton Dickinson, San Jose, CA) and analyzed to determine both the proportion of cells containing transferrin and the geometric mean of the positive population.

For Dextran uptake experiments, 50 μg/ml of Dextran labeled with rhodamine B was used. Dextran uptake was measured with flow cytometry (Molecular Probes, Eugene, OR) using a Becton Dickinson FACS Vantage (Registered trademark) (San Jose, CA) instrument. Rhodamine B was detected using a 630/22 nm bandpass filter in front of FL5. Sample files containing at least 10,000 events were collected using CellQuest (Registered trademark) software (Version 3.1f, Becton Dickinson) and analyzed to determine both the proportion of cells containing Dextran and the geometric mean of the positive population.

Flow Cytometry Analysis of Apoptotic Cells

RAW cells treated with Bafilomycin A1 were harvested and incubated with Annexin V-PE according to manufacturer's instruction (BD Biosciences) and analyzed by flow cytometry (Becton Dickinson FACSCalibur (Registered trademark). Apoptotic cell numbers were determined as percentage of total cells that were subjected to Annexin V-PE staining and FACS analysis.

RESULTS

Effects of Bafilomycin A1 on Transferrin Uptake

To examine the effect of Bafilomycin A1 on receptor-mediated endocytosis, quantitative analysis using flow cytometry was performed. RAW cells were treated with various doses of Bafilomycin A1 for 18 or 3.5 h and then incubated with 40 μg/ml of transferrin for 30 min. The cells were harvested and then subjected to flow cytometric analysis to quantitate the total levels of transferrin (Fig. 1A,B). RAW Cells treated with Bafilomycin A1 for 3.5 h showed increased total levels of transferrin at doses ranging from 2.5 to 135 nM when compared to untreated cells (Fig. 1C). Again, when compared to untreated cells, RAW cells treated with Bafilomycin A1 for 18 h showed increased total levels of transferrin at doses ranging from 0.5 to 2.5 nM, however, they exhibited decreased levels of transferrin at doses ranging from 12.5 to 62.5 nM (Fig. 1D). These results show that Bafilomycin A1 interfered with the pathway for receptor-mediated endocytosis.

Effects of Bafilomycin A1 on Dextran Uptake

To examine the effect of Bafilomycin A1 on fluid phase endocytosis, a dextran uptake experiment was performed. RAW cells were incubated overnight with 50 μg/ml of dextran rhodamine B, in the presence of various doses of Bafilomycin A1. The treated cells were then harvested and subjected to quantitative measurement of the total level of dextran by flow cytometry (Fig. 2A,B). Figure 2C shows that cells treated with Bafilomycin A1 exhibited reduced total intracellular dextran levels when compared with untreated cells during 16 h of dextran uptake (Fig. 2C). In a time course analysis, RAW cells were incubated with 50 μg/ml

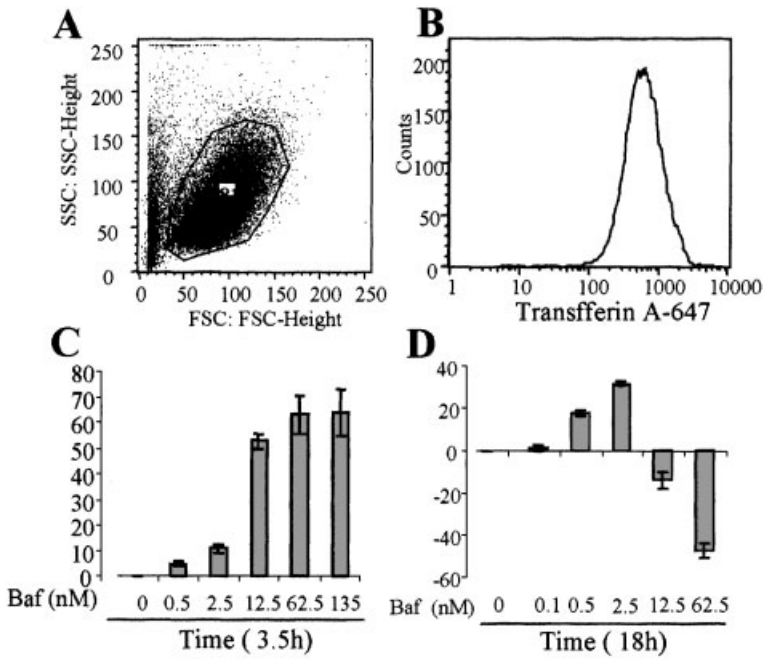

Fig. 1. The effect of Bafilomycin on the uptake of Transferrin Alexa 647, receptor-mediated endocytosis in $RAW_{246.7}$ cells. Approximately 1.5×10^5 $RAW_{246.7}$ cells seeded on 6-well plates were incubated in the presence of Bafilomycin A1. Twenty μg/ml of Transferrin Alexa 647 was added and incubated for 30 min. The treated cells were then subjected to FACS analysis. **A**: A representative window indicates cells sorted for analysis. **B**: A representative diagram displays the level (geometric means) of Transferrin Alexa 647. A dose-dependent response analysis of the effect of Bafilomycin A1 on Transferrin Alexa 647 in $RAW_{246.7}$ cells following the treatment of Bafilomycin at 3.5 h (**C**) and 18 h (**D**). Total levels (geometric means) of Transferrin Alexa 647 in percentage relative to mock control were presented.

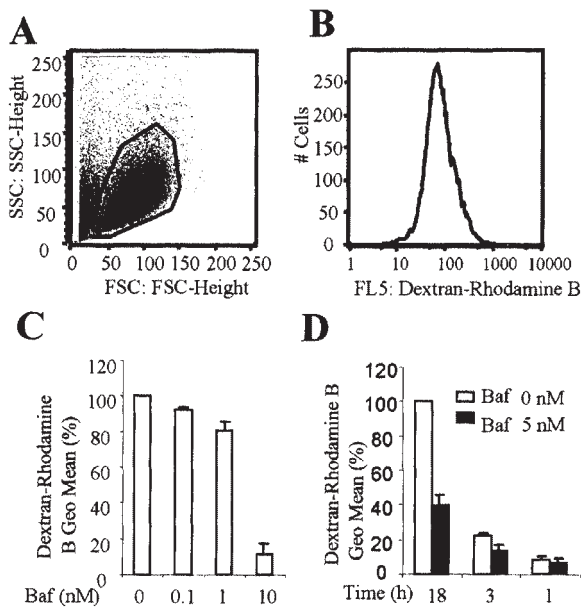

Fig. 2. The effect of Bafilomycin on the uptake of Dextran in $RAW_{246.7}$ cells. Approximately 1.5×10^5 $RAW_{246.7}$ cells seeded on 6-well plates were incubated in the presence of Bafilomycin A1 and 50 μg/ml of Dextran rhodamine B. The treated cells were then subjected to FACS analysis. **A**: A representative window indicates cells sorted for analysis. **B**: A representative diagram displays the intracellular level (geometric means) of Dextran rhodamine B. **C**: A dose-dependent response and (**D**) a time course analysis of the effect of Bafilomycin A1 on Dextran uptake in $RAW_{246.7}$ cells. Total levels (geometric means) of Dextran rhodamine B in percentage relative to mock control were presented.

of dextran in the presence (5 nM) or absence of Bafilomycin A1 for 18, 3, or 1 h. The total intracellular levels of dextran in cells treated with Bafilomycin A1 displayed a consistent reduction but of varying degree (Fig. 2D). These results indicate that Bafilomycin A1 interfered with the uptake or intracellular passage of dextran-Rhodamine.

Bafilomycin A1-Induced Apoptosis of RAW Cells

RAW cells treated with Bafilomycin A1 ranging from 12.5 to 62.5 nM, displayed morphological changes characteristic of cell shrinkage, chromatin compaction, and cytoplasmic condensation (Fig. 3). The dose response effects of Bafinomycin A1 on apoptosis were then examined in RAW cells by flow cytometric analysis. As shown in Figure 4A, at doses less than 5 nM and for 18 h of incubation, no significant effect on rates of apoptosis was detected. At doses between 5 and 10 nM, however, Bafilomycin A1 induced apoptosis in more than 60% of cells, but only caused necrotic cell death in approximately 10%. In a time course study carried out at a concentration of 10 nM, no significant change in apoptosis was observed at the 4 h time point. However, induction of apoptosis was observed between 12 and 24 h of treatment (Fig. 4B). These results indicate that Bafilomycin A1

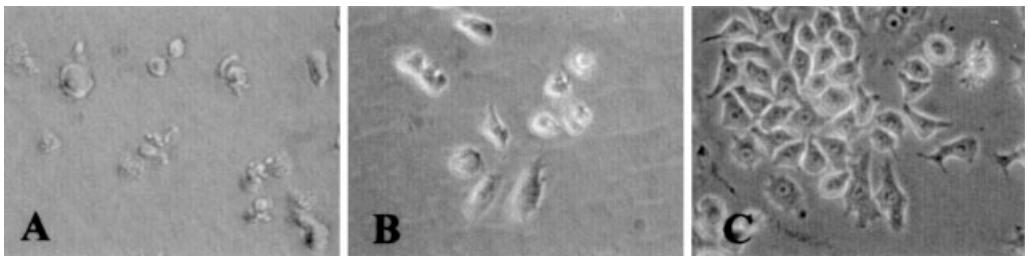

Fig. 3. Light microscopy images showing the effect of Bafilomycin on RAW cells. RAW cells were treated with Bafilomycin A1 at a concentration of 10 nM (**A**), 5 nM (**B**), or left untreated (**C**) overnight. Note that Bafilomycin-treated RAW cells exhibited morphological changes characteristic of cell shrinkage, chromatin compaction, and cytoplasmic condensation.

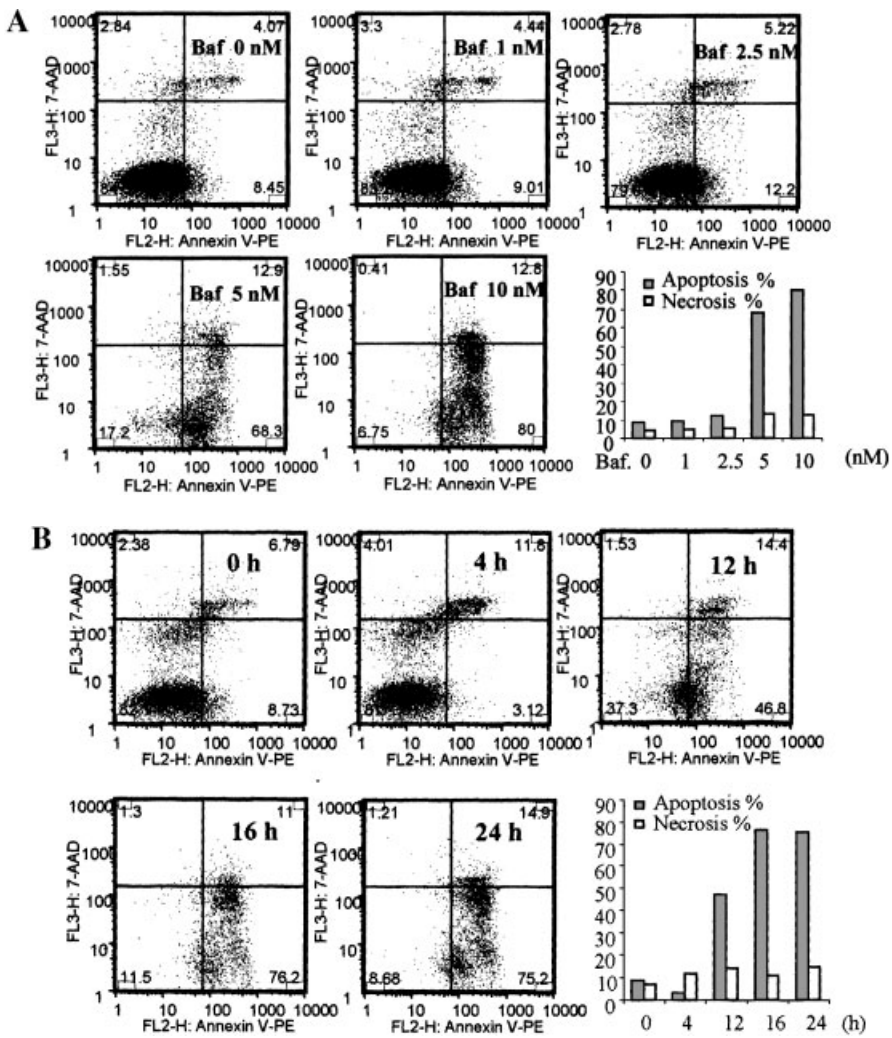

Fig. 4. Flow cytometry analysis showing the effect of Bafilomycin A1 on the apoptosis of RAW cells. **A**: A dose response analysis of the effect of Bafilomycin A1 on apoptosis of $RAW_{246.7}$ cells. **B**: A time course analysis of the effect of Bafilomycin A1 on apoptosis of $RAW_{246.7}$ cells. Approximately 1.5×10^5 $RAW_{246.7}$ cells were seeded on 6-well plates and were treated with 10 nM of Bafilomycin A1. At various time points, total cells were harvested and subjected to flow cytometry analysis using the Annexin V-PE. Representative results of three independent experiments are shown.

induced apoptosis of RAW cells in a dose- and time-dependent manner. Compared with the dose- and time-effects of Bafilomycin A1 on endocytosis, the induction of apoptosis seems to have occurred after interruption of endocytosis.

Effect of Bafilomycin on Endocytosis and Apoptosis in RAW Cell-Derived Osteoclasts

To investigate the effect of Bafilomycin A1 on osteoclasts, RAW cells were treated with GST-rRANKL to generate multinucleated TRAP positive osteoclasts [Xu et al., 2000]. The RAW-derived osteoclasts express calcitonin receptor and are able to resorb bone when seeded on dentine slices (data no shown). The osteoclasts were then incubated with various doses of Bafilomycin overnight, and then Transferrin Alexa 647 was added for 30 min. The cells were then fixed and stained with Hoechst. The results revealed that Bafilomycin A1-treated RAW cell-derived osteoclasts exhibited distinct cytoplasmic foci of Transferrin Alexa 647 localization together with obvious fragmentation of some nuclei when compared with the untreated controls (Fig. 5), indicating that Bafilomycin A1 has an effect on both endocytosis and apoptosis in these cells.

Gene Expression of Bafilomycin A1 Binding Subunit of V-ATPase During RANKL-Induced Osteoclastogenesis

The effect of Bafilomycin A1 on apoptosis and endocytosis in RAW cells and RAW cell-derived osteoclasts indicates that these cells are sensitive to treatment with Bafilomycin A1. The binding site of Bafilomycin A1 has been shown to reside on the 100 kDa subunit of the Vo domain of V-ATPases [Zhang et al., 1994] which is encoded by three isoforms a1, a2, and a3 [Nishi and Forgac, 2000]. It appears that *a3* (or the *Atp6i* gene) is unique and necessary for osteoclast-mediated extracellular acidification [Li et al., 1999]. Therefore, we next examined the expression of a3 in RAW cells during osteoclastogenesis. RAW cells were treated with RANKL at various time periods and total RNA was isolated. Semi-qauntitative RT-PCR was performed to assess the level of gene transcription. Cycle dependent RT-PCR was first carried out to establish linear amplification curves. Thirty-two cycles of PCR amplification for the *a3* gene were then chosen to determine the level of mRNA expression. The results showed that V-ATPase subunit a3 transcripts were upregulated during the differentiation of RAW cells into osteoclasts (Fig. 6). 36B4 was used as an internal control and the calcitonin receptor, *TRAP* and *cathepsin K* genes as controls for osteoclast differentiation (Fig. 6). In addition, TRAP activity and bone resorbing assays were carried out to confirm the presence of osteoclasts during RAW cell osteoclastogenesis (data not shown).

DISCUSSION

Development of strategies to control and modulate the formation or activity of osteoclasts has been a focus of bone research [Rodan and Martin, 2000; Teitelbaum, 2000]. V-ATPases have been found to be involved in bone resorption [Chatterjee et al., 1992] and therefore could be used as drug targets for bone anti-resorption agents. Bafilomycin A1 has a high potency and specificity for the inhibition of all the essential V-ATPases, but its selective inhibition for osteoclast V-ATPase has yet to be proven

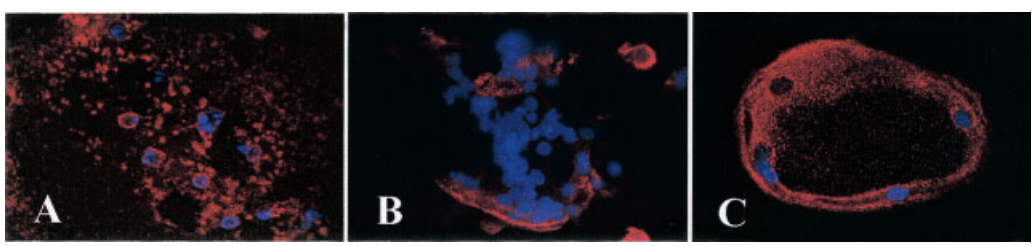

Fig. 5. Confocal images showing the effects of Bafilomycin A1 on endocytosis and apoptosis in osteoclasts. Osteoclasts cultured on glass coverslips were treated with Bafilomycin A1 at a concentration of 10 nM (**A**), 5 nM (**B**), or left untreated (**C**) overnight. Transferrin Alexa Fluor 546 was added at a final concentration of 50 μg/ml and incubated for 30 min. The cells were fixed with 4% paraformaldehyde in PBS and stained with 1/10,000 dilution of Hoechst. Fluorescent images were recorded from single osteoclast using a Confocal Laser Scanning Microscope (MRC-1000 Biorad) at a magnification of 40 ×. Red color images represent the cellular location of transferrin Alexa Fluor 546, whereas blue color images represent the nuclei staining. Note that some fragmented nuclei were observed in panel A and B.

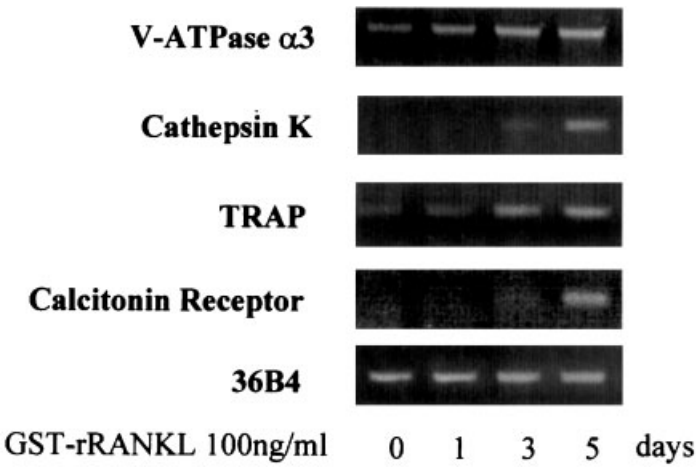

Fig. 6. Expression V-ATPase subunit a3 mRNA during osteoclastogenesis. **A**: RAW cells were cultured in the presence or absence of 100 ng/ml of GST-rRANKL $_{160-318}$ for 5 days. Total cellular RNA was extracted and cDNA was synthesized using 2 μg of total RNA with oligo-dT. PCR amplification was performed using specific primers to V-ATPase-subunit a3 calcitonin receptor *Cathepsin K, TRAP,* and *36B4* genes. PCR products were separated on a 1.2% of agarose gel. Representative results of three independent experiments are shown.

[Keeling et al., 1997; Farina and Gagliardi, 1999; Farina et al., 2001]. Early studies have found that Bafilomycin caused apoptosis in osteoclasts but not osteoblasts by TUNEL assay [Okahashi et al., 1997]. However, the mechanism of action and its relationship with endocytosis has not been elucidated. Understanding the mechanisms by which Bafilomycin A1 affects molecular and cellular processes in osteoclasts may facilitate the development of novel and selective inhibitors for the treatment of osteoporosis.

V-ATPase-mediated acidification is required for endocytosis, macromolecular processing, degradation, and intracellular transport of small molecules [Nelson, 1992; Stevens and Forgac, 1997; Forgac, 1999]. We have demonstrated that treatment with Bafilomycin A1 affects dextran uptake, a fluid phase endocytosis. At concentrations as low as 1 nM, Bafilomycin A1 caused a reduction of dextran uptake in RAW cells. It has been shown that Bafilomycin A1 could block the movement of a fluid phase marker from endosomes to lysosomes [van Weert et al., 1995], as well as inhibit the formation of carrier vesicles that mediate transport between early and late endosomes [Clague et al., 1994]. Therefore, the reduced uptake of dextran in RAW cells is likely due to the effect of Bafilomycin A1 on intracellular transport processes mediated by V-ATPases. In receptor-mediated endocytosis, acidification of endosomes is required to activate the release of internalized ligands from their receptor. The free receptors are then recycled to cell surface where they can be reutilized, whereas the ligands are transported to lysosomes for degradation [Stevens and Forgac, 1997]. We have shown that Bafilomycin A1 at lower doses (0.1–2.5 nM, 18 h treatment) increased total transferrin levels by FACS analysis. Other studies have examined the effect of Bafilomycin A1 on the uptake and release of ^{125}I transferrin, and shown that Bafilomycin A1 prevents a complete depletion of transferrin from the plasma membrane and inhibits the release of membrane-bound transferrin into the medium [van Weert et al., 1995]. The increased levels of transferrin after the treatment with Bafilomycin A1 is likely due to the reduced release of membrane-bound transferrin from RAW cells, and our results are consistent with previous studies [van Weert et al., 1995]. However, at higher doses (12.5–62.5 nM, 18 h treatment), Bafilomycin A1 triggered apoptosis of RAW cells, which could contribute to decreased levels of transferrin uptake. Taken together, Bafilomycin A1 affects both receptor-mediated and fluid phase endocytosis in RAW cells. Given that endocytosis is a critical process of osteoclast function [Nesbitt and Horton, 1997; Palokangas et al., 1997; Salo et al., 1997], interruption of both receptor-mediated and fluid phase endocytosis of osteoclasts by Bafilomycin A1 might contribute to inhibition of osteoclastic bone resorption.

The mechanism of Bafilomycin-induced apoptosis has not been well characterized. It appears that Bafilomycin A1-induced apoptosis occurs independently of intracellular pH in rat phreochromocytoma PC12 and HL-60 cells [Kinoshita et al., 1996]. In Bafilomycin A1-treated myocytes, increased accumulation of p53 protein and p53-dependent transactivation of gene expression, including a persistent upregulation of p21/wild-type p53 activated fragment 1/cyclin kinase inhibitor protein-1 mRNA appears to

play an important role [Long et al., 1998]. Given that inhibition of endocytosis by Bafilomycin A1 occurred earlier than apoptosis, it is possible that disruption of the endocytic pathway may contribute to the apoptosis. In support of this view, it has been shown that deregulation of vesicular transport could result in reduced capacitative calcium entry which may then induce apoptosis [Jayadev et al., 1999]. It is also possible that Bafilomycin A1 at lower doses could be used to inhibit bone resorption through interrupting endocytosis but without causing apoptosis.

Using semi-quantitative PCR analysis, we found that V-ATPase-subunit a3 transcripts were increased during RANKL-induced osteoclastogenesis. Our results are consistent with other findings that a3 protein was preferentially expressed in bone marrow cells cultured in the presence of 1,25(OH)2 D3 [Toyomura et al., 2000]. Further more, it has been shown that *a3* (or the *Atp6i* gene) is unique and necessary for osteoclast-mediated extracellular acidification [Li et al., 1999]. The binding of Bafilomycin A1 to a3 subunit of V-ATPases might contribute to the inhibition of endocytosis and induction of apoptosis in osteoclasts and RAW cells. Understanding the biological effect and underlining mechanisms is of important relevance to the development of drugs that will selectively inhibit osteoclast function.

In summary, we showed that Bafilomycin A1 increased the total transferrin levels and decreased the total uptake of intracellular Dextran-rhodamine B levels in RAW cells. Furthermore, Bafilomycin A1 induced apoptosis of RAW cells and RAW cell-derived osteoclasts. Given that endocytosis and apoptosis are important processes during osteoclastic bone resorption, the potent effect of Bafilomycin A1 on endocytosis and apoptosis of osteoclasts and their precursor cells may in part account for Bafilomycin A1 inhibited bone resorption. Our studies on the effects of Bafilomycin A1 on endocytosis and apoptosis of RAW cells and RAW cells-derived osteoclasts might extend our understanding on the molecular and cellular processes of action of Bafilomycin A1 in these cells.

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