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Protein Synthesis Is Required for Caspase Activation and Induction of Apoptosis by Bisphosphonate Drugs

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ABSTRACT

The exact mechanisms of action of antiresorptive bisphosphonate drugs remain unclear, although they may inhibit bone resorption by mechanisms that can lead to osteoclast apoptosis. These drugs also cause apoptosis in J774 macrophages, probably as a consequence of inhibition of protein prenylation. However, the molecular pathways that lead to apoptosis are not known. In some cells, apoptosis induced by statins (other inhibitors of protein prenylation) is dependent on protein synthesis. The aim of this study was to further characterize the kinetics and biochemical features of bisphosphonate-induced apoptosis, including the dependence on protein synthesis. Alendronate-induced apoptosis in J774 cells occurred after ~16 hr of treatment, although shorter exposures to the drug followed by incubation in bisphosphonate-free medium also committed cells to apoptosis. The appearance of apoptotic cells was associated with the appearance of caspase-3-like activity. Apoptosis induced by bisphosphonate or mevastatin was found to be dependent on protein synthesis because cycloheximide inhibited chromatin condensation, DNA fragmentation and activation of caspase-3-like protease or proteases. Protein synthesis was required for events that lead to commitment to apoptosis but not for the execution phase because cycloheximide did not prevent apoptosis when added ≥15 hr after the start of alendronate treatment. Furthermore, staurosporine-induced caspase-3-like activity and apoptosis in J774 cells could not be prevented by cycloheximide. These observations demonstrate that activation of caspase-3-like proteases and inhibition of commitment to apoptosis by cycloheximide are common features of apoptotic cell death induced by inhibitors of protein prenylation such as bisphosphonates.

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Excessive osteoclast-mediated bone resorption is an important feature of many common diseases affecting the skeleton, including postmenopausal osteoporosis, Paget's disease, and tumor metastasis. BPs, a class of nonhydrolysable pyrophosphate analogues (reviewed by Fleisch, 1988), have become the most important treatment for these skeletal disorders because they are powerful pharmacological inhibitors of bone resorption (Fleisch, 1991; Kanis *et al*., 1994; Liberman *et al*., 1995); BPs with a nitrogen-containing moiety, including PAM and ALN, being particularly potent.

The exact molecular mechanisms by which BPs inhibit osteoclastic bone resorption are currently the subject of intense debate (Rodan and Fleisch, 1996). Although BPs can cause osteoclast apoptosis (Hughes *et al*., 1995; Selander *et al*., 1996), the exact targets for BPs and the molecular mechanisms that lead to osteoclast apoptosis have not been clarified. Due to the difficulty in isolating large numbers of osteoclasts, we are using the macrophage-like cell line J774 as a convenient model with which to identify the molecular targets for BPs because these cells also undergo apoptosis after treatment with BPs (Rogers *et al*., 1996). We recently proposed that nitrogen-containing BPs such as PAM and ALN induce J774 apoptosis as a result of inhibition of enzymes of the mevalonate pathway and, hence, loss of post-translational protein prenylation (Luckman *et al*., 1998b). There is a strong correlation between the structure-activity relationships of nitrogen-containing BPs for inhibiting protein prenylation and inducing apoptosis in J774 cells *in vitro* and for inhibiting bone resorption *in vivo* (Luckman *et al*., 1998b), suggesting that these BPs do indeed act by inhibiting protein prenylation. Other drugs that inhibit the mevalonate pathway and prevent protein prenylation, such as lovastatin and mevastatin, can also induce apoptosis in a number of cell types (Perez-Sala *et al*., 1994; Padayatty *et al*., 1997; Marcelli

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ABBREVIATIONS: BP, bisphosphonate; ALN, 4-amino-1-hydroxy-butylidene-1,1-bisphosphonate (alendronate); PAM, 3-amino-1-hydroxy-propylidene-1,1-bisphosphonate (pamidronate); PBS, phosphate-buffered saline; CHX, cycloheximide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; DAPI, 4,6-diamidino-2-phenylindole; STP, staurosporine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin.

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et al., 1998) including J774 macrophages (Luckman *et al.*, 1998b). Apoptosis induced by lovastatin or BPs occurs after a delay of ≥12 hr (Perez-Sala *et al.*, 1994; Rogers *et al.*, 1996; Padayatty *et al.*, 1997). This contrasts with apoptosis induced by other agents such as glucocorticoids or anti-Fas antibody, which can be detected much sooner (Wyllie, 1980; Trauth *et al.*, 1989). The length of time taken for lovastatin and BPs to cause apoptosis could be related to the time required to internalize sufficient quantities of drug or to the rate of turnover of prenylated proteins that prevent apoptosis (Cortez *et al.*, 1996; Moorman *et al.*, 1996). We therefore investigated more closely the time of onset of BP-induced apoptosis in J774 cells and whether apoptosis still occurred when the cells were exposed to BPs for shorter periods of time.

Apoptosis induced by certain agents, including lovastatin, seems to be dependent on protein synthesis because CHX can prevent the characteristic morphological and biochemical features of apoptosis (Cohen and Duke, 1984; Wyllie *et al.*, 1984; Perez-Sala *et al.*, 1995). However, the exact stage of the apoptotic pathway that it is blocked by CHX is not known. Because we have recently shown that BPs probably cause apoptosis by inhibiting the same metabolic pathway that is inhibited by lovastatin and mevastatin, we therefore examined whether protein synthesis is also required for BP-induced J774 apoptosis and, if so, at which stage of the apoptotic process (i.e,. for commitment of cells to, or for execution of, apoptosis) (Lazebnik *et al.*, 1995). For this purpose, the effect of STP on J774 apoptosis was also studied because it has been reported that STP can induce apoptosis in other cells independently of protein synthesis, suggesting that the effector proteins of the apoptotic process are constitutively expressed (Weil *et al.*, 1996).

A common feature of the execution phase of apoptosis is the activation of members of the ICE/CED3 family of caspases (Hale *et al.*, 1996; Cohen, 1997), which cleave protein substrates such as poly(ADP-ribose) polymerase and nuclear lamins (Nicholson *et al.*, 1995; Cohen, 1997) and are thought to represent the irreversible step toward cell death. The activation of caspase-3-like proteases is thought to play a central role in apoptosis (Fernandes-Alnemri *et al.*, 1994; Cohen, 1997; Marcelli *et al.*, 1998) but can be prevented in some cells by CHX (Inayat-Hussain *et al.*, 1997; Medina *et al.*, 1997). We therefore also examined whether BPs cause activation of caspase-3-like enzymes and whether this could be inhibited by CHX.

Experimental Procedures

Materials. PAM was from Gentili S.p.a. (Pisa, Italy). ALN was provided by Procter and Gamble Pharmaceuticals (Cincinnati, OH). Stock solutions (10 mM) of BPs were prepared in PBS, pH 7.4, and filter-sterilized using a 0.2-μm filter. [^{14}C]Mevalonolactone was from Amersham (Aylesbury, Buckinghamshire, UK). Unless stated otherwise, all other reagents were from Sigma Chemical (Poole, UK).

Cell culture. The murine macrophage-like cell line J774.2 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Paisley, UK) containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM L-glutamine in a 5% CO_2 atmosphere.

Effect of short exposures of J774 macrophages to bisphosphonates. To determine the onset of ALN-induced apoptosis, cells were seeded onto 24-well plates (Costar, Cambridge, MA) at a density of 5×10^4 cells/well and treated the next day in triplicate with 100 μM ALN for 14, 16, 18, 20, 22, and 24 hr. Adherent and nonadherent cells were harvested and pooled, and then the proportion of apoptotic cells was determined on the basis of morphology after staining nuclei with DAPI (Rogers *et al.*, 1996).

The effect on cell viability of shorter exposures to BPs was assessed by MTT assay after a total culture period of 48 hr. Continuous treatment with 100 μM ALN or PAM results in >50% apoptosis by this time (Rogers *et al.*, 1996). J774 cells were seeded at a density of 10^4 cells/well onto 96-well plates (Costar) and then treated the next day with 100 μM PAM or ALN in replicates of six wells. Cells were exposed to BPs for 1, 2, 3, 5, 7, or 19 hr (or treated continuously) before rinsing with PBS and replacement of culture medium with BP-free medium. At 45 hr after commencing treatment with BP, the reduction of MTT reagent was measured as described previously (Rogers *et al.*, 1996).

Effect of protein synthesis inhibitors on apoptosis in J774 cells. The effect of CHX and actinomycin D on ALN- and mevastatin-induced apoptosis was examined by assessing changes in the proportion of apoptotic cells and the presence of oligonucleosome-sized DNA fragments. The proportion of morphologically apoptotic cells present after 48-hr cotreatment with 100 μM ALN or 20 μM mevastatin and either 0.5–1 μM CHX or 0.5 nM actinomycin D was determined after staining nuclei with DAPI. To determine effects on DNA fragmentation, DNA was isolated from J774 cells after treatment for 48 hr with 100 μM ALN or 30 μM mevastatin and either 0.5–1 μM CHX or 0.5 nM actinomycin D, according to Rogers *et al.* (1996). Aliquots of isolated DNA were electrophoresed on 1.2% agarose gels containing 1 μg/ml ethidium bromide, and bands were visualized and photographed under UV transillumination. Experiments also were undertaken in which 0.25 μM CHX was added 3–24 hr after commencing treatment with 100 μM ALN. The proportion of morphologically apoptotic cells was finally determined after a total incubation period of 48 hr.

In addition, the effect of STP on J774 cells was investigated. Cultures were incubated with 1 μM STP, 0.25 μM CHX, and 100 μM ALN (alone and in combination) for 48 hr; then, apoptosis was assessed by counting the proportion of apoptotic cells after staining with DAPI.

Measurement of caspase-3-like enzyme activity. Caspase-3-like enzyme activity was measured by proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC. J774 cells were treated in six-well plates with or without 100 μM ALN for 10, 13, 16, or 24 hr. Adherent and nonadherent cells then were harvested, washed in PBS and lysed in 150 μl of lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 10 mM EGTA, and 0.5% CHAPS). For the assay, a solution of 100 μl of cell lysates was made up to 3 ml with lysis buffer containing 5 mM cysteine plus 40 μM substrate and incubated at 37° for 1 hr. The release of AMC from the substrate was measured fluorimetrically using a Perkin-Elmer Cetus (Norwalk, CT) fluorimeter with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The results were corrected for protein content of the lysates [determined using the Pierce Chemical (Rockford, IL) BCA assay] and expressed as change in fluorescence units per μg protein. The effect of 0.5 μM CHX on caspase-3-like activity induced by 100 μM ALN or 1 μM STP also was investigated after treatment of J774 cells for 16 hr. In an additional experiment, J774 cells were treated with 100 μM ALN alone or with 0.5 μM CHX added 3, 6, 9, or 12 hr after the start of treatment with 100 μM ALN. Cell lysates were prepared after a total of 24 hr, and then caspase-3-like activity was determined and expressed as a percentage of control.

Metabolic labeling with [^{14}C]mevalonolactone. The effect of CHX on protein prenylation in J774 cells was investigated by studying the metabolic incorporation of [^{14}C]mevalonolactone into proteins post-translationally modified with farnesyl and geranylgeranyl groups, as described previously (Luckman *et al.*, 1998b). Briefly, cells were incubated for 16 hr with 5 μM mevastatin and 7.5 μCi/ml [^{14}C]mevalonolactone (specific activity, 57 mCi/mmol) in the pres-

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ence or absence of 1 μM CHX. The cells then were lysed in RIPA buffer [PBS, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) sodium deoxycholate, 10 μg/ml phenylmethylsulfonyl fluoride]. The protein concentration of the lysates was determined, and equal quantities of protein (50 μg) were electrophoresed on SDS-12% polyacrylamide gels. Gels were dried, and radiolabeled bands were visualized after exposure to a high sensitivity BioRad (Hercules, CA) phosphorimaging screen for 3 days.

Statistical analysis. Statistical analysis of the data was carried out using one-way analysis of variance followed by the Scheffé *F* test or by using Student's *t* test as indicated in the figure legends.

Results

Short exposure of J774 macrophages to bisphosphonates causes apoptosis. An increase in J774 apoptosis (cells with condensed, marginated chromatin or fragmenting nuclei) could be detected after 14 hr of continuous treatment with 100 μM ALN. After 16 hr, this effect was statistically significant (9% of the cells were apoptotic compared with <1% in the control cells, $p < 0.05$). The proportion of apoptotic cells steadily increased from 16 hr onward, until after 24 hr of treatment, >30% of the remaining cells were apoptotic ($p < 0.001$ compared with controls, Fig. 1).

Although a statistically significant increase in apoptosis could not be detected until after ~16 hr of treatment, cells that were exposed to BPs for shorter periods of time and then washed and incubated in the absence of BPs still underwent apoptosis. In this case, an increase in apoptosis also began to occur ~16 hr after commencing BP treatment (not shown). An exposure of 2 hr to 100 μM PAM or of 5 hr to 100 μM ALN was sufficient to cause a significant loss of cell viability ($p < 0.001$), assessed after a total incubation period of 48 hr (Fig. 2). Similar results were obtained by counting the proportion of apoptotic cells rather than measuring cell viability (not shown).

Induction of apoptosis by bisphosphonates or mevastatin requires protein synthesis. Coincubation of J774 cells with 100 μM ALN plus 0.25–1 μM CHX or 0.5 nM actinomycin D for 48 hr significantly reduced the proportion of apoptotic cells present after 48 hr (Fig. 3A) compared with cells treated with ALN alone ($p < 0.001$). In addition, both 1 μM CHX and 0.5 nM actinomycin D prevented internucleosomal DNA fragmentation caused by treatment with 100 μM ALN for 48 hr (Fig. 3B). Similarly, 0.25–0.5 μM CHX completely inhibited the induction of apoptosis caused by treatment of cells with 20 μM mevastatin for 48 hr ($p < 0.001$), assessed by counting the proportion of morphologically apoptotic cells (Fig. 4A) and by electrophoretic analysis of DNA fragmentation (Fig. 4B). CHX (0.25–1 μM) or actinomycin D (0.5 nM) also maintained the viability of ALN- and mevastatin-treated cells, measured with an MTT assay (not shown).

Protein synthesis is not required for the execution phase of bisphosphonate-induced apoptosis. Delayed addition of CHX was less effective at inhibiting apoptosis than coincubation with ALN and CHX for the entire culture period. The ability of 0.25 μM CHX to prevent ALN-induced apoptosis became progressively less as the time of addition of CHX was delayed after commencing treatment with 100 μM ALN (Fig. 5), although the addition of CHX after as long as 12 hr still resulted in a significant reduction in the proportion of apoptotic cells compared with treatment with ALN alone ($p < 0.05$). However, CHX was ineffective at preventing apoptosis when added ≥15 hr after the start of ALN treatment (Fig. 5B).

STP (1 μM), a protein kinase C inhibitor, was a more potent inducer of apoptosis in J774 cells than ALN, but in contrast to ALN-induced apoptosis, this could not be prevented by coincubation with 0.25 μM CHX (Fig. 6). Furthermore, STP-induced apoptosis occurred more rapidly than ALN-induced or mevastatin-induced apoptosis (not shown). The inhibitory effect of 0.25 μM CHX on apoptosis induced by 100 μM ALN could also be overcome by the addition of 1 μM STP (Fig. 6).

CHX prevents activation of caspase-3-like proteases. Apoptosis in J774 cells induced by treatment with 100 μM ALN was associated with a time-dependent increase in caspase-3-like enzyme activity (the ability to cleave the fluorogenic substrate Ac-DEVD-AMC). Caspase-3-like activity was slightly greater in lysates from ALN-treated cells than

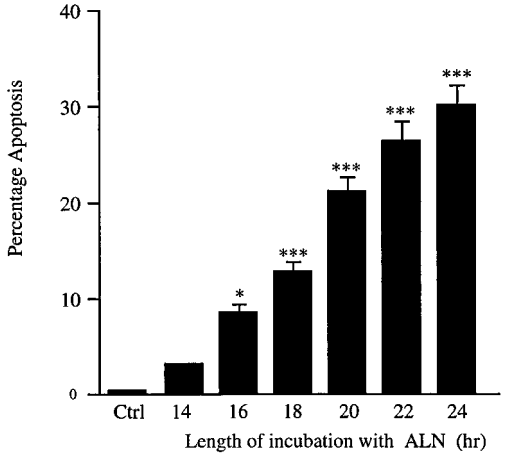

Fig. 1. Time-dependent induction of apoptosis by ALN in J774 cells. The proportion of apoptotic cells was determined on the basis of nuclear morphology after treatment with 100 μM ALN for 14–24 hr. *Ctrl*, proportion of apoptotic nuclei in untreated cells after 24 hr. Values are mean ± standard error ($n = 3$). *, $p < 0.05$ compared with control, ***, $p < 0.001$ compared with control (analysis of variance). Some error bars are not visible at this scale.

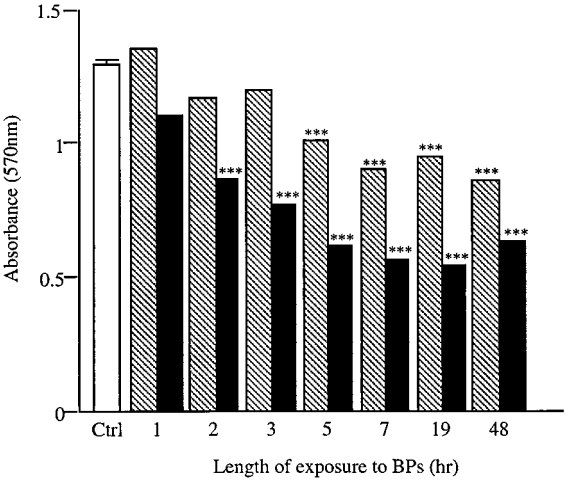

Fig. 2. Induction of apoptosis in J774 cells after a short exposure to BPs. Cells were exposed to 100 μM ALN (*cross-hatched bars*) or PAM (*solid bars*) for the times indicated and then washed and cultured in BP-free medium until 45 hr after the start of BP treatment. Control (*Ctrl*) cultures were not treated with BP. Cell viability was assessed using the MTT assay. Values are mean ± standard error ($n = 6$). ***, $p < 0.001$ compared with control (analysis of variance). Some error bars are not visible at this scale.

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lysates from control cells after 10 and 13 hr of treatment (Fig. 7A). This activity then increased until after 24 hr, the level of caspase-3-like activity was >20-fold higher in lysates from ALN-treated cultures than lysates from control cells (Fig. 7A). The increase in caspase-3-like activity therefore is coincident with the appearance of increased numbers of morphologically apoptotic cells, which were detected after ~14 hr (Fig. 1).

The appearance of caspase-3-like enzyme activity was completely inhibited ($p < 0.001$) when the cells were coincubated with 100 μM ALN plus 0.5 μM CHX for 16 hr (Fig. 7B). CHX did not inhibit caspase activity directly because 0.5 μM CHX did not inhibit cleavage of Ac-DEVD-AMC when added directly to the cell lysates (not shown). Apoptosis induced by 1 μM STP also was associated with an increase in caspase-3-like protease activity (Fig. 7B). This was >2-fold greater than ALN-induced caspase-3-like activity after 16 hr, reflecting the greater extent of apoptosis induced by STP at this time point. However, as with STP-induced apoptosis, STP-induced caspase-3-like activity was not prevented by 0.5 μM CHX (Fig. 7B).

The ability of CHX to inhibit the appearance ALN-induced caspase-3-like activity became progressively less as the time of addition of CHX was delayed (Fig. 8), which is in accord with the decreasing ability of CHX to inhibit apoptosis when addition of CHX was delayed (Fig. 5). Thus, although the addition of 0.5 μM CHX 12 hr after the start of ALN-treatment still inhibited the induction of caspase-3-like activity,

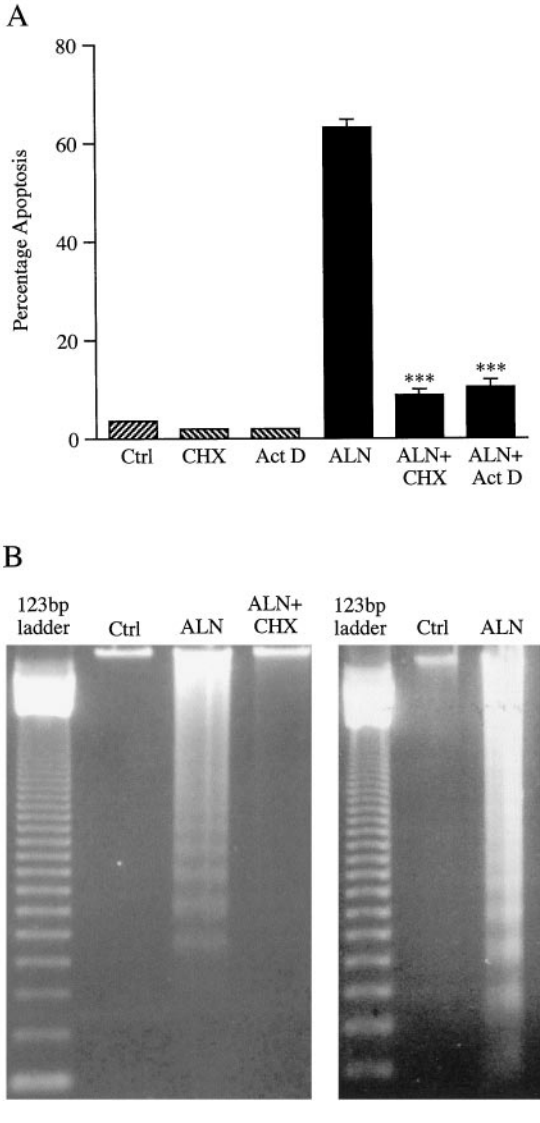

Fig. 3. BP-induced apoptosis is prevented by inhibitors of protein synthesis. J774 cells were treated with 100 μM ALN with or without 1 μM CHX or 0.5 nM actinomycin D. *Ctrl*, control. A, After 48 hr, apoptosis was assessed by determining the proportion of apoptotic cells. Values are mean ± standard error ($n = 3$). ***, $p < 0.001$ compared with ALN alone (Student's t test). Some error bars are not visible at this scale. B, Apoptosis also was assessed by analysis of DNA fragmentation after electrophoresis of DNA on a 1.2% agarose gel.

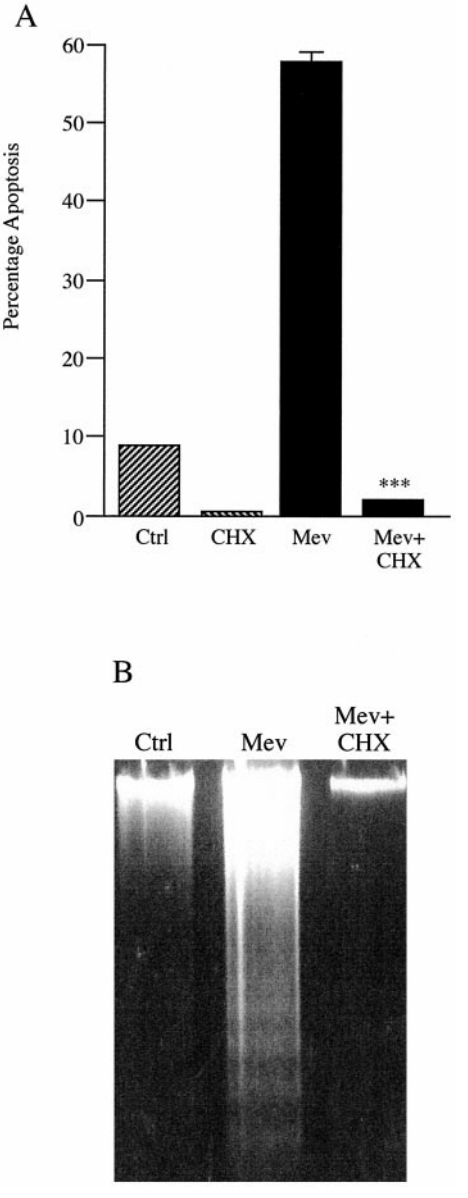

Fig. 4. Mevastatin-induced apoptosis is prevented by CHX. Cells were treated with 20 μM mevastatin (*Mev*) in the presence or absence of 0.5 μM CHX for 48 hr. *Ctrl*, control. A, Apoptosis then was assessed by counting the proportion of apoptotic cells. Values are mean ± standard error ($n = 3$). ***, $p < 0.001$ compared with mevastatin alone (Student's t test). Some error bars are not visible at this scale. B, Apoptosis also was assessed by analysis of DNA fragmentation after treatment of cells for 48 hr with 30 μM mevastatin with or without 0.5 μM CHX, by electrophoresis of DNA on a 1.2% agarose gel.

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this was not as effective as the addition of 0.5 μM CHX at the start of ALN treatment (Fig. 8).

CHX prevents incorporation of [^{14}C]mevalonolactone into proteins. A possible route by which CHX inhibits apoptosis is by preventing the accumulation of nonprenylated proteins, which may exert a dominant negative effect on signaling (Lerner *et al*., 1995). To test this hypothesis, we investigated whether CHX was able to prevent the incorporation of [^{14}C]mevalonolactone into proteins, which would verify whether the concentration of CHX that is effective at inhibiting BP-induced apoptosis (1 μM) also inhibits the synthesis of proteins destined for prenylation. J774 cells were incubated with 1 μM CHX plus [^{14}C]mevalonolactone in the presence of 5 μM mevastatin. The latter depletes the intracellular pool of mevalonate and therefore ensures more efficient metabolic radiolabeling of prenylated proteins. The incorporation of [^{14}C]mevalonolactone into prenylated proteins of molecular weight of ~25 kDa (small GTPases) and 45–70 kDa was almost completely prevented by 1 μM CHX (Fig. 9). By contrast, the radiolabeled band at the migrating front, which most likely consists of nonproteinaceous isoprenoids (Luckman *et al*., 1998b), was unaffected by CHX treatment.

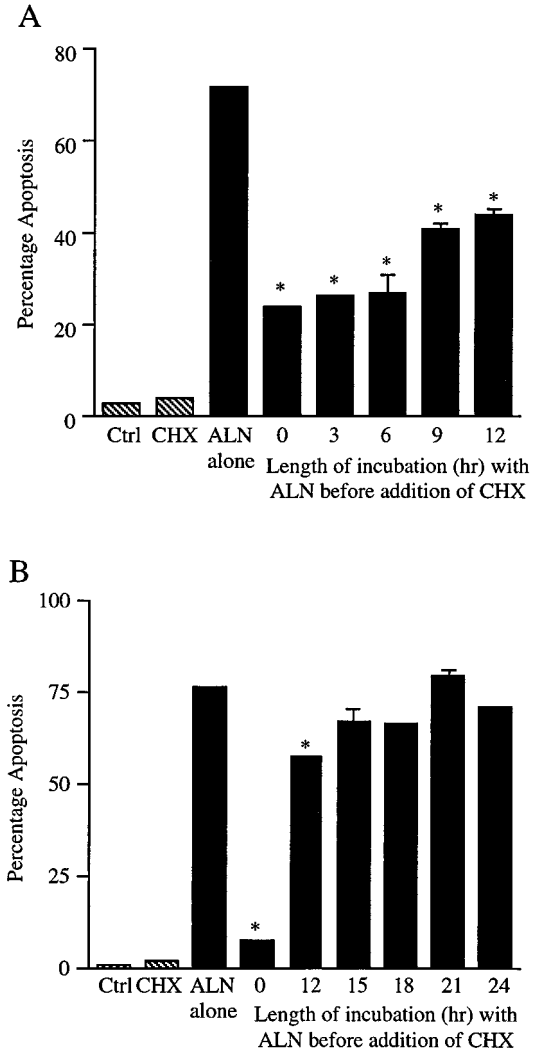

Fig. 5. Inhibition of ALN-induced apoptosis by CHX is dependent on the time of addition of CHX. *Ctrl*, control. J774 cells were treated with 100 μM ALN for 48 hr, with or without 0.25 μM CHX, which was added to the cells 0–12 hr (A) or 12–24 hr (B) after the start of ALN treatment. The proportion of apoptotic cells was determined on the basis of nuclear morphology. Values are mean ± standard error ($n = 3$). *, $p < 0.05$ compared with ALN alone (analysis of variance). Some error bars are not visible at this scale.

Discussion

We recently proposed that the BP drug clodronate inhibits bone resorption due to the accumulation of a cytotoxic metabolite within osteoclasts (Frith *et al*., 1997). However, the nitrogen-containing BP drugs such as ALN and PAM are not metabolized and seem to have a different mechanism of action (Frith *et al*., 1997). These BPs can cause apoptosis of osteoclasts (Hughes *et al*., 1995; Selander *et al*., 1996) and tumor cells *in vitro* (Shipman *et al*., 1997; Aparicio *et al*., 1998). We recently demonstrated that the cytotoxic effect of nitrogen-containing BPs toward macrophage-like J774 cells *in vitro* also is due to induction of apoptosis (Rogers *et al*., 1996). Cells with the nuclear morphology and fragmented DNA characteristic of apoptosis can be detected after treatment with BPs for 24 hr (Rogers *et al*., 1996). In the current study, more detailed analysis of the kinetics of BP-induced apoptosis demonstrated that a significant increase in the proportion of morphologically apoptotic J774 cells occurred after 16 hr of continuous treatment with 100 μM ALN. The appearance of apoptotic cells, with condensed and fragmenting nuclei, was associated with the appearance of caspase-3-like enzyme activity (the ability to cleave the fluorogenic peptide substrate Ac-DEVD-AMC; Nicholson *et al*., 1995).

Exposure of J774 cells to BPs for as little as 2 hr (i.e., with 100 μM PAM), followed by further incubation in BP-free medium, resulted in a similar extent of apoptosis to when cells were treated continuously with BP; apoptosis still occurred after a delay of ~16 hr after the first exposure to BPs. These observations suggest that the delay between the first exposure of the cells to BP (either continuous treatment or a short exposure) and the onset of apoptosis is not due to the time taken to internalize sufficient BP from the extracellular medium (which probably occurs by fluid-phase pinocytosis; Rogers *et al*., 1997). This conclusion is supported by the

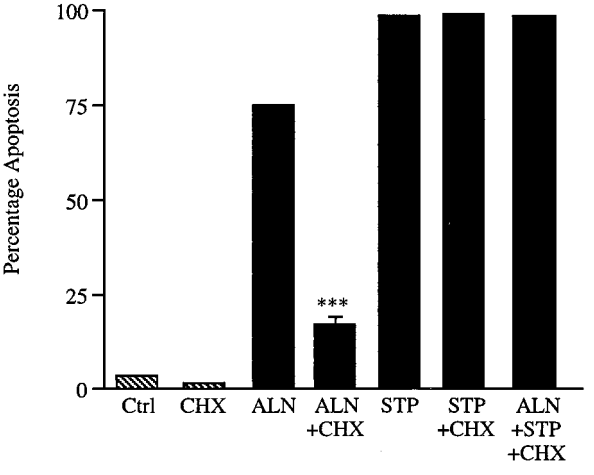

Fig. 6. STP induces apoptosis that cannot be prevented by CHX. J774 cells were treated with 0.25 μM CHX, 100 μM ALN, and 1 μM STP for 48 hr. *Ctrl*, control. The proportion of apoptotic cells then was determined on the basis of nuclear morphology. Values are mean ± standard error ($n = 3$). ***, $p < 0.001$ compared with ALN alone (Student's *t* test). Some error bars are not visible at this scale.

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observation that fluorescently labeled ALN is internalized into endocytic vacuoles by J774 macrophages and by osteoclasts *in vitro* within several minutes (Chestnut *et al.*, 1995). Because we recently proposed that ALN and other nitrogen-containing BPs may cause apoptosis by inhibiting the mevalonate pathway and hence by preventing protein prenylation (Luckman *et al.*, 1998a, 1998b), it is possible that the delay before the appearance of apoptotic cells is dependent on the rate of loss (i.e., the half-life) of prenylated proteins that may promote cell survival or maintain normal cell function, such as Ras or nuclear lamins (Perez-Sala *et al.*, 1994). Other compounds that inhibit the mevalonate pathway and prevent protein prenylation, such as lovastatin, also cause apoptosis after a delay of 12 hr (Perez-Sala *et al.*, 1994) or even 72 hr (Padayatty *et al.*, 1997). We have recently shown that bisphosphonate-induced apoptosis in human myeloma cell lines also occurs after a prolonged delay of ~48 hr (Shipman *et al.*, 1997).

BP-induced apoptosis in J774 cells was found to be dependent on *de novo* gene transcription and protein synthesis because the morphological features of apoptosis, as well as internucleosomal DNA fragmentation, could be completely prevented by 0.25–1 μM CHX or 0.5 nM actinomycin D. Inhibitors of protein synthesis can also prevent chromatin condensation, DNA fragmentation, and loss of cell viability in other cells (Cohen and Duke, 1984; Wyllie *et al.*, 1984), including lovastatin-treated HL-60 cells (Perez-Sala *et al.*, 1995). In agreement with this, we found that apoptosis induced in J774 cells by mevastatin, another inhibitor of protein prenylation, could be prevented by CHX. Thus, inhibition by CHX seems to be a common feature of apoptosis induced by agents that inhibit protein prenylation. This, together with the delayed onset of apoptosis that is observed with either bisphosphonate or mevastatin/lovastatin treatment (Perez-Sala *et al.*, 1994; Padayatty *et al.*, 1997), also supports our recent proposal (Luckman *et al.*, 1998a, 1998b) that the nitrogen-containing BPs such as ALN cause apoptosis by inhibiting protein prenylation.

BP-induced apoptosis was significantly inhibited by CHX when added within 12 hr from the start of treatment with BPs but had no significant effect when added after ≥15 hr from the start of BP treatment. Because apoptotic cells appeared only after ~14 hr and then increased in number throughout 48 hr of culture, the prevention of apoptosis by CHX seems to be the result of inhibition of synthesis of protein or proteins involved in the commitment of J774 cells to apoptosis, rather than inhibition of synthesis of proteins involved in execution of the apoptotic process itself. Medina

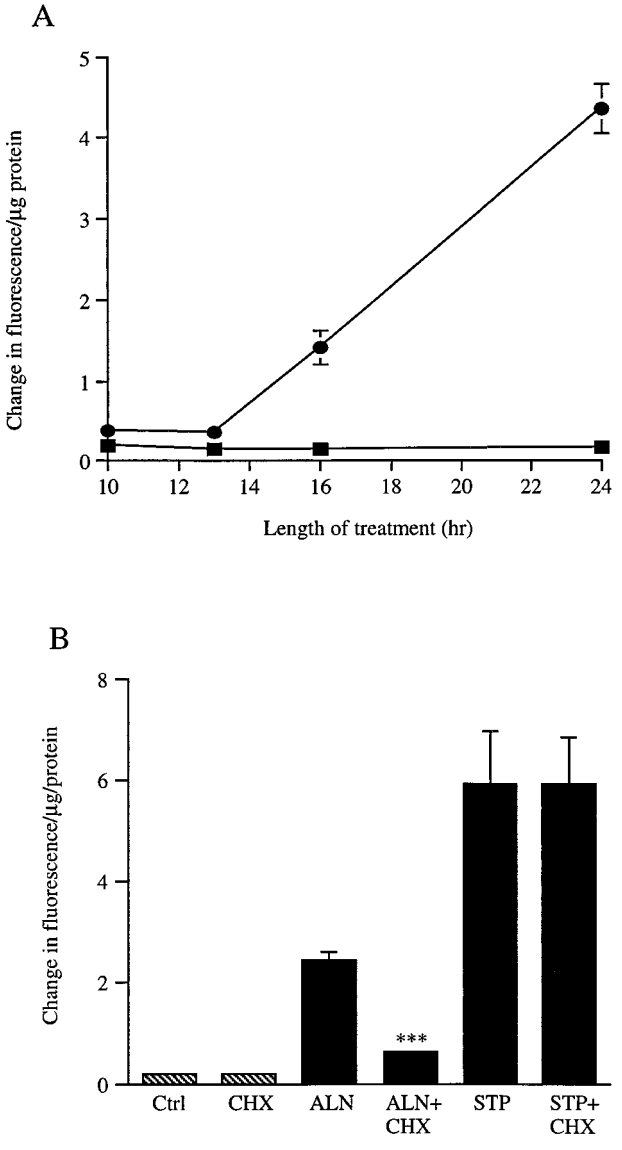

Fig. 7. BP-induced apoptosis is associated with the induction of caspase-3-like activity (A). J774 cells were treated with 100 μM ALN (●) or without ALN (■) for 10, 13, 16, or 24 hr, and then cell lysates were assayed for caspase-3-like activity by measuring proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC using fluorimetry. Values are mean ± standard error (n = 3). Caspase-3-like activity induced by ALN, but not caspase-3-like activity induced by STP, can be inhibited by CHX (B). *Ctrl*, control. J774 cells were treated for 16 hr with 100 μM ALN or 1 μM STP in the presence or absence of 0.5 μM CHX. Cell lysates were assayed for caspase-3-like activity by measuring proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC using fluorimetry. Values are mean ± standard error (n = 3). ***, $p < 0.001$ compared with ALN alone (Student's t test). Some error bars are not visible at this scale.

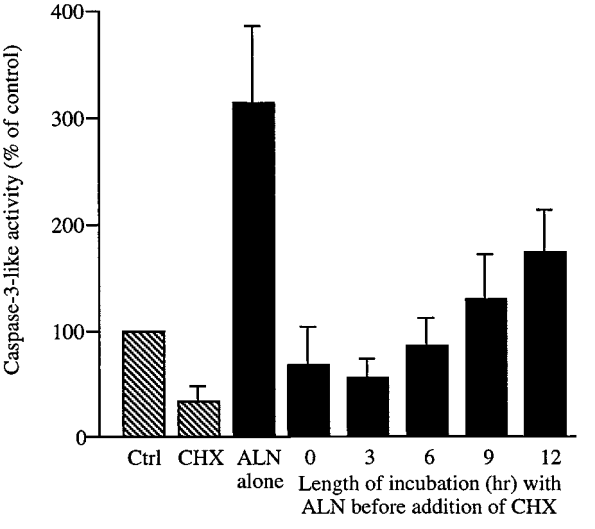

Fig. 8. Inhibition of ALN-induced caspase-3-like activity by CHX is dependent on the time of addition of CHX. *Ctrl*, control. J774 cells were treated with 100 μM ALN for 24 hr, with or without 0.5 μM CHX, which was added to the cells 0–12 hr after the start of ALN-treatment. Cell lysates were assayed for caspase-3-like activity by measuring proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC using fluorimetry. Values are mean ± standard error (n = 3).

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et al. (1997) also found that CHX inhibited butyrate-induced apoptosis in Jurkat cells only when added within 10 hr from the start of treatment and concluded that CHX inhibited commitment to apoptosis. Others also have suggested that CHX inhibits commitment of cells to apoptosis after the treatment of cells with inhibitors of protein prenylation (Borner *et al*., 1995; Perez-Sala *et al*., 1995). Furthermore, we found that the protein kinase C inhibitor STP could increase caspase-3-like activity and induce apoptosis in J774 cells in the presence of CHX (and hence in the absence of protein synthesis, similar to the finding of Weil *et al*., 1996). STP also overcame the inhibitory effect of CHX on ALN-induced apoptosis. These observations confirm that the execution phase of the apoptotic process does not require *de novo* protein synthesis, presuming that the execution phases of STP- and ALN-induced apoptosis are identical.

Our finding that the increase in caspase-3-like activity associated with ALN treatment could be completely prevented in the presence of CHX indicates that protein synthesis is required at a step in the pathway before caspase-3-like enzyme activation, which is concordant with the hypothesis that CHX prevents the commitment of cells to apoptosis. This is supported further by the fact that inhibition of ALN-induced caspase-3-like activity by CHX, like inhibition of apoptosis by CHX, becomes progressively less as the time of addition of CHX is delayed. These observations are in accord with those of Medina *et al*. (1997) and Inayat-Hussain (1997), who also found that CHX prevented activation of caspase-3-like proteases. Although the inhibitory effect of CHX on caspase activation could be due to inhibition of the synthesis of caspase-3-like enzymes (or other caspases that activate caspase-3-like proteases; reviewed by Cohen, 1997), this is unlikely because these enzymes generally are expressed constitutively in proenzyme form and are activated by proteolytic cleavage during apoptosis (Erhardt and Cooper, 1996). This was confirmed by the observation that CHX did not prevent the increase in caspase-3-like activity associated with STP treatment.

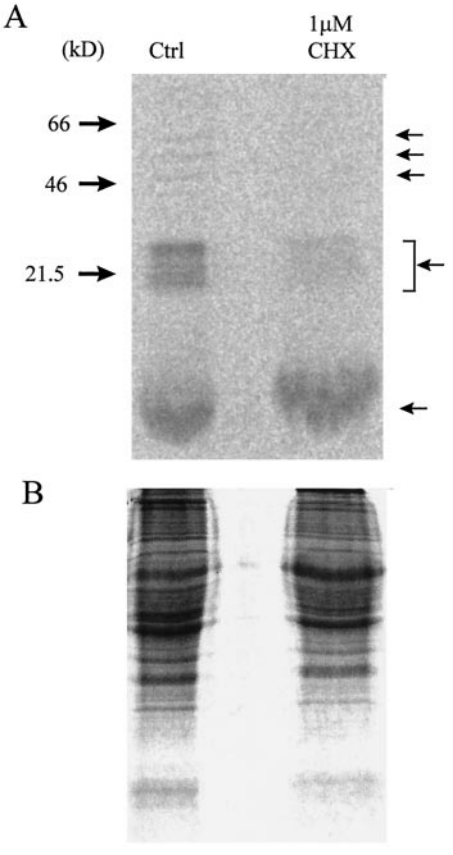

Fig. 9. CHX inhibits the incorporation of [^{14}C]mevalonolactone into proteins. J774 cells were treated with or without 1 μM CHX in the presence of 5 μM mevastatin and 7.5 μCi/well [^{14}C]mevalonolactone for 16 hr; then, lysates were prepared, and equal amounts of protein were electrophoresed on a 12.5% polyacrylamide gel. *Ctrl*, control. Radiolabeled bands were visualized by phosphorimaging (A), with the major labeled bands indicated (*arrows on the right*). Coomassie blue staining of the gel demonstrates that wells were equally loaded (B).

It is possible that the caspase-3-like enzyme activated after ALN treatment was actually caspase-7; Marcelli *et al*. (1998) recently reported that lovastatin-induced apoptosis in LNCaP prostate cancer cells is associated with activation of caspase-7 (which can cleave Ac-DEVD-AMC but to a lesser extent than caspase 3). Nevertheless, our observations, together with those of others (Borner *et al*., 1995; Perez-Sala *et al*., 1995; Marcelli *et al*., 1998), clearly demonstrate that activation of caspase-3-like protease or proteases and inhibition of commitment to apoptosis by CHX are common features of apoptotic cell death induced by inhibitors of protein prenylation.

The exact step of the apoptotic pathway that is affected by CHX remains to be identified. We and others have shown previously that the treatment of cells with inhibitors of protein prenylation such as lovastatin or ALN leads to accumulation of the nonprenylated forms of proteins such as Ras that would normally be prenylated (Luckman *et al*., 1998a; Repko and Maltese, 1989). Lerner *et al*. (1995) suggested that accumulation of nonprenylated, oncogenic Ras has a dominant negative effect on Ras signaling due to the sequestration of Raf (a Ras effector) in the cytoplasm. Hence, apoptosis of J774 cells induced by BPs or mevastatin may be a consequence of the accumulation of nonprenylated proteins such as Ras in the cytoplasm. Proteins normally are prenylated immediately after synthesis; Repko and Maltese (1989) demonstrated that there is no incorporation of [^{14}C]mevalonolactone (the precursor of isoprenoid groups) into proteins in the presence of CHX. We also found that 1 μM CHX effectively prevented the incorporation of [^{14}C]mevalonolactone into proteins in J774 cells (i.e., prevented the synthesis of proteins that would normally be prenylated). CHX therefore may prevent BP- and mevastatin-induced J774 apoptosis by preventing the accumulation of nonprenylated proteins. In support of this, CHX has been shown to prevent other effects associated with inhibition of prenylation, such as breakdown of the actin cytoskeleton after treatment of the cells with lovastatin (Fenton *et al*., 1992; Koch *et al*., 1997). Furthermore, Koch *et al*. (1997) demonstrated that the inhibitory effect of CHX is not downstream of prenylated proteins. This supports the idea that CHX also inhibits apoptosis induced by inhibitors of prenylation at the level of prenylated proteins rather than at a later stage in the apoptotic pathway.

Further studies are required to elucidate the exact mechanism by which CHX prevents BP-induced apoptosis. We expect that further characterization of the molecular events involved in the apoptotic cascade in J774 macrophages after BP treatment will lead to identification of the molecular targets for nitrogen-containing BP drugs, an issue that has

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remained unresolved since the discovery of this subclass of BPs >12 years ago (Shinoda *et al.*, 1983).

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