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Phytoestrogens Directly Inhibit TNF-α-Induced Bone Resorption in RAW264.7 Cells by Suppressing *c-fos*-Induced *NFATc1* Expression

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ABSTRACT

TNF-α-induced osteoclastogenesis is central to post-menopausal and inflammatory bone loss, however, the effect of phytoestrogens on TNF-α-induced bone resorption has not been studied. The phytoestrogens genistein, daidzein, and coumestrol directly suppressed TNF-α-induced osteoclastogenesis and bone resorption. TRAP positive osteoclast formation and resorption area were significantly reduced by genistein (10^{-7} M), daidzein (10^{-5} M), and coumestrol (10^{-7} M), which was prevented by the estrogen antagonist ICI 182,780. TRAP expression in mature TNF-α-induced osteoclasts was also significantly reduced by these phytoestrogen concentrations. In addition, in the presence of ICI 182,780 genistein and coumestrol (10^{-5}–10^{-6} M) augmented TNF-α-induced osteoclast formation and resorption. However, this effect was not observed in the absence of estrogen antagonist indicating that genistein's and coumestrol's ER-dependent anti-osteoclastic action normally negates this pro-osteoclastic effect. To determine the mechanism mediating the anti-osteoclastic action we examined the effect of genistein, coumestrol, and daidzein on caspase 3/7 activity, cell viability and expression of key genes regulating osteoclast differentiation and fusion. While anti-osteoclastic phytoestrogen concentrations had no effect on caspase 3/7 activity or cell viability they did significantly reduce TNF-α-induced *c-fos* and *NFATc1* expression in an ER dependent manner and also inhibited NFATc1 nuclear translocation. Significant decreases in *NFκB* and *DC-STAMP* levels were also noted. Interestingly, constitutive *c-fos* expression prevented the anti-osteoclastic action of phytoestrogens on differentiation, resorption and *NFATc1*. This suggests that phytoestrogens suppress TNF-α-induced osteoclastogenesis via inhibition of *c-fos*-dependent NFATc1 expression. Our data provides further evidence that phytoestrogens have a potential role in the treatment of post-menopausal and inflammatory bone loss directly inhibiting TNF-α-induced resorption. J. Cell. Biochem. 112: 476–487, 2011.

KEY WORDS: TNF-α; OSTEOCLAST; PHYTOESTROGENS; NFATc1

Post-menopausal osteoporosis is characterized by low bone mass and increased fracture risk. Worldwide, osteoporotic fractures are a major health concern especially in countries with aging populations. Prior to menopause osteoblastic bone formation and osteoclastic bone resorption are balanced such that there is little net bone loss during each remodeling cycle. Estrogen deficiency disrupts this equilibrium increasing bone turnover and skewing remodeling in favor of resorption. Recent evidence suggests that this is at least in part due to elevated levels of the potent pro-inflammatory cytokine TNF-α [Weitzmann and Pacifici, 2007]. Several studies show increased TNF-α production by bone marrow and T cells following estrogen deficiency in mice [Cenci et al., 2000; Grassi et al., 2007]. Furthermore, mice lacking T cells or TNF-α receptors are resistant to ovariectomy-induced bone loss [Roggia et al., 2001]. Human peripheral blood mononuclear cells (PBMC) also display estrogen dependent changes in TNF-α production; PBMC from post-menopausal or oophorectomised individuals synthesize elevated levels of TNF-α [D'Amelio et al., 2004], whereas estrogen replacement suppresses PBMC TNF-α production in-vivo [Bernard-Poenaru et al., 2001]. In addition to promoting post-menopausal bone loss TNF-α is also a key regulator of osteolysis associated with chronic inflammatory conditions such as rheumatoid arthritis [Boyce et al., 2005]. TNF-α promotes osteoclast differentiation by several actions, it augments receptor activator of NFκB ligand (RANKL) induced osteoclastogenesis and also directly stimulates osteoclast formation from human or mouse monocytes [Komine et al., 2001; Fox et al., 2008] and has been suggested to activate osteoclast formation independent of RANKL signaling [Kudo et al., 2002]. Thus, TNF-α has a central role in post-menopausal bone loss directly promoting

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osteoclast formation and augmenting the response to other resorptive stimuli.

Post-menopausal women are typically prescribed bisphosphonates to prevent bone loss, however, complications such as induction of the acute phase response and osteonecrosis of the jaw can occur [Cole et al., 2008]. As a consequence, other potential therapeutic interventions have been examined including phytoestrogens. Phytoestrogens are a diverse group of plant derived compounds with a structure and function similar to estradiol that are used clinically to control tumor progression. Some epidemiological studies suggest that diets with high phytoestrogen content may be associated with a more robust skeleton. Positive associations have been noted between soy protein intake and spinal and hip bone mineral density (BMD) in Asian women [Horiuchi et al., 2000; Mei et al., 2001; Kim et al., 2002] and women with the highest dietary soy levels have the lowest resorption rates [Mei et al., 2001]. Intervention studies have also noted beneficial actions of phytoestrogens on post-menopausal women's spinal [Alekel et al., 2000; Atkinson et al., 2004], trochanter [Chen et al., 2003b] and Ward's triangle BMD [Wu et al., 2006]. Decreases in resorption markers have also been described following phytoestrogen supplementation [Weaver et al., 2009]. However, not all studies have observed beneficial actions of phytoestrogens on the skeleton [Gallagher et al., 2004], suggesting that the response may be dependent on the nature of the treatment regime or is modified by other lifestyle factors. While phytoestrogens may have a beneficial action on skeletal mass, the cellular and molecular mechanism mediating this response is not fully understood and may differ depending on the phytoestrogen examined. Previous studies have shown that phytoestrogens reduce osteoclast formation by directly suppressing the response of monocytes to osteoclast-inductive stimuli and also in-directly by reducing osteoblastic RANKL expression [Gao and Yamouguchi, 1999; Gao and Yamaguchi, 2000; Rassi et al., 2002; Li and Yu, 2003; Garcia Palacios et al., 2005; Bandyopadhyay et al., 2006; Uchiyama and Yamaguchi, 2007]. Surprisingly, in spite of evidence showing that TNF-α has a crucial role in post-menopausal and inflammatory bone loss no study has examined the direct effect of phytoestrogens on TNF-α-induced osteoclast differentiation. This is of importance as while there is an overlap between the intracellular signals activated by TNF-α and RANKL the early events in the their transduction cascades are dissimilar, each binding to a distinct receptor linked to different groupings of TRAF signaling factors. RANKL activates TRAFs 2, 3, and 6 while TNF-α predominantly signals via TRAFs 2 and 3. Furthermore, RANKL activates a broader range of signal transduction pathways than TNF-α, which requires other co-stimulatory factors to facilitate osteoclast formation [Fox et al., 2008]. This raises the possibility that TNF-α and RANKL-induced osteoclasts may respond differently to anti-osteoclastic compounds that target the initial stages of osteoclast differentiation. In keeping with this the anti-resorptive effect of bisphosphonates is blunted in inflammatory conditions associated with high TNF-α levels such as rheumatoid arthritis [Zhang et al., 2005] and estrogen at least partly suppresses RANKL-induced osteoclast formation via a TRAF6 dependent action [Robinson et al., 2009]. Thus, to determine if phytoestrogens are able to directly inhibit TNF-α-induced osteoclastogenesis and establish the molecular mechanism of any anti-osteoclastic action we examined the effect of genistein, coumestrol, and daidzein on TNF-α-induced osteoclast formation from the monocytic cell line RAW264.7.

MATERIALS AND METHODS

MEDIA AND REAGENTS

RAW264.7 monocytes (ATCC, UK) were incubated in phenol red free Dulbecco's minimum essential medium supplemented with 10% charcoal stripped fetal calf serum (Autogen Bioclear, UK), 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin (all from Sigma, UK). Incubations were performed at 37°C in 5% CO_2, and cultures fed every 2–3 days by replacing half of the culture volume with fresh medium. The non-selective estrogen antagonist ICI 182,780 was obtained from Tocris Biosciences (Bristol, UK). Recombinant murine TNF-α was purchased from Insight Biotechnology (Wembley, UK). All other reagents were obtained from Sigma (Poole, Dorset, UK) unless stated.

OSTEOCLAST FORMATION

To examine the direct effect of phytoestrogens on TNF-α-induced osteoclast differentiation, RAW264.7 cells were transferred to 24-well plates at a density of 10^5 cells per well and cultured with combinations of TNF-α (50 ng/ml), genistein (10^{-5}–10^{-9} M), daidzein (10^{-5}–10^{-9} M), or coumestrol (10^{-5}–10^{-9} M) for 4 days with or without the estrogen antagonist ICI 182,780 (10^{-5} M). To determine the effect of phytoestrogens on mature TNF-α-induced osteoclasts RAW264.7 cells were incubated in the presence of TNF-α (50 ng/ml) for 4 days to generate osteoclast cultures which were then incubated for 24 h with phytoestrogen concentrations shown to inhibit osteoclast formation in the initial experiment with or without the estrogen antagonist ICI 182,780 (10^{-5} M). Osteoclast formation was evaluated by staining for the specific osteoclastic marker tartrate resistant acid phosphatase-positive (TRAP positive) using a modification of the method of Burstone [1958] using naphthol AS-BI phosphate as a substrate. The number of TRAP-positive cells was counted using an eyepiece graticule at a magnification of ×100 and the results expressed as the number of cells per cm^2. All experiments were performed in triplicate.

BONE RESORPTION

To assess the effect of phytoestrogens on TNF-α-induced bone resorption RAW264.7 cells were seeded onto 20 mm^2 slices of devitalised bovine bone (kind gift of Dr Karen Fuller) in 24 well plates at a density of 10^5 cells per well. Cells were incubated in TNF-α (50 ng/ml) with or without phytoestrogens for 8 days in the presence or absence of ICI 182,780 (10^{-5} M). After incubation cells were removed from the surface of bone slices by immersion in 10% (v/v) sodium hypochlorite for 10 min, followed by washing in distilled water and dehydration in 70% ethanol. After drying slices were mounted onto glass slides and stained with 1% toluidine blue to enable visualization of resorption pits. The percentage of bone surface resorbed was quantified by reflected light microscopy using an eyepiece graticule and magnification of ×100 on an Olympus BHB microscope with a Schott KL1500 light source.

MEASUREMENT OF CELL VIABILITY AND APOPTOSIS IN MONOCYTES AND MATURE TNF-α-INDUCED OSTEOCLASTS

The effect of phytoestrogens on cell viability was assessed using a commercial proliferation assay. Cells were incubated with TNF-α (50 ng/ml) or TNF-α plus genistein, coumestrol, or daidzein (10^{-4}–10^{-9} M) for 4 days. Proliferation was then assessed using an AQueous one solution cell proliferation assay (Promega, UK) according to manufacturer's instructions. The effect of anti-osteoclastic concentrations of phytoestrogens on apoptosis was assessed by incubating RAW cells with TNF-α (50 ng/ml) or TNF-α plus genistein (10^{-7} M), coumestrol (10^{-7} M) or daidzein (10^{-5} M) for 24 h. Caspase 3/7 activity was then measured using the apo-One homogeneous caspase assay (Promega, UK) according to manufacturer's instructions.

REAL TIME QUANTITATIVE PCR ANALYSIS OF MRNA EXPRESSION

RAW264.7 cells (5×10^5) were incubated in 25 cm^2 flasks for 24, 48, or 96 h with combinations of TNF-α (50 ng/ml), genistein, coumestrol, or daidzein. Total RNA was extracted from these cultures using a Sigma genelute RNA isolation kit and reversed transcribed with M-MLV reverse transcriptase using random nonamer primers. Real time-PCR was performed on a StepOne PCR system (Applied Biosytems, UK) using the DNA-binding dye SYBR green for detection of PCR products. A total of 2 μl of external plasmid standard or cDNA was added to a final reaction volume of 25 μl containing 0.05 U/μl Taq, SYBR green and specific primers (0.2 μM). Primers used were as follows Murine *β-Actin* Forward GTCATCACTATTGGCAACGAG, Reverse CCTGTCAG-CAATGCCTGGTACAT; Murine *NFATc1* Forward CCGTTGCTTCCA-GAAAATAACA, Reverse TGTGGGATGTGAACTCGGAA; Murine *c-fos* Forward CCATCAAGAGCATCAGCAA, Reverse AAGTA GTGCAGCCCGGAGTA; Murine *DC-STAMP* Forward AAAA CCCTTGGGCTGTTCTT, Reverse GTTCCTTGCTTCTCTCCACG; Murine *NFκB* Forward GTGGAGGCATGTTCGGTAGT, Reverse GTCCA-GAAGGCTCAGGTCAG; Murine *p38* Forward CGACCACGTT-CAGTTTCTCA, Reverse AGGTCAGGCTCTTCCACTCA. For the generation of standard curves, the corresponding cDNA was cloned into pGEM-T Easy (Promega). The concentration of DNA plasmid stock was determined by the OD at 260 nm. Copy number for each plasmid was calculated from these measurements. The linear range of the assay was determined by the amplification of log serial dilutions of external plasmid standard from 500 to 5×10^6 copies. The progress of the PCR amplification was monitored by real-time fluorescence emitted from SYBR Green during the extension time. Reaction conditions were 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. At the end of each PCR run, a melt curve analysis was performed to show the absence of non-specific bands. For each sample, mRNA levels were expressed as an absolute copy number normalized against β-actin mRNA. The mRNA copy number was calculated for each sample from the standard curves by the instrument's software. Samples were analyzed in triplicate.

NFATc1 IMMUNOFLUORESCENT STAINING

RAW 264.7 cells were seeded overnight onto glass coverslips and then incubated in TNF-α (50 ng/ml) with or without genistein (10^{-7} M), coumestrol (10^{-7} M), or daidzein (10^{-5} M). The distribution of NFATc1 protein 24 and 48 h after stimulation was assessed according to previously published protocols [Evans and Fox, 2007]. Coverslips were removed, washed in PBS, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with 1% goat serum and incubated with a specific anti-mouse NFATc1 monoclonal antibody (1:50, Santa Cruz, USA) for 1 h. Cells were washed in PBS, incubated for 2 h with biotinylated goat anti-mouse secondary (Vector Labs, USA) and then incubated for 15 min with fluorescein conjugated streptavidin (Vector Labs, USA). Fluorescence was visualized using a Leica HC microscope. The percentage of cells displaying nuclear staining was then quantified, 100 cells per group were measured from 3 separate coverslips per group. Photographs were taken with a JVC digital camera linked to image pro-plus at a magnification of ×400.

GENERATION OF *c-fos* EXPRESSING RETROVIRAL VECTOR

Constitutively active c-fos (N-core-cfos) was transduced using a retroviral vector, pBabe puro, which expresses cDNA inserts under the control of a retroviral enhancer-promoter [Morgenstern and Land, 1990]. The coding region for the n terminal and core regions of *c-fos* were PCR amplified from total mouse RNA and cloned into PGEM-T easy (Promega, UK). EcoRI digest fragments were then sub-cloned into pBabe puro. The resulting plasmid pBabe-cfos was sequenced (MWG eurofins). The pBabe-cfos and pBabe-empty (control) vectors were then transfected into the Phoenix retroviral packaging cell line using FuGene (Roche, Indianapolis). After 48 h stably transfected cells were selected by incubating with 2.5 μg/ml puromycin. Stably transfected clones were then picked 4–7 days later and grown to confluence in 25 cm^2 flasks. After incubation in fresh medium for an additional 2 days, stably transfected cells were selected with puromycin for 2 days.

INFECTION OF RAW264.7 CELLS WITH *c-fos* EXPRESSING RETROVIRUSES

Raw cells were added to the wells of 96 well plates (2×10^4 cells per well) containing thermonax coverslips or slices of devitalized bovine bone and cultured for 24 h prior to infection. Medium was then removed and replaced with filtered (0.45 μm) supernatant from pBabe-cfos or pBabe-empty virus-producing Phoenix cells in the presence of 8 μg/ml polybrene. Cultures were then incubated for 16 h before the addition of TNF-α (50 ng/ml) with or without concentrations of phytoestrogens shown to suppress osteoclast formation and bone resorption (genistein 10^{-7} M, daidzein 10^{-5} M, or coumestrol 10^{-7} M). After incubation for a further 2 days stably infected cells were selected by the addition of puromycin (2.5 μg/ml) for 2 days. Cells were then stained for TRAP and bone resorption assessed as described earlier. To examine the effect of pBabe-c-fos on NTATc1 expression Raw cells were seeded into 25 cm^2 flasks and then incubated with supernatant from pBabe-cfos or pBabe-empty virus-producing Phoenix cells in the presence of 8 μg/ml polybrene. Cultures were then incubated for 16 h before the addition of TNF-α (50 ng/ml) with or without genistein (10^{-7} M), daidzein (10^{-5} M), or coumestrol (10^{-7} M) in the presence of puromycin (2.5 μg/ml) for 48 h. Total RNA was then isolated as described above.

STATISTICAL ANALYSIS

Differences between groups were assessed using Fisher's analysis of variance (Statview; Abacus concepts). A difference of $P < 0.05$ was considered statistically significant.

RESULTS

GENISTEIN, COUMESTROL, AND DAIDZEIN SUPPRESS TNF-α-INDUCED OSTEOCLAST DIFFERENTIATION AND BONE RESORPTION

All phytoestrogens at 10^{-4} M significantly reduced cell viability whereas lower concentrations had no detrimental effect on proliferation and for this reason initial experiments used phytoestrogen concentrations from 10^{-5} to 10^{-9} M (Fig. 1). TNF-α directly stimulated the formation of strongly TRAP positive mononuclear and multinuclear osteoclasts within 4 days (Fig. 1), which similar to previous findings readily formed resorption pits on bone slices (Fig. 2) [Fuller et al., 2002]. Genistein, coumestrol, and daidzein all directly suppressed TNF-α-induced osteoclast formation and bone resorption. Genistein at 10^{-7} M significantly reduced TNF-α-induced osteoclast formation (11% of TNF-α treated group, 10^{-7} M; $P < 0.001$) (Fig. 1) and inhibited TNF-α-induced bone resorption (19% of TNF-α treated group; $P < 0.001$) (Fig. 2). Similar suppressive effects on osteoclast formation and resorption were noted with 10^{-7} M coumestrol (TRAP positive osteoclast formation 12% of TNF-α treated group $P = 0.0022$, bone resorption area 22% of TNF-α treated group $P = 0.008$) (Figs. 1 and 2). In contrast only the highest concentration of daidzein (10^{-5} M) suppressed osteoclast formation and bone resorption (TRAP positive osteoclast formation 13% of TNF-α treated group $P = 0.0024$, bone resorption area 74% of TNF-α treated group $P = 0.0017$) (Figs. 1 and 2). In addition anti-osteoclastic concentrations of genistein, coumestrol, and daidzein also significantly reduced TRAP expression in cultures of mature TNF-α-induced osteoclasts (Table I).

To determine if the anti-osteoclastic effect of phytoestrogens was mediated through an ER dependent mechanism cells were cultured in the presence of the estrogen antagonist ICI 182,780. ICI 182,780 prevented the suppressive action of genistein (10^{-7} M), coumestrol (10^{-7} M), and daidzein (10^{-5} M) on TNF-α-induced bone resorption and TRAP positive osteoclast formation, suggesting that phytoestrogens suppress TNF-α-induced osteoclastogenesis via an estrogen receptor dependent mechanism. In addition, in the presence of ICI 182,780 genistein and coumestrol (10^{-5}–10^{-6} M) also displayed a pro-osteoclastic effect, significantly augmenting TNF-α-induced osteoclast formation and bone resorption (Figs. 1 and 2). In contrast, daidzein had no augmentative action on osteoclast differentiation or bone resorption at any concentration in the presence or absence of ER antagonist. Thus, genistein and coumestrol possess multiple antagonistic actions on osteoclast formation; however, it is clear

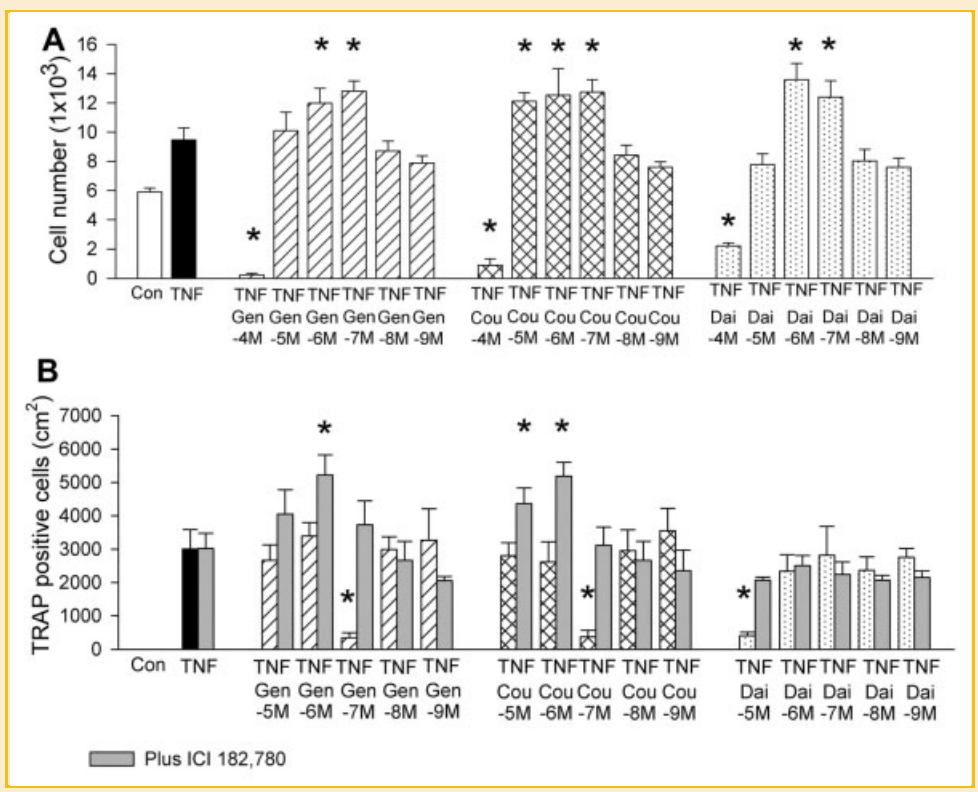

Fig. 1. Genistein, daidzein, and coumestrol (10^{-4} M) significantly decreased RAW264.7 cell viability after 4 days of incubation all other concentrations had no detrimental effect on monocyte number (A). Genistein, coumestrol, and daidzein suppressed TNF-α-induced osteoclast formation (B). Genistein (10^{-7} M), coumestrol (10^{-7} M), and daidzein (10^{-5} M) significantly reduced TNF-α-induced TRAP positive osteoclast formation, which was prevented by the ER antagonist ICI 182,780 (solid grey bars). In the presence of ICI 182,780 higher concentrations of genistein (10^{-6} M) and coumestrol (10^{-5} and 10^{-6} M) increased osteoclast formation. Values are expressed as the mean±SEM of 3 separate experiments n = 18, $^{*}P < 0.05$ versus TNF-α treated group.

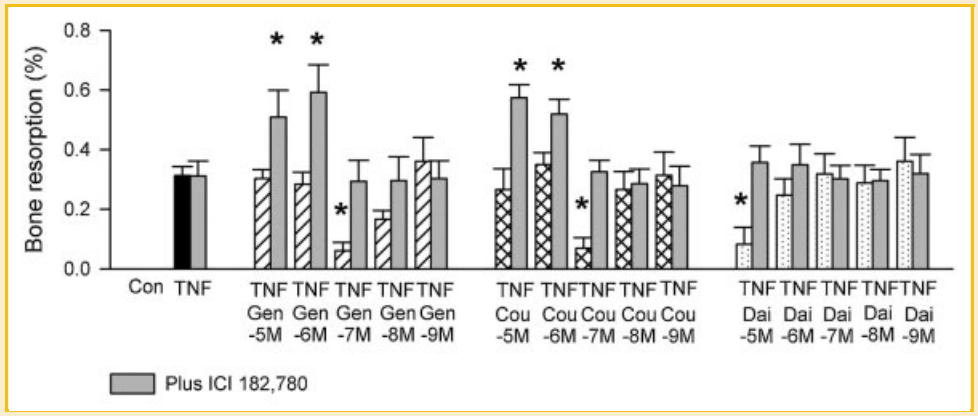

Fig. 2. Genistein, daidzein, and coumestrol significantly suppressed TNF-α-induced bone resorption in an ER dependent manner. RAW264.7 cells were incubated on bone slices in the presence of TNF-α (50 ng/ml) and genistein, coumestrol, or daidzein (10^{-5}–10^{-9} M) for 8 days and the percentage of bone surface displaying resorption pits was analyzed by reflected light microscopy. Higher concentrations of genistein and coumestrol (10^{-5} and 10^{-6} M) augmented osteoclast differentiation in the presence of ICI 182,780. Values are expressed as the mean±SEM of 3 separate experiments. $^{*}P < 0.05$ versus TNF-α treated group.

that when ER signaling is intact the anti-osteoclastic action counteracts any potential pro-osteoclastic effect (Figs. 1 and 2). Therefore, all the phytoestrogens studied only displayed a suppressive effect on TNF-α-induced osteoclast differentiation when delivered in isolation.

ANTI-OSTEOCLASTIC CONCENTRATIONS OF PHYTOESTROGENS HAVE NO EFFECT ON CELL VIABILITY OR APOPTOSIS

We then went on to determine the cellular and molecular mechanism through which genistein, coumestrol, and daidzein suppressed TNF-α-induced osteoclast formation. As shown in Figure 1 anti-osteoclastic phytoestrogen concentrations (genistein 10^{-7} M, daidzein 10^{-5} M, and coumestrol 10^{-7} M) had no detrimental effect on cell viability. Furthermore, caspase 3/7 activity was also unaffected by these phytoestrogen concentrations (Fig. 3), suggesting that the reduction in osteoclast formation and activity noted was mediated via an action on osteoclast differentiation.

GENISTEIN, COUMESTROL, AND DAIDZEIN SUPPRESS THE EXPRESSION OF REGULATORS OF OSTEOCLAST DIFFERENTIATION

Osteoclast differentiation is controlled by a network of signaling factors that regulate the expression of genes typical of osteoclasts such as *TRAP* and *DC-STAMP*. Central to this is the c-fos dependent induction of NFATc1 expression. Mice deficient in either NFATc1 or c-fos lack osteoclasts and are severely osteopetrotic as a consequence. Evidence also suggests that NFATc1 is sufficient stimulus on its own to promote osteoclast formation [Matsuo et al., 2004]. Therefore, to determine the effect of phytoestrogens on this master regulatory system we analyzed changes in *c-fos* and *NFATc1* expression using real time quantitative PCR. TNF-α induced a significant 3.82-fold increase in *NFATc1* expression and a significant 2.82-fold increase in *c-fos* expression within 48 h (Fig. 4). TNF-α-induced *NFATc1* and *c-fos* expression was significantly suppressed in the presence of concentrations of genistein (10^{-7} M), coumestrol (10^{-7} M), or daidzein (10^{-5} M) shown to reduce osteoclast formation and bone resorption in our earlier experiments (Fig. 4). These values were not significantly different from non-treated control. No other concentrations had any significant inhibitory effect. The suppressive action of genistein, coumestrol, and daidzein was prevented by ICI 182,780 suggesting that the reduction in mRNA expression was ER dependent. In keeping with the decrease in *NFATc1* mRNA expression genistein, coumestrol, and daidzein also suppressed TNF-α-induced NFATc1 nuclear translocation as shown by a significant reduction in the number of nuclei displaying TNF-α-induced NFATc1 immunostaining (Fig. 5).

Interestingly, in the presence of ICI 182,780 genistein and coumestrol (10^{-5}–10^{-6} M) significantly augmented TNF-α-induced *NFATc1* expression again suggesting that high concentrations of these compounds have additional ER independent actions (Fig. 4). However, in keeping with the changes in osteoclast differentiation and bone resorption described earlier no augmentation of *NFATc1* expression was noted in the absence of ICI 182,780, suggesting that genistein's and comestrol's ER-mediated inhibitory action negates any stimulatory effect. Furthermore, no increase in *c-fos* expression

TABLE I. Phytoestrogens Significantly Reduce the Number of TRAP Positive Mature Osteoclasts

Group	Control	Control ICI 182,780	TNF	TNF ICI 182,780	TNF Genistein	TNF Genistein ICI 182,780	TNF Coumestrol	TNF Coumestrol ICI 182,780	TNF Daidzein	TNF Daidzein ICI 182,780
TRAP positive cells (cm^2)	0 ± 0	0 ± 0	317 ± 55	296 ± 42	14 ± 8*	238 ± 34	0 ± 0*	250 ± 63	160 ± 35*	307 ± 45

*$P < 0.05$ versus TNF.

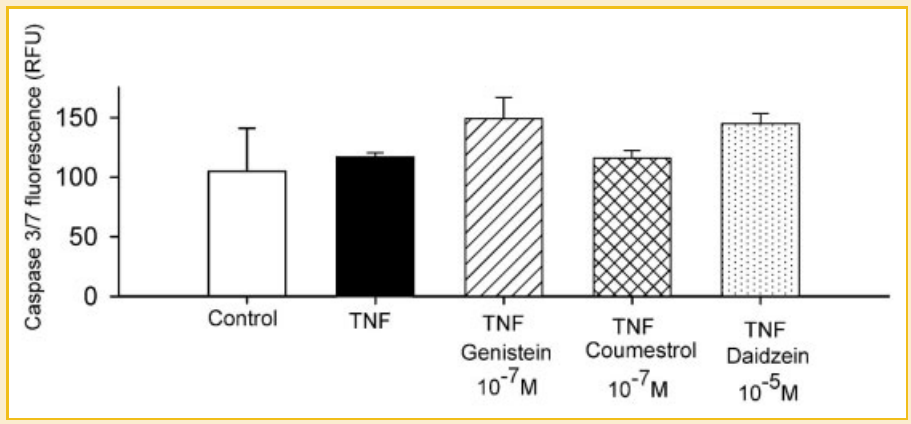

Fig. 3. Anti-osteoclastic concentrations of phytoestrogens had no significant effect on monocyte caspase 3/7 activity. Values are expressed as the mean ± standard error from 5 replicate experiments.

was noted with any combination of phytoestrogen and estrogen antagonist suggesting that the increase in *NFATc1* expression generated by high concentrations of genistein and coumestrol was not mediated via changes in *c-fos* transcription or turnover.

While c-fos and NFATc1 are master regulators of osteoclast differentiation other signaling pathways have been shown to modify osteoclast differentiation. Therefore, to determine the potential role of these pathways we examined the effect of anti-osteoclastic concentrations of genistein, coumestrol, and daidzein on *p38* and *NFκB* expression. TNF-α-induced a significant 2.6-fold increase in *NFκB* expression within 24 h (Fig. 6) which was prevented in the presence of genistein (10^{-7} M), coumestrol (10^{-7} M), or daidzein (10^{-5} M) in an ER dependent manner (Fig. 6). In contrast anti-osteoclastic concentrations of phytoestrogens had no effect on TNF-

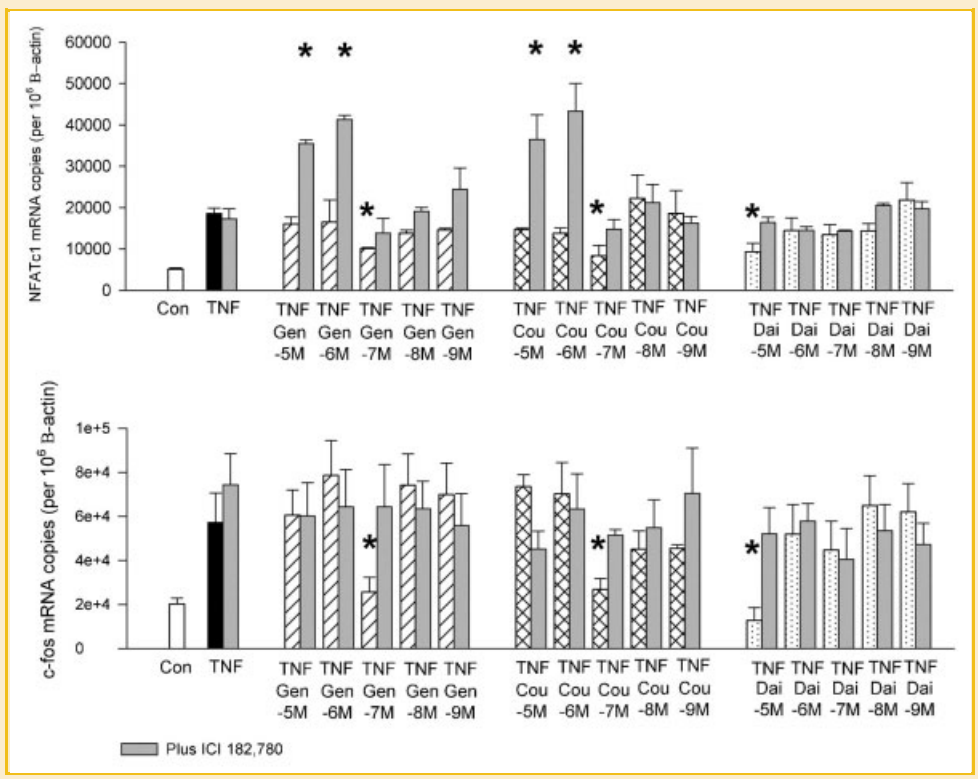

Fig. 4. TNF-α-induced *NFATc1* and *c-fos* expression is suppressed by genistein (10^{-7} M), coumestrol (10^{-7} M), or daidzein (10^{-5} M) in an ER dependent manner. RAW264.7 cells were incubated in combinations of TNF-α (50 ng/ml), genistein, coumestrol, or daidzein in the presence or absence of ICI 182,780 and the expression of *NFATc1* and *c-fos* was then assessed by quantitative real time PCR. Additionally, *NFATc1* but not *c-fos* expression was augmented by high concentrations (10^{-5}–10^{-6} M) of genistein or coumestrol in the presence of ICI 182,780. Data expressed normalized to 10^6 copies of β-actin and represent the mean ± SEM from 3 separate experiments. $^*P < 0.05$ versus TNF-α treated group.

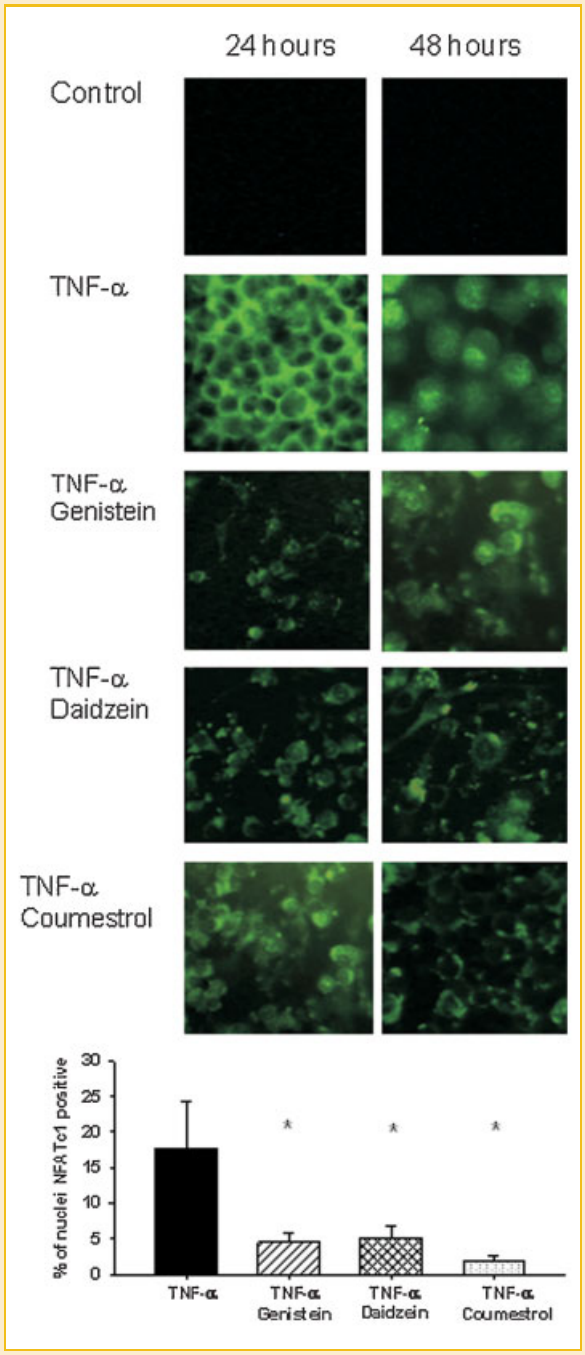

Fig. 5. NFATc1 immunofluorescent staining in RAW264.7 cells cultured for 24 or 48 h in the presence of combinations of TNF-α, genistein, coumestrol, or daidzein. Phytoestrogens reduced the intensity of TNF-α-induced NFATc1 cytoplasmic staining and significantly reduced the number of cells displaying NFATc1 nuclear localization. $^{*}P < 0.05$ versus TNF-α-treated group.

α-induced *p38* expression (Fig. 6). In addition to changes in transcription factor levels we also noted a significant inhibitory action on the essential regulator of osteoclast aggregation and fusion *DC-STAMP*. TNF-α induced a significant 4.3-fold increase in *DC-STAMP* expression 48 h after stimulation which was prevented in the presence of genistein (10^{-7} M), coumestrol (10^{-7} M), or daidzein (10^{-5} M) (Fig. 6).

RETROVIRAL *c-fos* EXPRESSION PREVENTS THE INHIBITORY EFFECT OF PHYTOESTROGENS ON TNF-α-INDUCED NFATc1 EXPRESSION, OSTEOCLAST FORMATION, AND RESORPTION

The initial stimulus for the cytoplasmic accumulation of NFATc1 is provided by c-fos. NFATc1 is subsequently activated by Ca^{2+} calmodulin dependent signals leading to nuclear translocation and auto-amplification of its expression. Without these separate stimuli insufficient NFATc1 levels are generated to promote osteoclast differentiation. The ability of phytoestrogens to substantially reduce c-fos expression raises the possibility that this represents a key molecular mechanism mediating their action on osteoclast formation. To assess this we generated monocytic precursors (c-fos-pBabe) expressing constitutively active c-fos under the control of a retroviral expression vector and exposed them to concentrations of phytoestrogens shown to suppress osteoclast formation in our earlier experiments. We found that retroviral driven c-fos expression increased basal NFATc1 mRNA levels 3.25-fold and led to a significant 4-fold increase in TNF-α-induced NFATc1 expression (Fig. 7). Moreover, constitutive c-fos expression prevented the inhibitory effect of anti-osteoclastic concentrations of phytoestrogens on TNF-α-induced osteoclastogenesis and bone resorption (Fig. 8), suggesting that phytoestrogens suppress TNF-α-induced osteoclast formation through a c-fos dependent action.

DISCUSSION

An increasing body of evidence implicates TNF-α-induced osteoclast formation in post-menopausal and inflammatory bone loss. TNF-α directly stimulates the formation of resorptive osteoclast from human monocytes in the presence of OPG [Kudo et al., 2002], whereas blockade of TNF-α using a range of biological agents reduces osteoclast number and prevents bone destruction in-vivo [Barnabe and Hanley, 2009]. Therefore, an understanding of how potential anti-resorptive therapies such as phytoestrogens modify TNF-α-induced osteoclastogenesis is essential. Genistein, coumestrol, and daidzein have previously been shown to suppress RANKL-induced osteoclast differentiation [Gao and Yamouguchi, 1999; Rassi et al., 2002; Garcia Palacios et al., 2005]. Comparable concentrations of these phytoestrogens also reduced TNF-α-induced osteoclast differentiation and bone resorption in our study, with genistein and coumestrol having a more potent effect than daidzein. This anti-osteoclastic action was mediated via a direct effect on monocyte differentiation and not via in-direct osteoblastic actions as suggested in previous studies using mixed cell populations [Chen et al., 2003a]. Genistein, coumestrol, and daidzein also directly reduced TRAP expression in mature TNF-α-induced resorptive osteoclasts. These results strengthen the data for the use of phytoestrogens as agents in the treatment of post-menopausal bone loss and inflammatory osteolytic diseases where they may suppress TNF-α-induced osteoclast formation and resorptive activity.

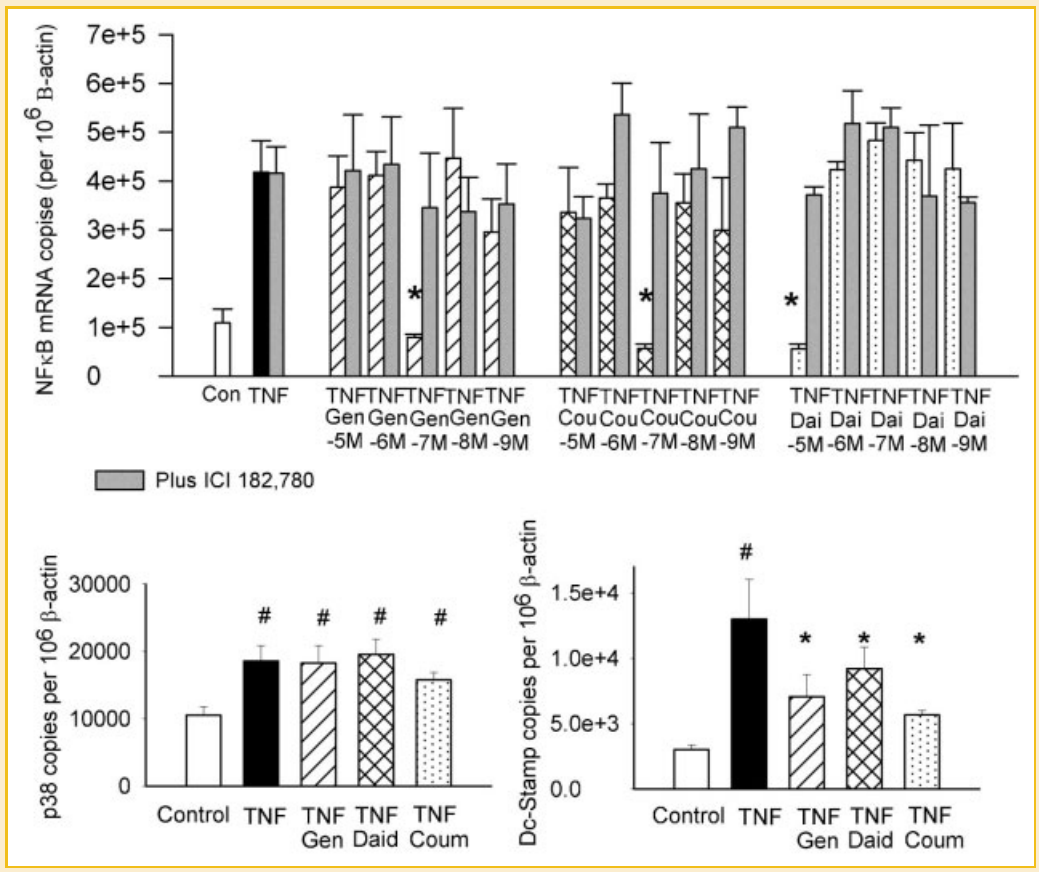

Fig. 6. Genistein, coumestrol, and daidzein inhibit *NFkB* and *DC-STAMP* but not *p38* expression. RAW264.7 cells were incubated in combinations of TNF-α (50 ng/ml), genistein, coumestrol, or daidzein and then mRNA expression was assessed by quantitative real time PCR. Data expressed normalized to 10^6 copies of β-actin and represent the mean ± SEM from 3 separate experiments. #$P < 0.05$ versus control; *$P < 0.05$ versus TNF-α treated group.

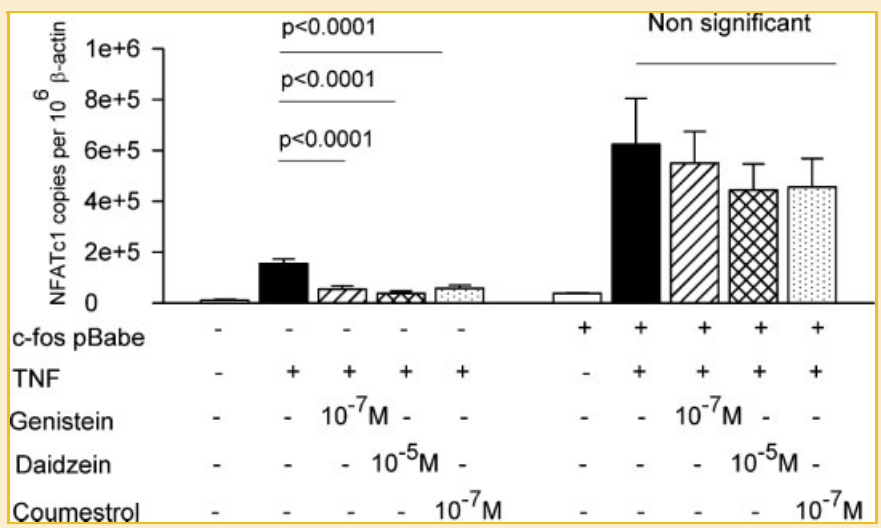

Fig. 7. Constitutive c-fos expression prevents the anti-osteoclastic effects of phytoestrogens on TNF-α-induced NFATc1 expression. RAW 264.7 cells were infected with control or c-fos (c-fos pBabe) expressing retroviruses and the effect of TNF-α (50 ng/ml), genistein (10^{-7} M), coumestrol (10^{-7} M), and daidzein (10^{-5} M) on NFATc1 expression was examined. Experiments were performed in triplicate.

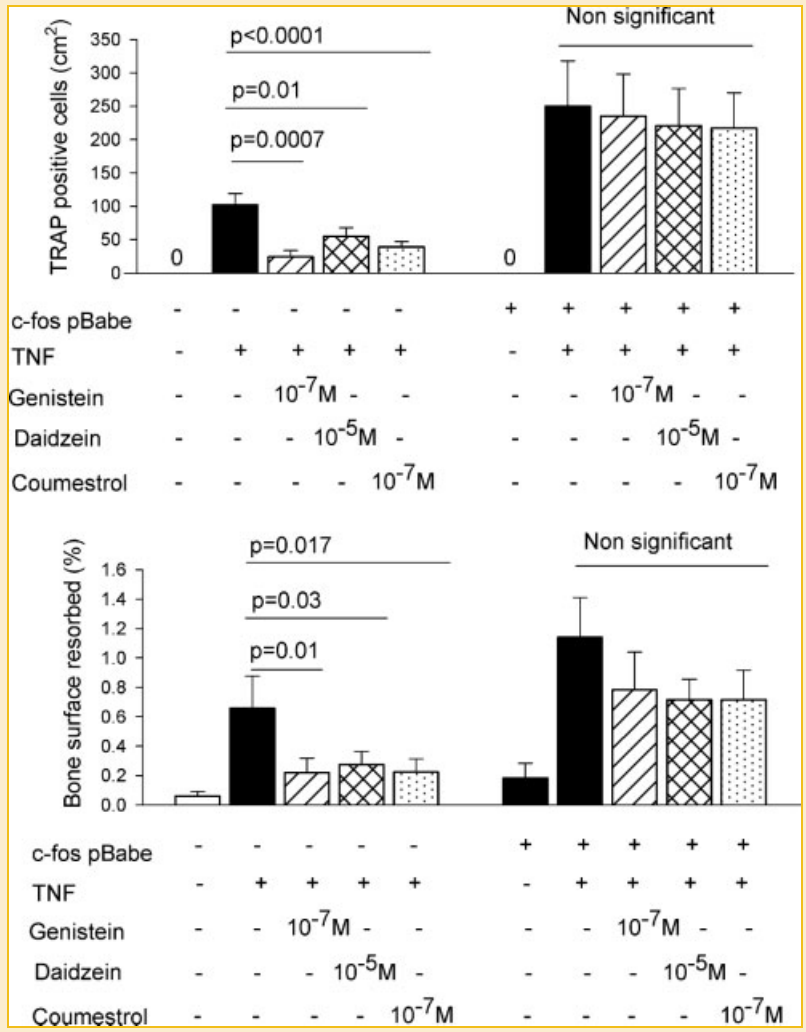

Fig. 8. Constitutive c-fos expression prevents the anti-osteoclastic effects of phytoestrogens on TNF-α-induced osteoclast formation and bone resorption. RAW 264.7 cells were infected with control or c-fos (c-fos pBabe) expressing retroviruses and the effect of TNF-α (50 ng/ml), genistein (10^{-7} M), coumestrol (10^{-7} M), and daidzein (10^{-5} M) on TRAP positive osteoclast formation and bone resorption was examined. Experiments were performed in triplicate.

Serum concentrations of phytoestrogens vary between populations with Asians having significantly higher levels than Westerners [Morton et al., 2002]. This has been suggested to contribute to the lower incidence of osteoporotic fractures in Asian women. Interestingly, the anti-resorptive concentration of genistein and coumestrol noted in our study is similar to levels measured in Asian populations but higher than those achieved by Western diets [Morton et al., 2002]. In contrast, both Asian and Western diets are unable to generate serum concentrations of daidzein similar to those shown to suppress TNF-α-induced osteoclastogenesis in our studies. However, these concentrations could be achieved with daidzein supplementation which generates tissue levels several orders of magnitude higher than dietary sources [Gardner et al., 2009]. The anti-resorptive effect of genistein and coumestrol was noted over a narrow concentration range with doses higher or lower than this having no effect on differentiation or resorption. This response likely reflects antagonistic interactions between genistein's and coumestrol's multiple biological actions. For instance in addition to its classical estrogenic effect genistein also inhibits tyrosine kinase activity [Aggarwal and Shishodia, 2006] and alters redox states which are known to have a bimodal effect on osteoclast formation [Kim et al., 2006]. Similarly, coumestrol activates MAPK activity which promotes osteoclast differentiation [Jeng et al., 2009]. Furthermore, the estrogen receptor-independent actions of genistein and coumestrol tend to have higher Ec50s, thus it is conceivable that while concentrations of 10^{-7} M stimulate estrogen receptor dependent anti-osteoclastic actions higher concentrations activate antagonistic non-estrogenic effects that promote osteoclast formation and bone resorption. This assertion is strengthened by the increase in osteoclast formation and resorption noted when ER signaling was inhibited with ICI 182,780 at higher concentrations of coumestrol and genistein (10^{-5}–10^{-6} M). These concentrations also promoted NFATc1 expression in the presence of estrogen antagonist indicating that increased osteoclast number most likely resulted from enhanced levels of this key osteoclastic transcription factor. In contrast, daidzein which acts exclusively via ER did not

augment osteoclast formation, bone resorption or NFATc1 expression in the presence of ICI 182,780 at any concentration examined. The precise nature of the ER independent action which augments NFATc1 transcription is unclear but would not appear to be mediated via an increase in *c-fos* or *NFκB* expression as neither genistein nor coumestrol increased levels of these transcription factors. Furthermore, while ICI 182,780 prevented the suppressive action of all phytoestrogens there is a possibility that other ER-independent actions may still have a role in the inhibitory action.

Importantly, while coumestrol and genistein appear to possess pro and anti-resorptive actions the current data suggests that the anti-resorptive action predominates with no augmentative effect apparent when ER signaling is intact. Genistein and coumestrol could therefore be used at relevant doses to decrease resorption in post-menopausal osteoporosis or inflammatory osteolysis with little theoretical chance of them enhancing resorption if this dose was exceeded. However, it does indicate caution in the use of genistein and coumestrol as anti-resorptive agents in women with ER-positive breast tumors that have preferentially metastasized to the skeleton. ER antagonists are commonly prescribed to these patients to reduce fracture risk and therefore in this setting high doses of genistein and coumestrol could potentially augment tumor associated osteolysis and increase the occurrence of skeletal related events.

Previous data suggest that phytoestrogens suppress RANKL-induced osteoclastogenesis by enhancing precursor apoptosis and disrupting intracellular signals regulating osteoclast differentiation [Gao and Yamouguchi, 1999; Rassi et al., 2002; Garcia Palacios et al., 2005; Uchiyama and Yamaguchi, 2007]. In contrast our data suggests that their suppressive action on TNF-α-induced osteoclast formation is predominantly mediated via an effect on differentiation with no impact on viability or apoptosis. This may relate to differences in the strength of survival signals stimulated by TNF-α and RANKL. Evidence suggests that TNF-α but not RANKL is able to induce levels of anti-apoptotic factors, such as Bcl-xL, sufficient to induce resistance to subsequent apoptotic stimuli [Zhang et al., 2005]. In keeping with this anti-resorptives such as alendronate and pamidronate are relatively ineffective at preventing focal inflammatory bone loss in rheumatoid arthritis where high TNF-α levels are the predominate resorptive driver [Lodder et al., 2003]. Alternatively, the phytoestrogens examined in the current study may induce apoptosis via a TRAF6 mediated action, which would be expected to modify RANKL but not TNF-α-induced survival signals. Whatever the answer it is clear that unlike their dual suppressive action on RANKL-induced osteoclastogenesis the predominant effect of phytoestrogens on TNF-α-induced resorption is mediated via suppression of osteoclast differentiation.

TNF-α-induced osteoclastogenesis is dependent on the coordinated expression of transcription factors that drive differentiation along the osteoclast lineage. Pivotal to this is NFATc1 which has been suggested to be sufficient for osteoclast formation due to its ability to promote expression of osteoclastic genes such as *Trap* and *DC-STAMP* [Matsuo et al., 2004]. NFATc1 displays a biphasic expression pattern during osteoclastogenesis; initially there is a small cytoplasmic increase followed by a larger increase in expression after nuclear translocation and autoregulation of its own gene. These processes are controlled by separate signals, cytoplasmic expression is c-fos dependent whereas nuclear accumulation is Ca^{2+} calmodulin dependent [Grigoriadis et al., 1994]. In addition autoamplification of NFATc1 expression is dependent on the presence of c-fos to stabilize NFATc1's interaction with its own promoter [Asagiri et al., 2005]. This places c-fos centrally in the regulation of NFATc1 and osteoclast differentiation. Our studies suggest that genistein, coumestrol, and daidzein may directly inhibit osteoclast differentiation by suppressing *c-fos* levels. The mechanism leading to a reduction in *c-fos* transcription is uncertain, but this could arise as a consequence of a direct ER mediated effect on the *c-fos* gene or indirectly via modification of an upstream regulator. The phytoestrogen-induced reduction in *c-fos* would in turn be expected to prevent NFATc1 reaching levels sufficient to enable osteoclast differentiation, maintaining precursors in a non-committed state or allowing them to differentiate towards alternative macrophage lineages. This conclusion is strengthened by the inability of all phytoestrogens to inhibit osteoclast formation and bone resorption in cells constitutively expressing high levels of c-fos. However, while it is clear that c-fos suppression is associated with the inhibitory action of phytoestrogens the levels generated by retroviral constructs are likely to be higher than those induced by TNF-α and may not therefore represent a physiological response.

While previous studies noted similar actions of genistein on NFATc1 expression during RANKL-induced osteoclast formation [Uchiyama and Yamaguchi, 2007]; this to our knowledge is the first report of an inhibitory effect of coumestrol, genistein or daidzein on monocytic *c-fos* expression. This is also the first report of an action of these compounds on *DC-STAMP*. DC-STAMP has a key role in later stages of osteoclast differentiation, its expression is elevated on mononuclear osteoclasts and it is enables their subsequent aggregation and fusion to form large multinuclear osteoclast. Mice lacking DC-STAMP develop a mild osteopetrosis and have no multinuclear osteoclasts [Kukita et al., 2004; Miyamoto, 2006]. The phytoestrogen-induced reduction in *DC-STAMP* most likely occurs as a secondary consequence of decreased *NFAT* and *c-fos* expression which would be expected to lower *DC-STAMP* promoter activity [Yagi et al., 2007]. Furthermore, in light of DC-STAMP's role in the formation giant cells during chronic inflammatory conditions such as tuberculosis this data is of interest to other groups and may provide a new avenue to modify granuloma formation.

In conclusion, we found that genistein, coumestrol, and daidzein directly suppressed TNF-α induced osteoclast formation and bone resorption. The concentration of genistein and coumestrol required to suppress resorption are achievable by diets containing high soy contents, whereas to achieve effective daidzein levels would require dietary supplementation. The anti-osteoclastic action is at least in part mediated by suppression of *c-fos* expression in osteoclast precursors which would prevent nuclear accumulation of NFATc1 a key regulator of osteoclast formation. Our results provide further evidence that phytoestrogens are potential therapeutic candidates in the prevention of bone loss associated with aberrant TNF-α levels in post-menopausal osteoporosis and inflammatory osteolysis.

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