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Caffeic Acid Phenethyl Ester, an Active Component of Honeybee Propolis Attenuates Osteoclastogenesis and Bone Resorption Via the Suppression of RANKL-Induced NF-κB and NFAT Activity

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Receptor activator NF-κB ligand (RANKL)-activated signaling is essential for osteoclast differentiation, activation and survival. Caffeic acid phenethyl ester (CAPE), a natural NF-κB inhibitor from honeybee propolis has been shown to have anti-tumor and anti-inflammatory properties. In this study, we investigated the effect of CAPE on the regulation of RANKL-induced osteoclastogenesis, bone resorption and signaling pathways. Low concentrations of CAPE (<1 μM) dose dependently inhibited RANKL-induced osteoclastogenesis in RAW264.7 cell and bone marrow macrophage (BMM) cultures, as well as decreasing the capacity of human osteoclasts to resorb bone. CAPE inhibited both constitutive and RANKL-induced NF-κB and NFAT activation, concomitant with delayed IκBα degradation and inhibition of p65 nuclear translocation. At higher concentrations, CAPE induced apoptosis and caspase 3 activities of RAW264.7 and disrupts the microtubule network in osteoclast like (OCL) cells. Taken together, our findings demonstrate that inhibition of NF-κB and NFAT activation by CAPE results in the attenuation of osteoclastogenesis and bone resorption, implying that CAPE is a potential treatment for osteolytic bone diseases.

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Osteoclasts are multinucleated bone-resorbing cells derived from precursors of the monocyte-macrophage lineage (Teitelbaum, 2000). The transcription factor nuclear factor kappa B (NF-κB) is known to play a pivotal role in osteoclast differentiation and survival (Xing et al., 2002; Novack et al., 2003; Chaisson et al., 2004; Dai et al., 2004; Ruocco et al., 2005; Xu et al., 2009). Most notably, mice lacking NF-κB p50/p52 are osteopetrotic due to a deficiency in osteoclastogenesis (Franzoso et al., 1997; Iotsova et al., 1997). Receptor activator of NF-kappa B ligand (RANKL) is a key factor in the differentiation and activation of osteoclasts in vitro and ex vivo (Lacey et al., 1998; Burgess et al., 1999; Kong et al., 1999; Xu et al., 2000). RANKL interacts with its receptor RANK, resulting in the recruitment of TNF receptor associated factor (TRAF) adapter proteins and activation of signaling pathways including NF-κB, JNK, ERK, and nuclear factor of activated T cells (NFAT) (Lee et al., 2003). Along the NF-κB pathway, RANKL induces the DNA binding of C-Rel, NF-κB1 (p50), and RelA (p65) NF-κB complexes (Wang et al., 2003). In addition, elevated activation of NF-κB is associated with lipopolysaccharide-induced bone destruction (Yip et al., 2004) and debris particle-induced periprosthetic osteolysis (Schwarz et al., 2000). It is therefore reasonable to propose that targeted modulation of NF-κB activity may prevent osteolysis that is secondary to enhanced osteoclast formation and activation.

Honeybee propolis is currently being used in a number of natural health products. Thus there is a growing need for evidence and mechanistic-based information to guide the use of honeybee propolis products for the prevention and treatment of osteolytic bone diseases. Caffeic acid phenethyl ester (CAPE), a phenolic antioxidant derived from the propolis of honeybee hives, is a potent and specific inhibitor of NF-κB and NFAT activation (Natarajan et al., 1996; Marquez et al., 2004). It is a natural compound derived from the bark of conifer trees and carried by honeybees to their hives (Natarajan et al., 1996; Marquez et al., 2004). CAPE has been shown to have anti-tumor (Chiao et al., 1995), anti-inflammatory (Michaluart et al., 1999; Orban et al., 2000), antiviral (Burke et al., 1995) and immunomodulatory properties (Natarajan et al., 1996).

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However, the action of CAPE on osteoclast formation, bone resorption and RANKL-activated signaling pathways remains ill defined.

In this study, we investigated the role of CAPE in the regulation of RANKL-induced osteoclastogenesis, osteoclast survival and function. Interestingly, we found that CAPE inhibits osteoclastogenesis and bone resorption via the suppression of RANKL-induced NF-κB and NFAT activities. Our findings suggest that CAPE might be a prototype for drugs that prevent osteolysis that is caused by enhanced osteoclast formation and activation.

Materials and Methods

Media and reagents

RAW264.7 cells were obtained from the American Type Culture Collection (Rockville, MD). Alpha Modification of Eagles Medium (α-MEM) and fetal bovine serum (FBS) were purchased from TRACE (Sydney, Australia). Caffeic acid phenethyl ester (CAPE) was purchased from Sigma–Aldrich (Sydney, Australia). The luciferase assay system was obtained from Promega (Sydney, Australia). Annexin V-PE apoptosis staining reagents was purchased from BD Biosciences PTY Ltd (Sydney, Australia). GST-rRANKL was generated as previously described (Xu et al., 2000).

In vitro osteoclastogenesis assay

For primary cell cultures, bone marrow cells isolated from C57/BL6 mice were cultured for 3 days in α-MEM supplemented with 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (complete medium) with the addition of 10 ng/ml macrophage-colony stimulating factor (M-CSF). Cells were kept in a humidified 37°C incubator that was gassed with 95% air/5% CO_2. Bone marrow derived macrophages (BMM) were then seeded (8×10^3 cells/well) into 96-well plates and incubated overnight before stimulation with RANKL (100 ng/ml) in the absence or presence of various concentrations of CAPE. Culture medium was replenished every second day. After 7 days culture, cells were fixed with 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRACP) activity (Xu et al., 2000). TRACP-positive multinucleated cells with >3 nuclei were scored as osteoclasts.

RAW264.7 cells were seeded (1.3×10^3 cells/well) in complete medium into 96-well plates and incubated overnight before stimulation with fresh medium containing RANKL (100 ng/ml) and various doses of CAPE. Medium was replaced every 2 days and after 5–7 days of culture, cells were fixed, TRACP stained and scored as described above.

Bone resorption pit assay

To examine the effect of CAPE on osteoclastic-bone resorption, human OCL cells isolated from giant cell tumor (GCT) of bone as previously described were used (Huang et al., 2000). Briefly, human GCT samples were freshly isolated from patients who have undergone surgery at the Sir Charles Gairdner Hospital (Nedlands, Western Australia). Tumor tissues were finely chopped in complete medium and the resultant cell suspension was passed through a 100 μm nylon cell strainer (BD Bioscience, MA), and approximately 200 OCL cells were seeded onto bovine bone slices (Huang et al., 2000). After cell attachment, bovine bone slices were either treated with CAPE (0.5 μM) or left untreated. After 48 h at 37°C incubation, bovine bone slices were further incubated for 2 h in 2 M NaOH and cells were removed by mechanical agitation and sonication. Resorption pits were visualized under a Philips XL30 scanning electron microscope and the percentage of bone surface area resorbed quantified using Scion Image software (Scion Cooperation, National Institute of Health, USA) (Yip et al., 2006).

NF-κB and NFAT activation in RAW264.7 cells

To examine the effects of CAPE on RANKL-induced NFAT and NF-κB activation, RAW264.7 cells were transiently transfected with luciferase reporter genes (p-NF-κB-TA-Luc, p-NFAT-TA-Luc, pTA-Luc (Clontech, Mercury Pathway Profiling System) or pGL3-promoter (Promega)) as previously described (Wang et al., 2003; Xu et al., 2003; Ang et al., 2007). Cells were seeded (3×10^5 cells/well) into 24-well plates, incubated at 37°C for 18 h, treated with CAPE (0.5, 1, or 2 μM) for 1 h and then stimulated with RANKL (100 ng/ml) for a further 8 h. Luciferase activities were measured in cell lysates using the Promega Luciferase Assay System according the manufacturer's instructions (Promega).

Immunoblotting analysis of IκBα

Proteins in extracts from appropriately treated RAW264.7 cells were separated by SDS–PAGE and electro-blotted onto nitrocellulose membranes (Bio-Rad, New South Wales, Australia). Membranes were blocked with 5% (w/v) skim milk powder (SMP) in 10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% [v/v] Tween-20 (TBST) and probed with primary antibodies for IκBα or α-tubulin (1:1,000: Santa Cruz Biotechnology, Inc., CA) in 1% (w/v) SMP in TBST. After three washes with TBST, membranes were incubated with HRP-conjugated secondary antibodies diluted 1:5,000 in 1% (w/v) SMP in TBST. The membranes were then developed using an ECL system (Amersham Pharmacia Biotechnology, Sydney, Australia).

p65 immunohistochemistry

BMM were seeded (2×10^4/well in 100 μl) into 96-well plates and incubated overnight. Cells were then pre-incubated with CAPE (1 or 5 μM) for 1 h before stimulation for up to 30 min with RANKL (100 ng/ml). After washing with 1 × PBS, cells were fixed with 4% paraformaldehyde for 20 min, washed three more times with 1 × PBS and then permeablized for 5 min with 0.1% Triton X-100 in PBS. Following two washes with 0.1% BSA-PBS, cells were incubated at 37°C for 45 min with 50 μl of anti-p65 antibody (Santa Cruz Biotechnology, Inc.) diluted 1:200 in 0.1% BSA-PBS. The cells were then washed four times with 0.1% BSA-PBS, four times with 1 × PBS, once with 0.1% BSA-PBS before the addition of streptavidin-horseradish peroxidase (Dako, Victoria, Australia). After 20 min incubation at room temperature and wash (four times with 0.1% BSA-PBS, four times with PBS, once with 0.1% BSA-PBS), Dako Liquid Dab (20 μl: Dako) was added for 30 min or until brown coloration appeared (Ang et al., 2009).

Confocal microscopy

For immunofluorescence studies, RAW264.7 cells (1×10^4) were seeded onto 13 mm glass coverslips in 24-well plates. After 24 h of incubation, cells were stimulated with RANKL (100 ng/ml) for 5 days to induce osteoclast formation. OCL cells were treated for 18 h with or without CAPE (10 μM), incubated overnight, washed twice with 1 × PBS, fixed for 15 min at room temperature with 4% paraformaldehyde in 1 × PBS (pH 7.4) and then washed extensively in 1 × PBS. Cells were then permeabilized in 1 × PBS containing 0.1% Triton X-100, washed twice in 1 × PBS containing 0.2% BSA (0.2% BSA-PBS) and incubated for 1 h at room temperature with a monoclonal anti-α-tubulin antibody (Sigma, St. Louis, MO) diluted 1:500 with 0.2% BSA-PBS. Following washing (4 × with 0.2% BSA-PBS, 4× with PBS and 2× with 0.2% BSA-PBS), cells were labeled for 45 min at room temperature with an Alexa Fluor 488 secondary antibody (Molecular Probes, Inc., Eugene, OR) diluted 1:500 in 0.2% BSA-PBS. Cells were then washed in 0.2% BSA-PBS and PBS as above, counter-stained for 3 min at room temperature with Hoechst 33258 (1:10,000: Molecular Probes, Inc., VIC, Australia), and then mounted for confocal microscopy (MRC-1000, Bio-Rad, CA) as previously described (Pavlos et al., 2005).

Apoptosis assay

Apoptosis assays were performed as previously described (Xu et al., 2003). In brief, RAW264.7 cells were seeded (5×10^5 cells per well) in 3 ml of complete medium into six-well plates, and left overnight before exposure to various doses of CAPE for 24 h. Cells were harvested and then resuspended in 0.5 ml of Annexin V Binding Buffer (BD-Pharmigen, NSW, Australia). Microfuge tubes containing resuspended cells (100 μl), Annexin V-PE (5 μl: BD-Pharmigen) and 7-Amino-Actinomycin (7-AAD) (5 μl: BD-Pharmigen) were gently vortexed and then incubated for 15 min at room temperature in the dark. Binding buffer (400 μl: BD-Pharmigen) was added to each tube and within 1 h, 10,000 cells analyzed by flow cytometry (Becton Dickinson FACSCalibur®, New South Wales, Australia). Results were expressed as the percentage of apoptotic cells within the population.

Caspase-3 assay

RAW264.7 cells were seeded (2×10^6 cells/well) into 24-well plates, cultured overnight, treated with various doses of CAPE for 24 h and then caspase 3 activities were measured as previously described (Yip et al., 2005). In brief, cells were harvested by trypsinization and vigorous pipetting, washed once in 1 × PBS and then lysed by three freeze thaw cycles in 20 μl of buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml pepstatin A and 10 μg/ml leupeptin. The protein content of the lysate was determined by the Bradford assay (Bio-Rad, New South Wales, Australia). The caspase-3 activities of lysates were determined using a kinetic assay, in buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA and 10% glycerol, by monitoring the cleavage of acetyl-DEVD-AFC in the presence or absence of the caspase-3 inhibitor Ac-DEVD-CHO (1 μM) (Promega). The changes in AFC fluorescence were measured at 510 nm after excitation at 400 nm in a multifunctional microplate reader (POLARstar OPTIMA, BMG, Germany).

Statistical analyses

Data presented were representative results from one of three independent experiments or the mean ± SEM of those experiments. Student's *t*-test was used to determine statistical significance between groups. A *P*-value of <0.05 was considered to be statistically significant.

Results

CAPE dose-dependently inhibits RANKL-induced osteoclastogenesis

BMM cells cultured in the presence of RANKL alone form multinucleated TRACP-positive OCL cells (Fig. 1A). However, BMM or RAW264.7 cells treated with CAPE demonstrated a dose-dependent inhibition of RANKL induced osteoclast formation (Fig. 1A–C). In addition, treatment of RANKL stimulated BMM or RAW264.7 cells with CAPE resulted in the formation of OCL cells that were smaller in size compared to those cultured in the presence of RANKL only (Fig. 1A and results not shown). Notably, higher concentrations of CAPE

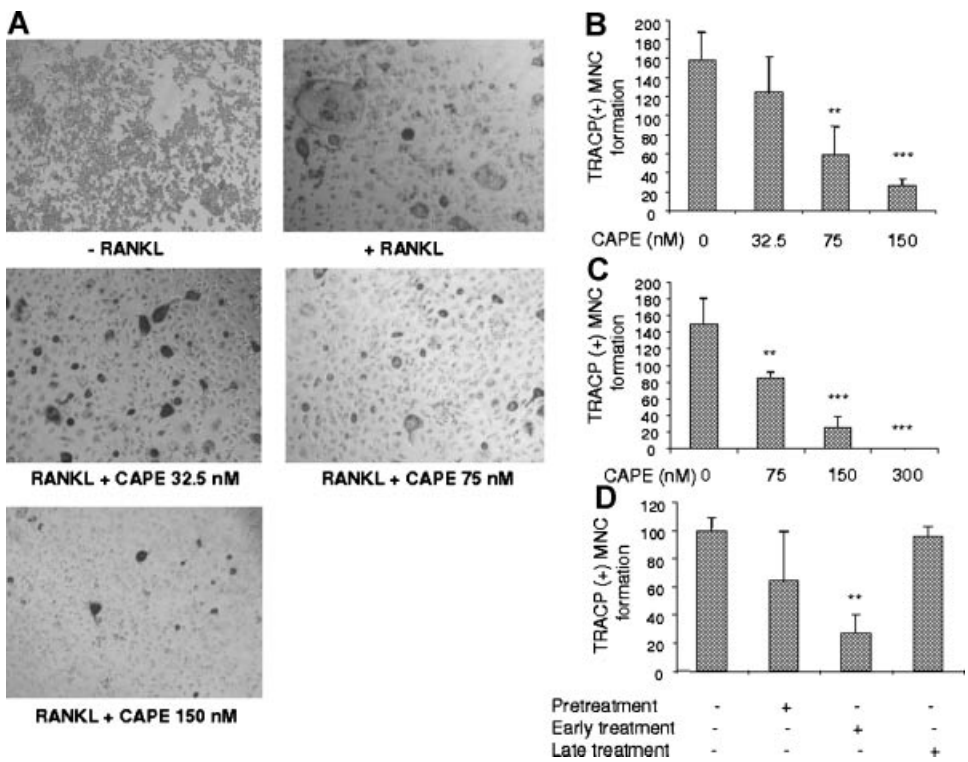

Fig. 1. CAPE inhibits RANKL-induced osteoclastogenesis. A,B: BMM and (C) RAW264.7 cells cultured in 96-well plates in the presence of RANKL (100 ng/ml) with or without CAPE (0–300 nM) for 7 days were fixed with 4% paraformaldehyde and TRACP positive multinucleated cells (MNC) with more than 3 nuclei counted. D: RAW264.7 cells were cultured for 5 days with RANKL (100 ng/ml) alone or were treated with CAPE (100 nM) after 1 or 2 days (early treatment) or after 3 or 4 days (late treatment) of the RANKL treatment. Additional RAW264.7 cell cultures were treated with CAPE for 24 h before 5 days of RANKL (100 ng/ml) treatment (pretreatment). Cells were fixed and counted as described above. Representative images from one of three experiments are shown. Values are expressed as mean ± SEM ($P < 0.01$, ***$P < 0.001$ compared to control).**

(1 μM to 10 μM) resulted in cellular detachment (results not shown).

In order to determine at which stage of osteoclast differentiation CAPE exerted its inhibitory effect, RANKL-stimulated RAW264.7 cells were treated with CAPE (100 nM) at various times points during osteoclast formation. CAPE almost exclusively inhibited RANKL-induced osteoclastogenesis when added in the early stages (i.e., 1–2 days) of osteoclast development (Fig. 1D). The pretreatment group also showed a trend towards inhibition of osteoclastogenesis. In contrast, CAPE had no inhibitory effect on committed pre-osteoclastic cells in the late treatment group, indicating that its specific inhibitory activity targets the early phases of osteoclast formation.

CAPE suppresses bone resorption in vitro

To investigate whether CAPE had an inhibitory effect on pathological human osteoclastic bone resorption, we isolated OCL cells from patients presenting with Giant cell tumors of bone. CAPE (0.5 μM) potently suppressed osteoclastic bone resorption (Fig. 2A,B). Importantly, this decreased bone resorption did not simply reflect apoptosis of osteoclastic cells as we observed no significant differences in the total number of TRACP-positive cells per bone slice at the end of the experiment (Fig. 2C). Together, these data indicate that in addition to their inhibitory effects on osteoclast formation, CAPE blocks osteoclastic resorptive function in pathological conditions.

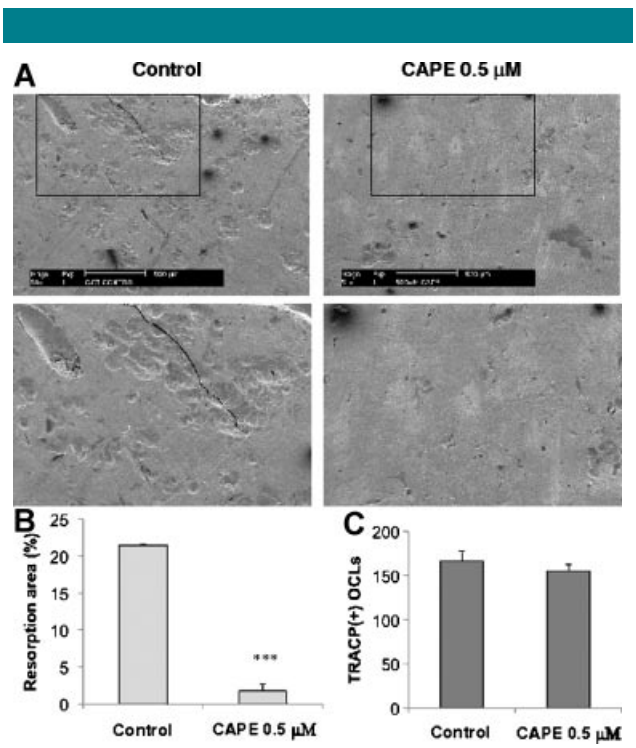

Fig. 2. CAPE suppresses bone resorption. Equal numbers of human OCL cells were seeded onto bovine slices and allowed to recover and attach for 3 h prior to 48 h of CAPE (0.5 μM) exposure. A: Representative images of bone resorption pits taken with a scanning electron microscope. B: Pit area expressed as a percentage of total bone area (mean ± SEM, $^{***}P < 0.001$). C: Total number of OCL cells per bone slice.

CAPE suppresses RANKL-induced activation of NF-κB and delays IκBα degradation

Bone homeostasis is regulated by cytokines such as RANKL by activating the NF-κB signaling pathways in osteoclastic cells (Xu et al., 2009). To explore the effects of CAPE on NF-κB signaling, RAW264.7 cells were transiently transfected with an NF-κB-driven luciferase reporter construct. As expected, RANKL induced a ninefold increase in NF-κB promoter driven luciferase gene expression compared to RAW264.7 cells cultured in medium without RANKL (Fig. 3A). Treatment of RAW264.7 cells with CAPE (0.5, 1, or 2 μM) strongly inhibited RANKL-induced NF-κB transcriptional activation. CAPE treatment also significantly suppressed constitutive levels of NF-κB activity in RAW264.7 cells (Fig. 3A).

Given the effect of CAPE on RANKL-induced NF-κB activation, we next examined its ability to block RANKL-induced IκBα-degradation. Cells treated with RANKL showed maximal loss of IκBα after 20 min followed by an NF-κB driven resynthesis of IκBα at 60 min. Preincubation with CAPE (1 μM) delayed the RANKL induced degradation of IκBα to 60 min (Fig. 3B).

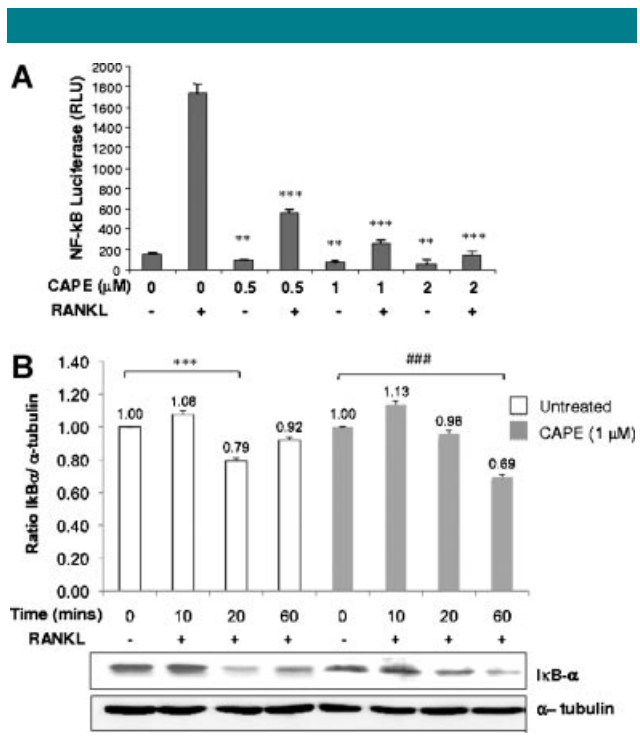

Fig. 3. CAPE suppresses RANKL-induced NF-κB-dependent transcription and delays IκBα degradation. A: RAW264.7 cells, transiently transfected with the p-NF-κB-TA-Luc reporter gene, were pretreated with CAPE (0, 0.5, 1, and 2 μM) for 1 h and then exposed to RANKL (100 ng/ml) or medium alone for 8 h before luciferase activity was measured. Results are expressed as means ± SEM of triplicate determinations ($^{**}P < 0.01$, compared to untreated control, t-test; $^{***}P < 0.001$, compared to RANKL treated cells, t-test). B: Whole cell extracts from RAW264.7 cells were pretreated with CAPE (1 μM) or left untreated for 2 h followed by treatment with RANKL (100 ng/ml) for 10, 20, and 60 min were subject to Western Blot analysis. The IκBα and α-tubulin bands were quantified using densitometry and the IκBα band intensity was divided by the α-tubulin band intensity. Ratios were then normalized to the unstimulated control ratio. The blot is a representative of three independent experiments ($^{***}P < 0.001$, compared to unstimulated controls in the absence of CAPE, t-test; $^{###}P < 0.001$, compared to RANKL treated cells in the presence of CAPE, t-test).

CAPE suppresses RANKL-induced p65 nuclear translocation

To further explore the influence of CAPE on NF-κB activity, we next evaluated the effect of CAPE on RANKL-mediated p65 nuclear translocation in BMMs. As demonstrated in Figure 4, pre-treatment of BMMs with 1 or 5 μM CAPE markedly decrease the nuclear translocation of p65 upon RANKL stimulation, as compared to vehicle pre-treated control cells (compare Fig. 4C,D–B).

CAPE inhibits RANKL-induced NFAT activation

In addition to its activation of NF-κB, RANKL signals via the calcium/calmodulin-dependent phosphatase, calcineurin, to the nuclear factor of activated T cells (NFAT) signaling pathway, which is now recognized as important in osteoclastogenesis (Hirotani et al., 2004; Matsuo et al., 2004). To examine the potential effect of CAPE on RANKL induction of NFAT signaling, RAW264.7 cells were transiently transfected with an NFAT-luciferase reporter gene construct. RANKL induced NFAT transcriptional activity by two folds whereas 1 h pretreatment with CAPE (0.5, 1, or 2 μM) markedly suppressed RANKL-induced NFAT activation (Fig. 5). CAPE also suppressed constitutive level of NFAT transcriptional activity in RAW264.7 cells, similar to its effects on constitutive NF-κB activity. This suppression of constitutive levels of NFAT and NF-κB transcriptional activity was also consistent with findings by Marquez et al. (2004).

The suppression of NF-κB and NFAT transcriptional activity seen with 0.5 and 1 μM CAPE treatment of RAW264.7 cells is unlikely to be due to a cytotoxic effect (such as necrosis or apoptosis) as these concentrations CAPE had no effect on constitutive transcriptional activity driven by the SV40 or TATA promoters (Supplementary Fig. S1). However, at 2 μM CAPE suppressed constitutive transcriptional activity in RAW264.7 cells by 40% suggesting that it may be toxic at this concentration (Supplementary Fig. S1).

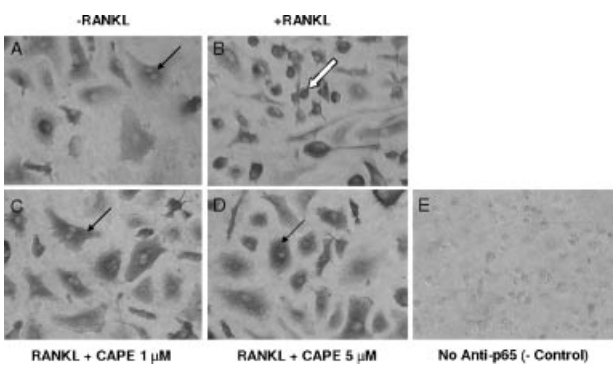

Fig. 4. CAPE inhibits RANKL-induced p65 nuclear translocation. BMM cells were pre-incubated with CAPE (1 and 5 μM) for 1 h followed by 30 min of RANKL (100 ng/ml) stimulation. The cells were fixed with 4% paraformaldehyde and stained with an anti-p65 antibody. A,B: Treatment with RANKL induces p65 translocation from the cytoplasm to the nucleus. C,D: RANKL-induced nuclear translocation was inhibited by pre-treatment with CAPE. E: Negative control cells treated as in (B), but in the absence of the anti-p65 antibody. Black arrows indicate the localization of p65 in the cytoplasm, and white arrow specifies the presence of p65 in the nucleus.

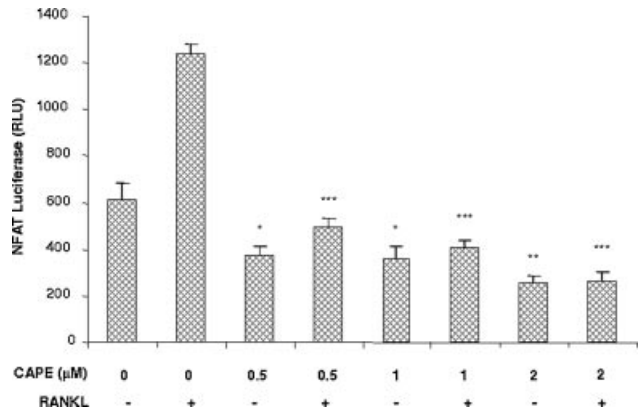

Fig. 5. CAPE suppresses RANKL-induced NFAT-dependent transcription in RAW264.7 cells. RAW264.7 cells, transiently expressing an *NFAT-luc* reporter gene construct (p-NFAT-TA-Luc), were left untreated, treated with or without RANKL (100 ng/ml), and with CAPE at 0.5, 1, and 2 μM for 8 h. Luciferase activities are the mean ± SEM of triplicate determinations ($^{*}P < 0.05$, $^{**}P < 0.01$ compared to unstimulated control, *t*-test; $^{***}P < 0.001$, compared to RANKL treated cells, *t*-test).

CAPE induces apoptosis of OCL cells

Since 2 μM CAPE suppressed constitutive transcriptional activity from NF-κB, NFAT, SV40, and TATA reporter genes (Figs. 3A, 5, and Fig. S1 respectively) we examined whether CAPE might induce OCL cell apoptosis. In the absence of CAPE, cells exhibited characteristic osteoclast morphology; including well-spread cytoplasm and intact nuclei (Fig. 6A). OCL cells subjected to 10 μM CAPE, however, exhibited membrane retraction, the formation of large cytoplasmic vacuoles and membrane blebbing, indicative of cellular apoptosis (Fig. 6B).

To investigate the morphological aberrations in greater detail, OCL cells were doubled stained with an antibody specific for α-tubulin and with Hoescht 33258 to visualize the cytoskeleton and nuclei respectively. Untreated osteoclasts display a typical well-organized cytoskeleton (Fig. 6C,E) and intact microtubule filaments radiating from each osteoclast nuclei (Fig. 6G). By comparison, osteoclasts treated with CAPE exhibited a disrupted microtubule-staining pattern with little microtubule organization and increased diffuse cytosolic staining, indicative of increased microtubule depolymerization (Fig. 6D,F). In addition, OCL cells treated with CAPE exhibited nuclear fragmentation consistent with apoptosis (Fig. 6H).

To confirm that the observed morphological effects were due to apoptosis and did not simply reflect cellular necrosis, we next examined the effect of various concentrations of CAPE on Annexin V-PE staining and caspase-3 activation. FACS analysis was carried out on cells stained with both Annexin V-PE and 7-AAD. Cells that were stained positive for Annexin V-PE and negative for 7-AAD accounted for early apoptosis, whereas cells, which were positive for both Annexin V-PE and 7-AAD, were considered to be undergoing late apoptosis/necrosis. Rates of apoptosis were low in RAW264.7 cells left either untreated or treated with 0.1 μM CAPE (Fig. 7A,B). However, at concentrations of CAPE higher than 1 μM, rates of apoptosis increased markedly, with more than 50% of cells undergoing early apoptosis at doses of 5 μM or above (Fig. 7A,B). Caspase-3 activity of RAW264.7 cells was not altered by 24 h of exposure to 0.1 μM CAPE but was significantly elevated by 1 μM or 5 μM CAPE treatment (Fig. 7C).

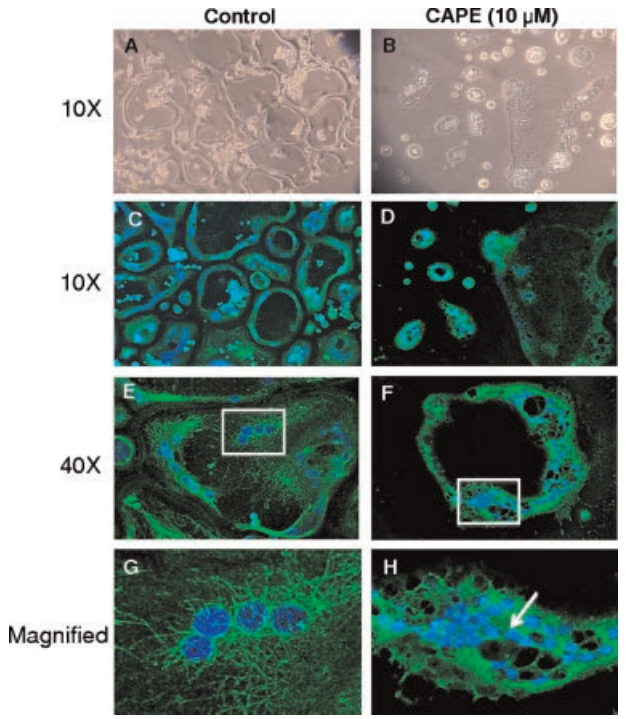

Fig. 6. CAPE induces apoptosis in OCL cells. Microscopic images of RAW264.7 cell derived OCL cells; either (A) untreated or (B) treated overnight with 10 μM CAPE. Similarly treated OCL cells were fixed with 4% paraformaldehyde and stained with Hoechst 33258 dye and α-tubulin antibody. C–H: Confocal images showing the staining of nuclei and α-tubulin structure. White boxes represent areas magnified in (G,H). White arrowhead shows nuclei fragmentation and the disruption of α-tubulin.

Discussion

In this study, we document the inhibitory effect(s) of CAPE on osteoclast formation and function through the suppression of RANKL-induced activation of NF-κB and NFAT. In addition, we found that the impact of CAPE on osteoclastogenesis occurs predominantly at the early stages of cellular differentiation. These observations mirror previous studies using selective modulators of NF-κB, TPA and (−)-DHMEQ, which largely inhibited osteoclastogenesis at an early stage of differentiation via suppression of RANKL-induced NF-κB activation (Wang et al., 2003; Takatsuna et al., 2005). Thus, the present findings add further weight to the notion that modulation of NF-κB is crucial for the initial stages of RANKL-induced osteoclast formation.

Previous studies have shown that CAPE can block the activation of NF-κB induced by TNF-α, phorbol esters, ceramide, okadaic acid, and hydrogen peroxide (Natarajan et al., 1996). Unlike classical NF-κB inhibitors, CAPE does not inhibit NF-κB activation by blocking IκB degradation (Natarajan et al., 1996; Marquez et al., 2004). CAPE does, however, delay IκBα resynthesis, as IκBα is transcriptionally regulated by NF-κB, so this is presumably a secondary effect (Natarajan et al., 1996). Several studies indicate that CAPE inhibits NF-κB activation by suppressing the interaction of NF-κB proteins with NF-κB binding motifs in DNA (Natarajan et al., 1996; Marquez et al., 2004). In our study, however, we revealed that CAPE inhibits RANKL-induced NF-κB activation and p65 nuclear translocation, which is consistent with the findings of Marquez et al. (2004).

Osteoclast apoptosis influences the life span of osteoclasts, which in turn alters osteoclastic bone resorption and remodeling. In this study, we found that CAPE is capable of initiating the onset of apoptosis in RAW264.7 cells and RAW264.7 cell-derived OCL cells. Consistently, osteoclasts treated with CAPE, displayed morphological hallmarks of apoptosis including the disruption of the microtubule network and nuclear fragmentation. Recent studies have demonstrated

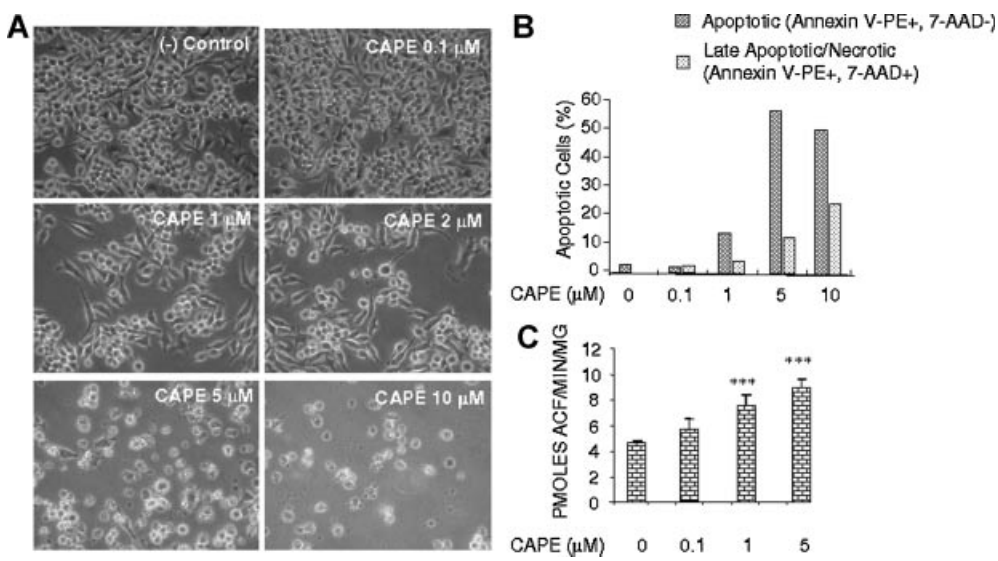

Fig. 7. CAPE dose dependently induces apoptosis of OCL cells. A: Light field microscopic images showing the effect of CAPE on RAW264.7 cells. B: RAW246.7 cells treated with varying concentrations of CAPE for 24 h were harvested, double-stained with Annexin V-PE and 7-AAD and 10,000 cells subjected to analysis by flow cytometry. Results, representative of three independent experiments, show the percentage of the total cell population displaying early apoptotic (Annexin V-PE (+), 7-AAD (−)) or necrotic/late apoptotic (Annexin V-PE and 7-AAD (+)) phenotypes. C: Caspase-3 activity of RAW264.7 cell lysates following 24 h CAPE treatment. (*$P < 0.001$, compared to untreated controls).**

that CAPE induces apoptosis in other cell types including human prostate cancer cell line (PC3) and breast cancer MCF-7 cells (Hung et al., 2003; McEleny et al., 2004; Watabe et al., 2004). It has been suggested that NF-κB inhibition underlies CAPE-induced apoptosis (Watabe et al., 2004). The role of NF-κB in suppressing apoptosis is well established (Kucharczak et al., 2003). In mice lacking RelA and c-Rel, B-lymphocytes are susceptible to apoptosis when induced by mitogens (Grossmann et al., 2000). NF-κB is most commonly involved in suppressing apoptosis by inducing the expression of anti-apoptotic genes which include inhibitor of apoptosis proteins (IAPs), TRAF1, TRAF2, Bcl-2 family, A20, and c-FLIP (Pahl, 1999; Kucharczak et al., 2003). The disruption of the microtubule network could negatively impact p65 nuclear translocation and thus in turn affect the NF-κB signaling pathway. Therefore, the suppression of NF-κB activity and stimulation of caspase-3 activity by CAPE is likely to be important in the induction of osteoclast apoptosis.

There in now increasing evidence indicating that the RANKL-induced NFAT signaling pathway plays a critical role in osteoclastogenesis (Hirotani et al., 2004; Matsuo et al., 2004). In this study, we have demonstrated that CAPE inhibited NFAT activity and hence this effect may contribute to the inhibitory action of CAPE on osteoclastogenesis. Consistent with this position, CAPE has been found to inhibit T-cell receptor-mediated T-cell activation by targeting both NF-κB and NFAT (Marquez et al., 2004). Furthermore, recent studies demonstrate that inhibition of NF-κB itself leads to the down-regulation of NFAT (Takatsuna et al., 2005). Whether the inhibitory effect of CAPE on RANKL-induced NFAT activation is an NF-κB-dependent or -independent event remains to be seen. In addition the effect of CAPE on the Akt, p38, ERK, and c-Jun pathways, which have all been implicated in osteoclast differentiation, warrants investigation (Matsumoto et al., 2000; Hotokezaka et al., 2002; Lee and Kim, 2003).

In summary, this study documents for the first time, that CAPE, via suppression of RANKL-induced NF-κB and NFAT activation has, dual effects on osteoclasts, inhibiting osteoclastogenesis as well as inducing apoptosis. Given that many pathological bone diseases are associated with enhanced osteoclast formation and activation, our studies imply that this remarkable natural compound might be useful for the prevention or treatment of osteolytic bone diseases.

Acknowledgments

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