Negative Regulation of RANKL-induced Osteoclastic Differentiation in RAW264.7 Cells by Estrogen and Phytoestrogens

Verónica García Palacios‡, Lisa J. Robinson‡, Christopher W. Borysenko‡, Thomas Lehmann‡, Sara E. Kalla, and Harry C. Blair‡§¶\

From the Departments of §Pathology and Cell Biology and ¶Physiology, University of Pittsburgh and the Veterans Affairs Medical Center, Pittsburgh, Pennsylvania 15243

We studied estrogen effects on osteoclastic differentiation using RAW264.7, a murine monocytic cell line. Differentiation, in response to RANKL and colony-stimulating factor 1, was evaluated while varying estrogen receptor (ER) stimulation by estradiol or nonsteroidal ER agonists was performed. The RAW264.7 cells were found to express ERα but not ERβ. In contrast to RANKL, which decreased ERα expression and induced osteoclast differentiation, 10 nM estradiol, 3 μM genistein, or 3 μM daidzein all increased ERα expression, stimulated cell proliferation, and decreased multinucleation, with the effects of estrogen ≥ daidzein > genistein. However, no estrogen agonist reduced RANKL stimulation of osteoclast differentiation markers or its down-regulation of ERα expression by more than ~50%. Genistein is also an Src kinase antagonist in vitro, but it did not decrease Src phosphorylation in RAW264.7 cells relative to other estrogen agonists. However, both phytoestrogens and estrogen inhibited RANKL-induced IκB degradation and NF-κB nuclear localization with the same relative potency as seen in proliferation and differentiation assays. This study demonstrates, for the first time, the direct effects of estrogen on osteoclast precursor differentiation and shows that, in addition to effecting osteoblasts, estrogen may protect bone by reducing osteoclast production. Genistein, which activates ERs selectively, inhibited osteoclastogenesis less effectively than the nonselective phytoestrogen daidzein, which effectively reproduced effects of estrogen.

Estrogen is a key regulator of skeletal mass. Most estrogen effects are mediated by estrogen receptors (ERs)α and β, which are ligand-dependent transcriptional regulators. ERα, the classical sex-related receptor, has a wide tissue distribution and is found in osteoclasts and osteoclast precursors as well as osteoblasts; ERβ is primarily found in epithelial and mesenchymal tissues, including the mesenchymal stem cell-derived bone-forming cells, osteoblasts. There are also estrogen-binding proteins that are not transcription factors, and some estrogenic effects are mediated by membrane receptors linked to calcium (1, 2). The effects of estrogens on skeletal cells are complex, and the mechanisms of action are controversial (reviewed in Ref. 3). However, transgenic and knock-out mice with varying ERα and ERβ expression established that estrogen effects on bone involve ERα and ERβ, which modulate signaling pathways involving Erk and nitric oxide and perform direct transcriptional activity (4). Estrogen responses in mesenchymal stem cell-derived bone-forming cells, osteoblasts, are extensively studied. The effects of estrogens on osteoblasts include regulation of synthesis of the osteoclast differentiation factor RANKL relative to its inhibitor osteoprotegerin (5, 6), which may secondarily regulate osteoclast formation and activity.

In contrast, primary estrogen effects on osteoclast differentiation and function are largely uncharacterized. ERα is present in osteoclasts (7), whereas ERβ is controversial. By immune localization, ERβ is reported in the nuclei of human osteoclasts (8, 9), but only ERα is reported to be found in isolated human osteoclast precursors or murine pre-osteoclastic RAW264.7 cells (10, 11). Available precedents suggest that the effects of estrogen on osteoclast progenitors and osteoclast differentiation may be more important than the effects on formed osteoclasts (12–14). How osteoclast precursors respond to estrogen was the focus of the present study.

We examined the effects of estrogen and nonsteroidal ER agonists genistein and daidzein on the proliferation and differentiation of murine RAW264.7 cells. Genistein (5,7,4-trihydroxyisoflavone) and daidzein (7,4-trihydroxyisoflavone) are naturally occurring plant substances that activate ER phytoestrogens. Substantial evidence shows that phytoestrogens have a protective effect against a variety of disorders related to steroid receptors or, in some cases, protein kinase-mediated signaling (15, 16). Phytoestrogens are alternatives to estrogen replacement, but the effects of phytoestrogens on osteoclast differentiation are not clear. By receptor competition, genistein and daidzein have on the order of 1% the solution affinity of estradiol-17β. When studied by comprehensive luciferase promoter assays, genistein has a higher affinity for ERβ, with relative selectivity for ERβ at lower concentrations (17). However, phytoestrogens give variable results depending on the cell type expressing the ER and on the target gene; in osteoblasts, genistein-regulated ERα-dependent transcription has been demonstrated (18). There is particular interest in the differential effects of genistein relative to other ER agonists because of the additional properties of genistein, which inhibits several enzymes. These enzymes include protein tyrosine kinases.
DNA topoisomerases I and II, and especially Src and ribosomal S6 kinase (16, 19). Other phytoestrogens, including daidzein, have no known enzyme inhibitory properties. The effect of genistein on kinase cascades is theorized to be the leading cause of the antiproliferative effects of genistein, with cell cycle arrest in G1/M. In contrast, daidzein can be pushed to toxic concentrations without altering the cell cycle (20).

Our study examined the effects of estrogen and phytoestrogens on osteoclast precursors using the murine monocytic line RAW264.7, and we induced differentiation with recombinant cytokines. Osteoclast features are induced in RAW264.7 by RANKL with much higher efficiency and reproducibility than can be achieved in untransformed cells, allowing subtle differences to be resolved. The in vitro differentiation model also avoids interference from mesenchymal family cells such as osteoblasts, which preclude separation of direct and indirect estrogen effects on osteoclast formation in vivo. Cell proliferation, fusion, and expression of osteoclast differentiation markers were studied as well as the effects of estrogen on key differentiation pathways. The experiments show that all of the estrogen agonists reduce RANKL-induced osteoclast differentiation, with the effects of daidzein being surprisingly similar to those of estradiol, but that genistein has a lower potency.

MATERIALS AND METHODS

Cell Culture—Murine monocytic RAW264.7 cells were from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco’s modified Eagle’s medium with 4 mM l-glutamine, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. Other cell lines used were as described previously (14). To assess the effects of steroids on growth or differentiation, media were charcoal-stripped and without phenol red. For osteoclast generation, cells at 2 × 10^5/cm^2 were supplemented with 50 ng/ml RANKL and 10 ng/ml CSF-1 (R&D Systems, Minneapolis, MN). Differentiation was induced by treatment with forskolin, which was added to Sigma. The Src inhibitor PP1 was from Biomol (Plymouth Meeting, PA). For proliferation assays, cells were plated at 1.25 × 10^3/cm^2 and incubated for 24 h prior to the addition of estrogens or phytoestrogens, which were diluted from ethanol solutions with ethanol at the same concentration, generally 0.1%, added to control cultures.

RNA-based Assays—Total RNA was isolated by guanidinium thiocyanate-acetic acid extraction (13). Total RNA was purified by oligo(dT) affinity (RNAeasy, Qiagen, Valencia, CA). First strand cDNA synthesis used oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Superscript, Invitrogen). Real-time PCR was performed using 1.3 numerical aperture 40× oil immersion objective of a Nikon TE2000 inverted fluorescence microscope equipped with a Spot 12-bit 1600 × 1200 pixel CCD camera. Green fluorescence was used excitation at 450–490 nm, a 510-nm dichroic filter, and a 520-nm barrier. Red fluorescence used excitation at 536–556 nm, a 580-nm dichroic filter, and a 590-nm barrier. Fluorescent labels were photographed using 1.3 numerical aperture ×40 or ×100 oil objectives.

RESULTS

Osteoclastogenesis Regulated by Estrogen Receptors

Estrogen Receptors in RAW264.7 Cells with or without Estragon Agonists and RANKL—Because ER responses are central to the study, we examined the effect of estrogen on ER expression. ERs can be detected by Western blot with enhanced chemiluminescence in some cells, including osteoblasts (14). This approach did not give an adequate signal in RAW264.7 cells, as PCR and quantitative real time PCR were used. No ERγ was amplified (to 40 cycles on PCR or 50 cycles on real time PCR) from over a dozen cDNAs from RAW264.7, in growth or differentiation media with or without estrogen or phytoestrogen treatment. These studies used three different probe sets that amplified ERγ in MG63 and MCP7 cells. In addition, a Taqman-pretreated ERγ real time PCR probe set (Applied Biosystems, Foster City, CA) was used with the same result (Fig. 1A). In contrast, ERα was uniformly positive, and quantitative assays showed ERα mRNA in RAW264.7 cells in growth or differentiation media, with or without estrogen agonists. As expected from reports that osteoclasts have less ER expression than precursor cells (11), differentiation medium reduced ERα cDNA ~75% relative to GAPDH (Fig. 1B). The estrogen agonists all counteracted this effect partially but significantly. In this case, the effects of the two phytoestrogens at 3 μM were slightly larger than those of estradiol at 10 μM, a high but physiologically estradiol concentration.
Effect of Estrogen Agonists on Growth and Differentiation of RAW264.7 Cells—All media were phenol red-free and charcoal-stripped to avoid interference by serum steroids. Differentiation medium, with 50 ng/ml RANKL and 10 ng/ml CSF-1, had a large negative effect on proliferation and, as expected, resulted in the formation of large numbers of TRAP-expressing polykaryons. Estradiol, 10 nM, had significant positive effects on cell proliferation by thymidine incorporation at 5 days (Fig. 2A) or on the total cell number, as determined with a Coulter counter (Fig. 2B). Estradiol had negative effects on RANKL-induced osteoclast differentiation, which was assessed by the number of TRAP-expressing cells (Fig. 2C). Genistein and daidzein, 3 μM, had smaller effects in the same direction as estradiol, which sometimes did and sometimes did not reach significance; in most cases, differences in the effect of genistein were smaller than those of daidzein. The increases in cell number relative to RANKL and CSF-1 only and the decreased formation of TRAP-expressing osteoclast-like cells with estradiol or phytoestrogens suggested that estradiol reduces osteoclast formation and may retain cells in the proliferating pool of mononuclear cells. However, the effects of genistein and daidzein were smaller than those of estradiol and could not be clearly resolved by these methods; subsequent analysis will show that these differences are, however, meaningful. The concentrations of phytoestrogens used were such that maximal effects could be seen. These effects were consistent with maximal concentrations for estrogen transcription effects of genistein or daidzein in plasmid reporter studies (17). At 10⁻⁷ M, the effects on RAW264.7 cell proliferation or differentiation were not measurable, whereas at 10⁻⁵ M, cell viability decreased. The concentration dependence of estradiol on Erk and NF-κB response was also determined directly. These determinations are described below.

Effect of Phytoestrogens on Osteoclast Differentiation—Beyond the modest effects of estradiol and phytoestrogen on cell number and TRAP-expressing cells, there were qualitative differences in the appearance of TRAP-positive cells, suggesting that estradiol, and to a lesser extent genistein and daidzein, reduced cell fusion and multinucleation (Fig. 3). Qualitative differences, such as the size of multinucleated cells, are notoriously difficult to determine by microscopy, so the cell cultures were analyzed by flow cytometry at 2-day intervals after the addition of differentiation agents. This method, which is illustrated in the supplemental material, used doublet discrimination analysis, Dean-Jett-Fox cell cycle analysis (23), and sub-G₁ populations to define single cells in the growth cycle, multinucleated cells, and apoptotic nuclei (22–24). Results of multinucleation, cell cycle, and apoptosis analysis for cells 2 days post-RANKL are shown (Fig. 4). This analysis showed a clear reduction in multinucleation and in total numbers of nuclei in multinucleated cells by genistein, daidzein, and estradiol. The effects of genistein were clearly intermediate between differentiation medium alone and the effect of estradiol, which was on the order of 60% at two days. The effects of daidzein were smaller in terms of total nuclei in multinucleated cells and were not statistically different from those of estradiol.

Estrogens and Phytoestrogens Reduce S-phase Cells and Modify the Apoptosis Rate—The effects of RANKL, phytoestrogens, and estradiol on the cell cycle are shown (Fig. 5A). Although many cells left the cell cycle with differentiation and multinucleation (Fig. 4), of cells remaining in the cell cycle (shown in supplemental material), RANKL had surprisingly little effect other than a clear suppression of apoptosis (Fig. 5B). Estradiol and daidzein reduced cells in the S-phase, with accumulation in G₀/G₁, whereas genistein also suppressed the S-phase but with increases in both G₀/G₁ and G₂/M. The effects of genistein on apoptosis were insignificant relative to RANKL alone, but daidzein slightly reduced, and estradiol slightly increased, the number of apoptotic cells. Differences in apoptotic cells were on the order of 1% relative to RANKL.

Osteoclastogenesis Regulated by Estrogen Receptors

FIG. 1. Effect of RANKL and estrogens on estrogen receptor expression. A. ERα was expressed in all cells tested. ERα in RANKL-containing media (far right curve) was decreased. As with the effect on cell proliferation, estradiol and the phytoestrogens had small but statistically significant counteracting effects. In this case, the effects of genistein and daidzein were greater than those of estradiol. In contrast, ERβ did not amplify in any sample (to 50 cycles), as indicated by plus signs on the ordinate. B. copy number of ERα relative to GAPDH. The quantitative ERα expression decreased ~75% with the addition of RANKL. Estrogen and both of the phytoestrogens had significant effects, increasing ERα relative to the differentiation medium alone. The mean ± S.D. for 3 determinations is shown.

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Effect of Estrogen and Phytoestrogens on Differentiation Signals—To determine how estrogen and phytoestrogens modulate RANKL-induced differentiation, we studied the key signaling pathways of Src kinase activity, which was hypothesized to be sensitive to genistein, and the Erk and NF-κB pathways, which are critical for osteoclast differentiation. Src phosphorylated at tyrosine 416 (enzymatically active Src) dramatically decreased with differentiation. Comparing Src phosphorylation in the presence and absence of estrogen agonists showed a trend toward slightly decreased phospho-Src, but changes were too small to be regarded as definite (for comparison, the effect of the Src-specific inhibitor PP1 is shown in Fig. 6A). The effects appeared similar for all estrogen agonists (Fig. 6A) in either growth or differentiation medium. A larger concentration-dependent decrease in Src phosphorylation was expected with genistein, a tyrosine kinase inhibitor. However, compared with other estrogen agonists, no additional effect of genistein was seen within ~20% confidence because of experimental variability. Phospho-Erk1/2, the p44/42 MAP kinases (Erk), was decreased by estrogen agonists with the same pattern of activity as seen in cell cycle data. That pattern is estrogen > daidzein > genistein (Fig. 6B). Similar results were obtained in both growth and differentiation media. Phospho-Erk was elevated by short term exposure to differentiation medium, which
estradiol

phytoestrogen effects were, however, consistent with higher resolution studies by flow cytometry (Fig. 5), which show the same order of potency, fewer TRAP-positive cells, but differences were modest and typically did not reach significance by manual cell counts. In cultures with RANKL and essentially no osteoclasts in growth medium (second bar), with a trend toward increased cell number (but not statistical significance) in genistein and daidzein and a statistically significant ($p < 0.05$) increase in estradiol (right bar). C, numbers of TRAP-positive cells in media. There were essentially no osteoclasts in growth medium (left bar) and many in RANKL-containing medium (second bar). The phytoestrogens had a trend toward fewer TRAP-positive cells, but differences were modest and typically did not reach significance by manual cell counts. In cultures with RANKL and estradiol (far right bar) there was, however, a significant decrease in the number of TRAP-positive cells relative to RANKL alone. The phytoestrogen effects were, however, consistent with higher resolution studies by flow cytometry (Fig. 5), which show the same order of potency, estradiol > daidzein > genistein.

**Fig. 2. Effect of RANKL and estrogen agonists on DNA synthesis, cell number, and TRAP expression.** A, DNA synthesis in growth media and differentiation media with and without phytoestrogens and estrogen. Relative to growth medium, differentiation media supported much lower rates of DNA synthesis (first versus second bar, note discontinuous scale). Estrogen agonists (3 μM genistein or daidzein, 10 nM estradiol-17β) had a modest but consistent counteracting effect (third, fourth, and fifth bars). In this determination, which is typical of several similar studies, thymidine incorporation in RANKL plus genistein or estradiol was significantly greater ($p < 0.05$) than that in differentiation medium alone, but differences in RANKL with and without daidzein did not reach significance. B, total cell number in 5-day cultures under the same conditions as in A. A Coulter counter was used to measure the cell number after 25,000 cells were incubated for 5 days. The cell number was dramatically reduced by the differentiation medium (second bar), with a trend toward increased cell number (but not statistical significance) in genistein and daidzein and a statistically significant ($p < 0.05$) increase in estradiol (right bar). C, numbers of TRAP-positive cells in media. There were essentially no osteoclasts in growth medium (left bar) and many in RANKL-containing media (second bar). The phytoestrogens had a trend toward fewer TRAP-positive cells, but differences were modest and typically did not reach significance by manual cell counts. In cultures with RANKL and estradiol (far right bar) there was, however, a significant decrease in the number of TRAP-positive cells relative to RANKL alone. The phytoestrogen effects were, however, consistent with higher resolution studies by flow cytometry (Fig. 5), which show the same order of potency, estradiol > daidzein > genistein.

replicates effects described previously (see the "Discussion"). Because these effects could easily be related to differentiation, the relationship of phospho-Erk in the presence of RANKL with the phytoestrogens or varying estrogen concentrations was studied at short intervals after the addition of RANKL (Fig. 6C). Studying these relationships showed that there was no significant effect of estrogens or phytoestrogens at short intervals (10 min) when the RANKL effect was clear, and only a small trend toward estrogen effects was seen at 20 min. Thus, it is likely that the Erk activation is downstream of earlier effects such as NF-κB activation.

Studies of NF-κB (Fig. 7) showed similar quantities of p65 with or without estrogen agonists. No effect on total NF-κB quantity was seen after short term (1 h) exposure to RANKL in four similar experiments (not illustrated). These results are not surprising because this nuclear factor is mainly regulated by nuclear translocation, which follows the tyrosine kinase or tumor necrosis factor receptor-dependent dissolution of complexes that permit nuclear translocation of free NF-κB. After short term exposure to RANKL, a large effect on NF-κB distribution was seen, with a clear increase in nuclear localization. RANKL-dependent nuclear localization was reduced by estradiol (Fig. 7, A–C) and by the phytoestrogens with the same order of activity seen in the cell cycle effects (Fig. 7D). This reduction correlated with increased IκB, which was clearly seen at estrogen concentrations of $10^{-8}$ M and higher (Fig. 7E), in accordance with effective concentrations of estrogen in differentiation experiments. Estrogen and phytoestrogens stabilized IκB in the presence of RANKL with efficacy similar to the relative effects of these substances (Fig. 7F).
DISCUSSION

In terrestrial vertebrates, the osteoclast performs the vital tasks of dissolving bone for calcium or pH homeostasis and removing bone for growth or to replace bone (25). It has been established that in the presence of CSF-1 sufficient to maintain cell growth and survival, RANKL, via its tumor necrosis factor receptor RANK, is sufficient to induce complete osteoclastic differentiation from hematopoietic precursors and that knock-out mice with defects in the RANK system cannot form osteoclasts (26). It is also established that estrogen withdrawal causes rapid skeletal degradation, an effect that certainly involves other cells, including osteoblasts, but that has also been hypothesized to involve direct effects on osteoclast formation (3–5). This report demonstrates that phytoestrogens and β-estradiol directly reduce osteoclastic differentiation in the murine RAW264.7 cell model.

In this cell model, we found that ERα is expressed and that ERα expression is greatly reduced by RANKL-induced differentiation. This is consistent with previous reports that estrogen receptors decline with osteoclastic differentiation (7, 11). With our sensitive PCR-based method, ~30% of ERα mRNA could still be detected in mature RANKL-treated cells. However, estrogen agonists, including both of the tested phytoestrogens, maintained higher levels of ERα mRNA when added to

FIG. 4. Effect of estrogens and phytoestrogens on multinucleation. A, comparison of the number of nuclei in multinucleated cells. Histograms for RANKL and CSF without or with phytoestrogens and estrogen are shown. The method is illustrated in the supplemental material. The average of two determinations and range are shown. The number of multinucleated cells without RANKL was small and is not illustrated. Relative to differentiation medium alone, all of the test substances reduced multinucleation, with estrogen being the most efficient, and with the largest number of multinucleated cells still having only two nuclei. In daidzein or genistein, the number of multinucleated cells was reduced but with smaller effects. B, number of nuclei in multinucleated cells by flow analysis. These data show the product of the number of cells for each nuclear number multiplied by the number of nuclei. The mean ± range for 2 determinations, each counting 25,000 events, is shown. All of the phytoestrogens had significantly reduced multinucleation, with effect of estrogen > daidzein > genistein. Differences between daidzein and estradiol did not reach significance, but all other differences were significant (p < 0.01).

FIG. 5. Effect of estrogens and phytoestrogens on cell cycle and apoptosis in 2-day cultures. A, effects of RANKL, phytoestrogens, and estrogen on the cell cycle. This was determined by Dean-Jett-Fox analysis (23) as described in the supplemental material. Many cells were taken out of the cell cycle by differentiation (see Fig. 4B). This figure shows distribution of mononucleated cells. RANKL had only small effects (Differentiation set of bars) relative to growth medium. In contrast, phytoestrogens had significant effects, reducing S-phase and increasing G0/G1, with estrogen > daidzein > genistein. Genistein also slightly increased the G2M population. B, effects of RANKL without and with phytoestrogens on apoptosis. Differentiation media with or without RANKL all reduced apoptotic nuclei relative to growth medium, which was determined from hypoploid cells as described in the supplemental material. Daidzein and estradiol further decreased or slightly increased apoptosis, respectively, relative to RANKL alone, on the order of 1%.
RANKL-containing differentiation medium. How this is reflected in longer-term effects of estrogen on bone turnover is unclear at this point, but estrogen-dependent maintenance of ERα receptors would be expected to facilitate the estrogen response. We were unable to detect ERβ in RAW264.7 cells, a finding that is consistent with results in both murine and human osteoclast precursors (10, 11). There is some controversy on this point because antibody-based studies have reported ERβ in human osteoclasts (8, 9). It is possible that there are species differences or that small quantities of ERβ were present but not detected, but it seems unlikely that there is an important ERβ-mediated response in the RAW264.7 cells.

Estradiol and the phytoestrogens were effective roughly in proportion to their reported efficacy in activating ERαs, and the order of efficacy was estradiol > daidzein > genistein. There were some variations in individual assays, including in ERα mRNA, where genistein and daidzein appeared slightly more effective than estrogen. On the other hand, key assays were performed using sensitive flow cytometric methods, and during differentiation at 2 days, the differences in multinucleation and osteoclast-forming cells, although protecting bone-producing cells, would have a bone-sparing effect and may be one reason why estrogen is a more effective bone-sparing agent than phytoestrogens, even at high concentrations.

There are few other studies of the effect of phytoestrogen on osteoclasts. There are animal studies, but these reflect the effects on osteoblasts, where phytoestrogens reduce the ratio of RANKL relative to osteoprotegerin (6), an indirect inhibitory effect that may mask any direct effects of phytoestrogens on osteoclast differentiation. Our earlier work showed the effects of genistein on acid secretion in avian osteoclasts (28), an effect unrelated to osteoclast differentiation. One study reported that genistein may suppress osteoclast formation via apoptosis at 10 μM (29). Our experience is consistent with that report, although because concentrations on the order of 10 μM appeared to increase cell death and our interest was in differentiation, we did not use concentrations above 3 μM (29). Our experience is consistent with that report, although because concentrations on the order of 10 μM appeared to increase cell death and our interest was in differentiation, we did not use concentrations above 3 μM. Apoptotic effects were avoided (Fig. 5B). This revealed small but consistent and significant effects of estrogen on osteoclast formation (Figs. 4 and 5). One report suggests that nanomolar daidzein increases pit formation by rodent osteoclasts (30). We have been unable to detect daidzein effects at nanomolar concentrations, and the effects that we measured at 3 μM were in the opposite direction, opposing osteoclast formation. In vivo effects require concentrations of daidzein on the order of 0.1% in the diet (31), conflicting with large effects at nanomolar levels. These effects are also indicated by clinical studies of weak estrogen agonists, in which it was difficult to attain effective concentrations of these substances. Results were variable and typically small (32). On the other hand, daidzein may be an effective alternative to estrogen if effective concentrations can be reached.

RANKL and CSF-1 mediate osteoclast differentiation by a number of pathways. These include several tyrosine phosphorylation steps, particularly initiated by CSF-1, and direct or indirect stimulation of Erk phosphorylation and NF-κB nuclear translocation (15). Because genistein has tyrosine kinase antagonist properties and can inhibit Src, we looked specifically for a genistein effect on Src phosphorylation. However, no effect large enough to be resolved by the Western blot methods was seen (Fig. 6A). The estrogen agonists generally appeared to have a slight negative effect on the amount of Src phosphorylation, but this was at the limit of resolution and much smaller than the effects of control inhibitors, such as PP1. Thus, we...
cannot exclude a direct effect of estrogen agonists on Src-dependent pathways, but such effects appear to be small at best. On the other hand, when both Erk phosphorylation and NF-κB nuclear translocation were examined (Figs. 6, B and C, and 7), the estrogen agonists appeared to counteract RANKL effects in close correspondence to their effects on osteoclast differentiation, which were estradiol > daidzein > genistein. Concentration dependence studies of IκB effects suggest that estrogen concentrations >1 nM are required to affect osteoclast differentiation. Additional studies of Erk phosphorylation at short intervals after RANKL stimulation suggested that although phospho-Erk effects are clear at long intervals after RANKL stimulation, these are probably secondary to differentiation, which was most clearly related to IκB. Together, these results strongly suggest that IκB is stabilized by cytoplasmic estrogen receptor complexes.

Although this mechanism will require further study, it is likely that a cytoplasmic event is critical, because estrogen agonists caused coordinated changes in multiple nuclear activating steps. The key change appears to be reduced translocation of NF-κB, although there were small differences in Src phosphorylation and larger, but probably indirect, effects on Erk phosphorylation. Src is a part of redundant pathways that are co-regulated by p130cas, which coordinates integrin-binding signals (33) and nongenomic ERs signals (34). Src is thus likely to be involved in the effects of estradiol and phytoestrogens on RAW264.7 osteoclastic differentiation, and this proximal mechanism would account for coordinated differences in Src phosphorylation and down-regulation of Erk and NF-κB. Estrogens are also known to activate Erk by non-genomic mechanisms, which may be important in the attenuation of apoptosis (35). The effect seen in this case, however, was the suppression of Erk phosphorylation, and apoptosis had a minor effect. Another candidate mechanisms for estrogen repression of these pathways is interference with binding of NF-κB to its promoter site (36), which could be involved in the effects observed but would not explain the inhibition of nuclear localization (Fig. 6C), which clearly points to an upstream mechanism. On the contrary, it is likely that the NF-κB effect relates to the stabilization of IκB in the cytoplasm (Fig. 7, E and F). This interesting finding could relate to intermediate signals or the

Fig. 7. Effect of RANKL, estrogens, and phytoestrogens on NF-κB p65 translocation. A–C, effect of RANKL and estrogen on nuclear localization of NF-κB. RAW264.7 cells were labeled for NF-κB and filamentous actin. The three left panels show NF-κB distribution, the middle panels are filamentous actin to show cellular detail, and the right panels are a merged image; all frames are at the same magnification and are 40 μm square fields. A, in growth medium (top), nuclei were not clearly distinguished from the cytoplasm, although there was some finely stippled NF-κB labeling in the nuclei (N), and most of the label was cytoplasmic (C). B, 1 h after the addition of 40 ng/ml RANKL, nuclei were strongly labeled, and cytoplasmic labeling was weak. C, when RANKL and 10 nM estradiol were added, most NF-κB was still nuclear (N), but the cytoplasm (C) had a greater proportion of the label than with RANKL alone. D, NF-κB nuclear localization at 1 h with the co-addition of phytoestrogens (3 μM) or estradiol. Nuclear and non-nuclear NF-κB were measured from preparations of RAW264.7 cells and calculated as a ration. Results are shown from cells without RANKL (left bar) or 1 h after the addition of 40 ng/ml RANKL (second bar), RANKL with 3 μM genistein or daidzein, or 10 nM estradiol as indicated. Differences in daidzein and estradiol were statistically significant (p < 0.01). Differences in daidzein had p ~ 0.5, n = 8, mean ± S.E. E, dependence of IκB on estradiol concentration. NF-κB was held in the cytoplasm in a complex with IκB, which was ubiquitinated and degraded in response to RANK activation. Thus, one mechanism for the effects of estrogen on NF-κB could be stabilization of IκB. Here, estradiol concentrations from 100 pM to 10 μM were included with RAW264.7 cells, and the amount of IκB was determined by Western blot analysis after a 1-h incubation. Note that the estradiol effect is seen at 10−8 M but reaches a plateau at this concentration. F, effect of estrogen and phytoestrogens on IκB after adding RANKL. IκB-α was measured in lysates of RAW264.7 cells after a 30-min (left panel) or 1-h (right panel) incubation with or without 50 ng/ml RANKL (left panel) and with or without phytoestrogens or estrogen with RANKL (right panel). This large concentration of RANKL reduces IκB very effectively; all of the estrogen agonists stabilized the IκB somewhat, with daidzein and estrogen being most efficient. The right panel shows a long development time to resolve these differences. Reprobing the blot for actin showed that protein loads were all within 10% (not illustrated).
reduction of ubiquitination of IκB and will be an avenue for further study.

In summation, we found that in the RAW264.7 cell model, ERα but not ERβ is expressed, and estrogen agonist effects were consistent with their expected effects on ERα from transcriptional activation studies (17). The estrogen receptor agonists appeared to oppose the effects of RANKL via its Erk and transcriptional activation studies (17). These results make a case for a relatively straightforward negative effect of estrogen on bone degradation at the level of osteoclast formation.

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