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Estrogen Stimulates Protein Tyrosine Phosphorylation and Src Kinase Activity in Avian Osteoclasts

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Abstract The estrogen, 17β-estradiol, stimulated a profound increase in phosphotyrosine immunostaining of proteins that localized along the site of attachment in avian osteoclasts within 1 min of treatment. By 10 min, this rapidly occurring event had returned to basal levels. Pretreatment with 1 μM herbimycin A, a tyrosine kinase inhibitor, prevented the response. Immunoblotting revealed that Src kinase was one of the phosphorylated intermediates. Src kinase also appeared to translocate to the periphery of the cells during the 1 min 17β-estradiol treatment and became dispersed by 10 min. Src kinase activity measurements indicated an increase in phosphotransferase activity after the 1 min estradiol treatment; this effect diminished with longer exposures to estrogen. Pretreatment of osteoclasts with 1 μg/ml cytochalasin B, an inhibitor of actin polymerization, delayed the appearance of increased phosphotyrosine immunostaining at attachment sites, possibly through inhibition of Src kinase translocation. These findings demonstrate that estrogen stimulates rapid tyrosine phosphorylation in osteoclasts, a process that involves activation and translocation of Src kinase to the plasma membrane. J. Cell. Biochem. 76:206–216, 1999.

Key words: osteoclasts; 17β-estradiol; phosphotyrosine; Src kinase; actin; confocal laser scanning microscopy

Classically, steroid hormones are believed to act through direct binding to a nuclear receptor that dimerizes and acts as a transcriptional regulator [Fuller 1991; Carson-Jurika et al., 1990]. By contrast, peptide hormones and growth factors act through receptors in the plasma membrane that stimulate rapid signaling pathways. Recently, it has been suggested that steroid receptor and growth factor pathways overlap. Aronica and Katzenellenbogen [1993] reported that IGF-1 and other agents that increase cyclic adenosine monophosphate (cAMP) levels, increased estrogen receptor activation. Protein kinase activators were also shown to increase the transcriptional activity of the estrogen receptor [Katzenellenbogen, 1996]. In addition, steroids have been found to produce rapid nongenomic responses that are similar to those evoked by peptide hormones and growth factors. Estrogen has been shown to regulate Ca^{2+} fluxes and/or cAMP levels in various cell types, including enterocytes [Picotto et al., 1996], granulosa cells [Morley et al., 1992], neurons [Mermelstein et al., 1996], and smooth muscle cells [Farhat et al., 1996], and to stimulate an increase in phosphotyrosine levels in MCF-7 breast cancer cells within 10 s of treatment [Migliaccio et al., 1993]. More recently, Migliaccio et al. [1996] demonstrated that estrogen stimulated the Ras/mitogen-activated protein (MAP) kinase pathway through the direct activation of Src kinase in MCF-7 cells. Endoh and colleagues [1997] reported that 17β-estradiol stimulated MAP kinase in ROS 17/2.8 osteoblast-like cells within 5 min. These studies provide solid evidence of estrogen regulation through rapid signaling pathways commonly used by peptide hormones and growth factors.

We report that 17β-estradiol increases phosphotyrosine levels in avian osteoclasts within 1 min of treatment. The distribution of newly phosphorylated proteins was detected along the actin/podosome ring at cell attachment sites. Estrogen treatment stimulated both the activity and translocation of Src kinase to the plasma

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membrane. We suggest that this is the beginning of a rapid signaling pathway that may include the Ras/MAP kinase cascade.

MATERIALS AND METHODS

Materials

Synthetic salmon calcitonin, heat-inactivated calf serum (CS), heat-inactivated fetal bovine serum (FBS), minimum essential medium (MEM, Eagle's modification with nonessential amino acids and Earle's salts), neutral red, penicillin-streptomycin, L-glutamine, Trizma base (Tris), glycine, Triton X-100, gelatin (Teleostean), donkey serum, sodium orthovanadate (Na_3VO_4), Tween-20, trichloroacetic acid (TCA), paraformaldehyde, rabbit anti-actin polyclonal Ab, rabbit anti-whole mouse polyclonal Ab, casein acid hydrolysate, herbimycin A, Hepes (Na^+ free), tamoxifen, 17β-estradiol, and glycerol were purchased from Sigma Chemical Co. (St. Louis, MO). Worthington Biochemicals (Freehold, NJ) was the source of trypsin (bovine pancreatic, 3× crystalized). SDS, acrylamide, TEMED, dithiothreitol (DTT), molecular-weight markers and ammonium persulfate were obtained from Bio-Rad (Hercules, CA). Complete™ protease inhibitor cocktail was purchased from Boehringer Mannheim (Indianapolis, IN). Mouse anti-phosphotyrosine monoclonal Ab (clone 4G10), mouse anti-Src monoclonal and mouse anti-chicken Src (avian-specific monoclonal) and Src kinase activity kit (#17–131) were obtained from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase (HRP)-linked anti-mouse and rabbit antibodies, enhanced chemiluminescence (ECL) kit, and hyperfilm-ECL were obtained from Amersham (Arlington Heights, IL). γ-^{32}P-ATP was purchased from Dupont NEN (Boston, MA). Protein A-Sepharose beads were obtained from Zymed (San Francisco, CA). Donkey anti-rabbit-Cy3-conjugated polyclonal Ab was purchased from Calbiochem (Temecula, CA). Texas Red-phalloidin was obtained from Molecular Probes (Eugene, OR). Immobilon-P was purchased from Millipore (Bedford, MA). The BCA protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL).

Isolation and Cell Culture

Osteoclasts were isolated from tibias of 2.5- to 3-week-old chicks (Peterson-Arbor Acre) injected subcutaneously with calcitonin (30 mU/100 g body weight) using a combination of earlier methods [Gay et al., 1983; Hunter et al., 1988]. Calcitonin was employed to enhance detachment of osteoclasts from bone surfaces. Thirty min following calcitonin injection, the birds were euthanized by decapitation. Tibias were removed and cleaned with sterile gauze to remove attached muscle, split longitudinally, and the bone marrow was lifted out with forceps. The bone halves were placed in MEM + 10% CS and flushed vigorously to remove any remains of marrow, then subjected to a 20-min digestion in 0.03% trypsin in MEM at 37°C, 5% CO_2. The bones were then placed in neutral red (0.1 mg/ml) in MEM + 10% CS for 5 min at 37°C, 5% CO_2 to allow the phagocytotic osteoclasts to take up the dye. After rinsing the bones in MEM + 10% CS, cells were gently scraped off the endosteum with a rubber policeman (VWR Scientific Products, Baltimore, MD), and the final suspension was filtered through 250- and 150-μm polypropylene meshes (Small Parts, Miami Lakes, FL), centrifuged at 625*g* in a swinging bucket rotor for 10 min at room temperature and resuspended. The pelleted cells were resuspended in MEM + 5% FBS and plated as follows: 1 ml per well for 12-mm coverslips, 3 ml per well for 24-mm coverslips and 4 ml per Lab-tek single well chambered coverglass (Nunc, Naperville, IL), where 2 × 1-ml wells were plated per chick. Cell density was approximately 10^4 cells per well. The cells were incubated overnight at 37°C, 5% CO_2, then rinsed in MEM and placed in MEM + 0.5% FBS for the next 3 days at 37°C, 5% CO_2. Purity assessment using a histochemical stain for tartrate-resistant acid phosphatase (TRAP), a marker for osteoclasts was between 60–85% by counting cells in a representative number of fields of view. Cells not stained for TRAP were fibroblast-like and did not stain for alkaline phosphatase, a marker for osteoblasts.

Cell Treatment and Sample Preparation for Immunoblotting

Osteoclasts plated on Lab-tek chambered coverglass were pre-rinsed three times with MEM and either pretreated with 1 μM herbimycin A or with 1 μM tamoxifen for 5 min or not pretreated, then exposed to vehicle or 10 nM 17β-estradiol for 0, 1, 5, 10 or 30 min at 37°C, 5% CO_2. At the end of each treatment, media along with any additives were removed and cells were lysed on ice with 250 μl of lysis buffer (1%

Triton X-100, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, and 10 mM Tris, pH 7.2) to which 1 mM sodium orthovanadate and Complete™ protease inhibitor cocktail were freshly added. The lysates were centrifuged at 16,000*g* at 4°C and the supernatants were collected.

SDS-PAGE and Immunoblotting

Osteoclast lysates, mixed with 6× sample buffer (125 mM Tris-Cl, pH 6.8, 10% sodium dodecyl sulfate [SDS], 30% glycerol, 9.3% DTT) for a final concentration of 1× sample buffer, were boiled for 5 min and separated by SDS-polyacrylamide gel electrophoresis (PAGE), using 7.5–10% acrylamide gels and the Hoefer SE 600 gel electrophoresis apparatus. Two gels (1.5 mm thick) were typically run at 65-mA constant current in buffer containing 25 mM Tris, 192 mM glycine and 0.1% SDS. Immunoblots were prepared by electroblotting proteins separated by SDS-PAGE onto Immobilon-P PVDF transfer membrane using a FisherBiotech FB-SDB-2020 semi-dry blotter. Transfer was performed for 2 h at 120 mA constant current with Towbin transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol, 0.01% SDS). Blots were dried and stored at −80°C until needed.

Blots were placed in 100% methanol briefly, then rinsed twice with 50 ml Tris-buffered saline (10 mM Tris pH 7.4, 50 mM NaCl) plus 0.1% Tween 20 (TTBS). Blots were then blocked with 3% Teleostean gelatin in TTBS for 1 h with shaking, then rinsed twice with TTBS for approximately 1 min each rinse. The blots were incubated in primary antibody for 1 h at the following dilutions: anti-phosphotyrosine, 1 µg/ml; anti-Src, 1 µg/ml, and anti-actin, 1:1,000. The first two antibodies were used individually on separate blots, while the anti-actin antibody was used to reprobe blots as an internal control for each lane. After primary antibody incubation, the blots were rinsed in 100 ml TTBS (3 × 15 min). After rinsing, the blots were probed with either HRP-linked anti-mouse or rabbit antibodies at a dilution of 1:5,000 for 1 h. Blots were rinsed in the same manner as after exposure to primary antibodies. Detection was performed using the Amersham ECL kit. Molecular weights were determined by Coomassie blue staining the blot after reprobing blots for actin. Most blots contained two sets of samples to facilitate making side-by-side comparisons.

Immunocytochemistry

Osteoclasts on coverslips were pre-rinsed three times in MEM before adding vehicle or 17β-estradiol (10 nM) for 0, 1, 5, 7.5, 10, and 30 min. Some cells were pretreated with 1 µM herbimycin A for 5 min or 1 µg/ml cytochalasin B for 10–30 min before adding estrogen or vehicle. Cells were then fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2 (PBS) for 10 min, then rinsed three times in PBS. Cells were permeabilized with 0.025% Triton-X-100 in blocking solution (1% gelatin, 0.5% casein in PBS) for 10 min. Primary antibodies to anti-phosphotyrosine (10 µg/ml), and anti-chicken Src (10 µg/ml, 1 h in blocking solution) were applied to coverslips. To stain for actin, cells were exposed to Texas Red-phalloidin (1:50, 20 min). Cells were then rinsed in buffer (0.1% gelatin, 0.05% casein, 0.025% Tween-20 in PBS; 3 × 15 min). The coverslips, except for those stained for actin, were then incubated in rabbit-anti-mouse whole antibody (1:5,000, 30 min) and rinsed once again with buffer (3 × 15 min). The coverslips were then exposed to donkey anti-rabbit Cy3-conjugated antibody (1:250, 1 h) in blocking solution plus 1% donkey serum. After rinsing three times, the coverslips were inverted and mounted on slides with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). The secondary and tertiary antibodies had been presorbed on fixed cells for 15 min before use, to minimize nonspecific staining. The cells were analyzed with the Bio-Rad MRC 600 confocal microscopy system using the 564-nm laser line. The same gain and contrast levels were maintained throughout each experiment, to permit valid comparisons. Cells were optically sectioned by 0.5-µm steps, using a 63× oil immersion lense (Leitz Wetzlar, Germany, 1.4 NA). After optical sections were projected, average pixel intensity values were measured by tracing the fluorescent actin/phosphotyrosine ring in each osteoclast using Bio-Rad software to compute the average pixel intensity of selected areas.

Src Kinase Assay

Osteoclasts plated on 24-mm glass coverslips were treated with 10 nM 17β-estradiol or vehicle (0.01% EtOH) for 0, 1, 10, and 60 min before being lysed with 250 µl lysis buffer (0.25% Triton X-100, 50 mM NaCl, 30 mM sodium

pyrophosphate, 50 mM NaF, 0.25% sodium deoxycholate, 4.0 mg/ml CHAPS, and 10 mM Tris, pH 7.4) to which 1 mM Na_3VO_4 and Complete™ protease inhibitors were freshly added. After scraping the coverslips with a rubber policeman, the lysates were transferred to microcentrifuge tubes, and a 10-µl aliquot was used for protein analysis using the BCA protein assay kit. Samples, in triplicate, containing 75 µg protein were incubated with 0.5 µg mouse anti-Src antibody for 1 h on ice with shaking. Then, 2.2 µg rabbit-anti-mouse whole antibody was added to each tube for 30 min. Sixty microliters of a 50% slurry of Protein A-Sepharose beads, pre-rinsed (3×) in lysis buffer, was added to each tube to capture the Src kinase/ antibody complex for 1 h on ice with shaking. After centrifuging at 16,000*g*, the beads were rinsed twice with 400 µl lysis buffer and three more times with 400 µl Src kinase reaction buffer (100 mM Tris, pH 7.2, 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 0.25 mM Na_3VO_4, 2 mM DTT). Src kinase activity was measured according to kit instructions. Controls included omission of the primary antibody; a sample containing Protein A-Sepharose only; and a set of samples that did not receive the substrate peptide, to demonstrate endogenous phosphorylation.

Statistics

The data were analyzed by analysis of variance (ANOVA) in SAS version 6.0.

RESULTS

Osteoclasts treated with 10 nM 17β-estradiol, fixed with 2% paraformaldehyde and immunostained for phosphotyrosine were examined by confocal microscopy as shown in Figure 1. The intensity of phosphotyrosine staining increased markedly along sites of osteoclast attachment following the 1-min treatment (Fig. 1C). The phosphotyrosine staining decreased to basal levels after 10 min (Fig. 1E). Figure 1A,B,D,F represents controls (as defined in the figure legend). Along with the increased phosphotyrosine staining, a second ring of fluorescence appeared at the cell periphery after the 1-min treatment (Fig. 1C, arrow), a phenomenon not detected at longer incubation times. We did not observe an increase in phosphotyrosine staining in the fibroblast-like cells present (not shown). Figure 2 summarizes the average pixel intensity of phosphotyrosine specific fluorescence in osteoclasts treated with 17β-estradiol depicted in Figure 1. Fluorescence attributable to phosphotyrosine immuno-

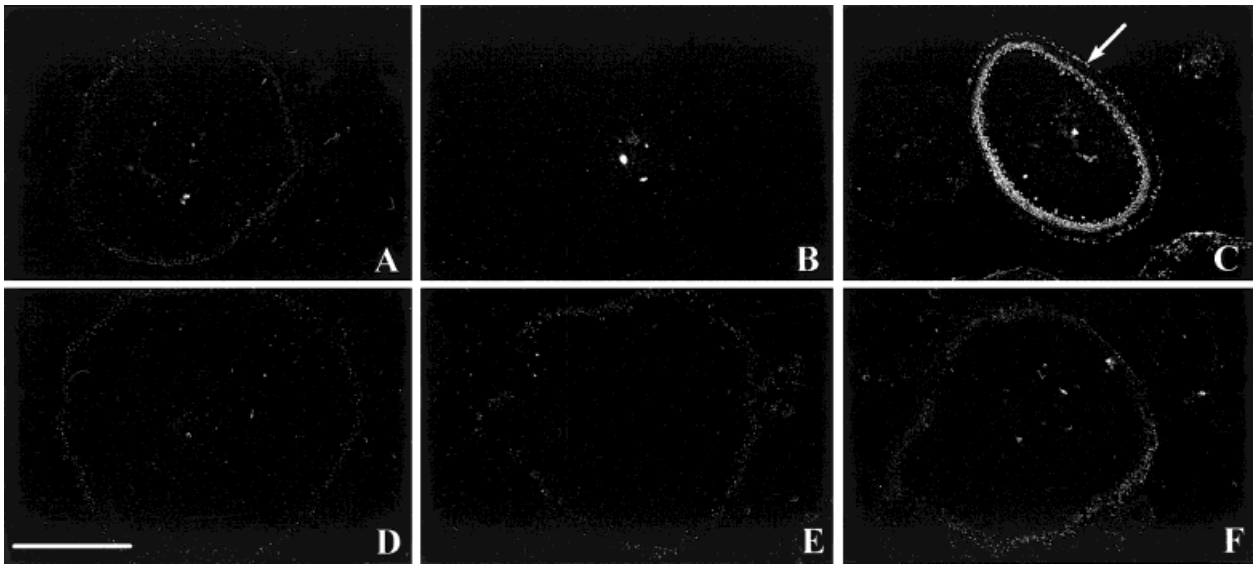

Fig. 1. Immunostaining of phosphotyrosine in estrogen and vehicle treated osteoclasts. Osteoclasts were either untreated or treated with 10 nM 17β-estradiol or vehicle for 1 or 10 min before fixation in 2% paraformaldehyde. Cells were labeled using a triple antibody protocol, using a mouse anti-phosphotyrosine. **A:** Untreated cell (all antibodies applied). **B:** Secondary and tertiary antibody only. **C:** 1 min 17β-estradiol treatment, arrow points to the outer ring of fluorescence that appears at this time point. **D:** 1 min vehicle; **E:** 10 min 17β-estradiol; **F:** 10 min vehicle. Scale bar = 50 µm.

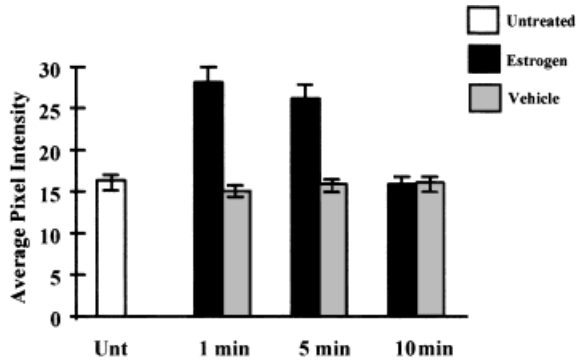

Fig. 2. Average intensity of osteoclasts immunostained for phosphotyrosine following estrogen treatment. Osteoclasts were treated with 10 nM 17β-estradiol or vehicle for 1, 5, or 10 min before fixation in 2% paraformaldehyde. Cells were immunostained as in Fig. 1. Each bar represents the average pixel intensity ±SEM for a minimum of 45 cells from three experiments. The 1 and 5 min 17β-estradiol treatments were found to be significantly different ($P < 0.001$) from all other treatments (except each other), using analysis of variance (ANOVA). UNT, untreated control exposed to vehicle (0.01% ethanol).

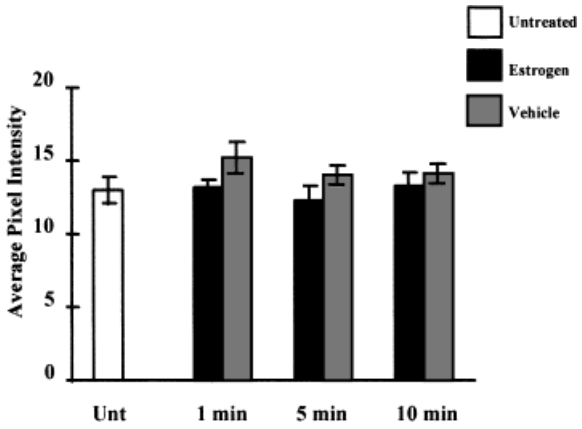

Fig. 3. Average intensity of phosphotyrosine immunostaining of osteoclasts during tyrosine kinase inhibition. Osteoclasts were pretreated with 1 μM herbimycin A for 5 min before exposure to 10 nM 17β-estradiol or vehicle for 0, 1, 5, and 10 min. Cells were then fixed and immunostained as in Fig. 1. Each bar represents the average pixel intensity ±SEM for a minimum of 45 cells from three experiments. There were no significant differences among treatments.

staining nearly doubled at 1 min of treatment and remained at that level for at least 5 min before returning to basal levels by 10 min. Pretreatment with herbimycin A, a tryrosine kinase inhibitor, blocked the increase in phosphotyrosine immunofluorescence (Fig. 3). Immunoblotting with anti-phosphotyrosine antibody demonstrated that several proteins ranging in size from 40 to 120 kDa were tyrosine phosphorylated in response to the 1-min treatment, including Src kinase (Fig. 4A). Pretreatment with 1 μM herbimycin A for 5 min blocked the increased phosphotyrosine levels associated with the 1 min treatment (Fig. 4A, lane G). Pretreatment with 1 μM tamoxifen, a nonsteroidal estrogen antagonist, also blocked the 1 min estrogen response (Fig. 4A, lane F). Blots were reprobed for actin as a loading control and the average of the ratio of tyrosine-phosphorylated Src kinase to actin is presented in Figure 4B and confirms that Src kinase is phosphorylated above basal levels at the 1 and 5 min treatment times.

When osteoclasts were stained with an antibody directed against chicken Src kinase (Fig. 5), the enzyme was found to be distributed along sites of attachment, as well as in the center of the cell where vesicles are located (Fig. 5A), confirming an earlier report [Boyce et al., 1992]. Cells treated with 17β-estradiol for 1 min showed increased Src kinase immunostaining at the periphery of the cell (Fig. 5C); peripheral staining disappeared with longer incubation (Fig. 5E).

Figure 6 shows that Src kinase immunoprecipitated by an anti-Src monoclonal antibody reacts with both anti-chicken Src kinase and anti-phosphotyrosine antibodies. A band of approximately 58 kDa was labeled. To demonstrate Src kinase activation by 17β-estradiol treatment, the enzyme was immunoprecipitated from lysates of cells treated with 17β-estradiol or vehicle and activity was measured. The assay is based on the incorporation of a ^{32}P-labeled phosphate group into a Src kinase specific peptide corresponding to residues 6–20 of p34cdc2. Src kinase from cells treated with 17β-estradiol for 1 min had approximately a twofold increase in phosphotransferase activity, as shown in Figure 7. This activity was inhibited by pretreatment of cells with herbimycin A; furthermore, activity diminished to basal levels by 10 min of treatment.

Osteoclast inactivation by calcitonin, an inhibitor of bone resorption, is associated with shape changes and the loss of the actin ring at the site of attachment [Lakkakorpi and Väänänen, 1990]. To assess whether 17β-estradiol affects the actin/podosome ring, cells treated with 17β-estradiol were stained with Texas Red-conjugated phalloidin, which binds actin filaments. The actin ring in osteoclasts treated with 17β-estradiol for 0–60 min remained intact (Fig. 8). Figure 9 exhibits actin staining of

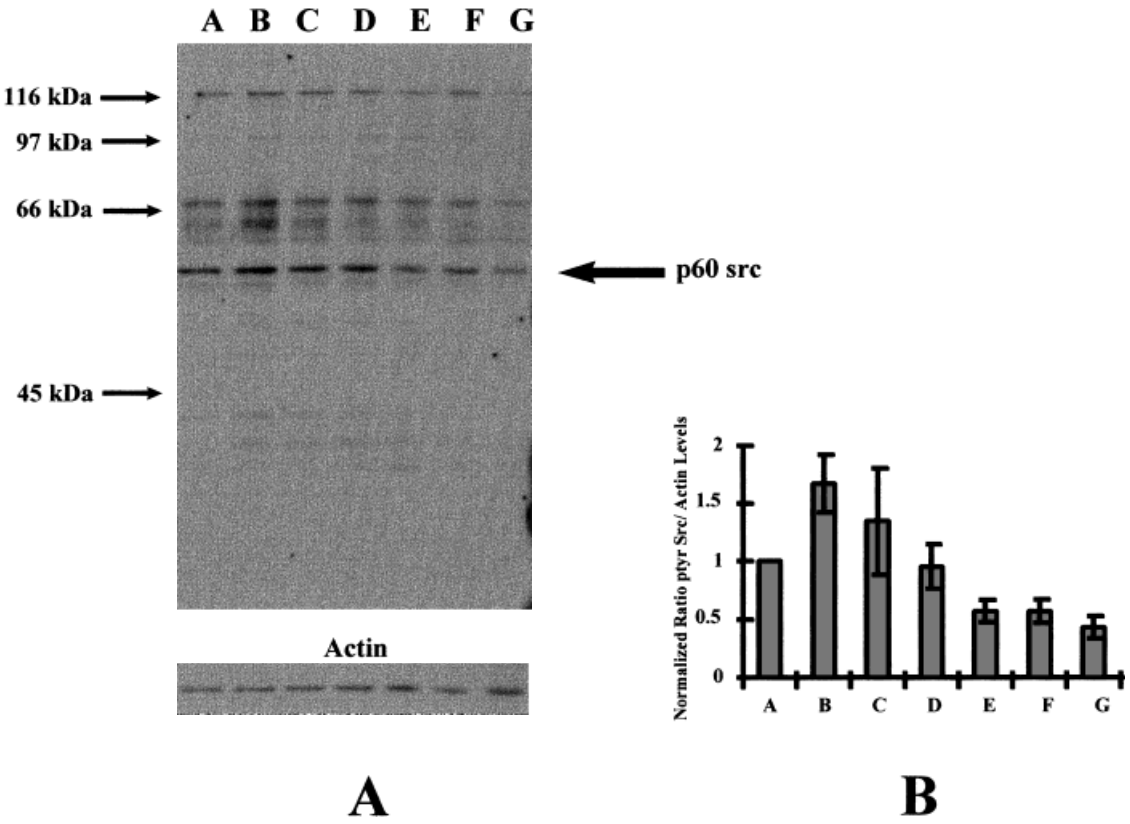

Fig. 4. **A:** Phosphotyrosine immunoblot analysis of osteoclast whole cell lysates. **Lane A**, 1 min vehicle treatment; **lanes B,C,D,E**, 1, 5, 10, and 30 min 10 nM 17β-estradiol treatments, respectively; **lane F**, 5 min 1 μM tamoxifen pretreatment, followed by 1 min 17β-estradiol; **lane G**, 5 min 1 μM herbimycin A, followed by 1 min 17β-estradiol. The band indicated as p60src is so labeled on the basis of the co-immunolabeling for Src kinase and phosphotryrosine shown in Fig. 6. Molecular-weight markers are indicated on the left. **B:** Averages of tyrosine phosphorylated Src kinase: actin ratios in immunoblots (three blots averaged). Band intensities were normalized to the 1 min vehicle values. **A–G** as described for **A**.

an untreated osteoclast (Fig. 9A) compared with one treated with cytochalasin B for 20 min (Fig. 9B). To determine whether disrupting the actin network would interfere with tyrosine phosphorylation, cells pretreated with cytochalasin B followed by 17β-estradiol were immunostained with the phosphotyrosine antibody. Although a response was observed, it was delayed (Fig. 10). Figure 11 quantifies the delayed phosphotyrosine response to 17β-estradiol of cytochalasin B-treated cells.

DISCUSSION

This investigation stemmed from earlier studies from this laboratory that suggested the existence of an estrogen plasma membrane receptor in osteoclasts, namely that acid production by osteoclasts was decreased in the presence of cycloheximide within minutes of 17β-estradiol treatment [Gay et al., 1993]. In addition, an estrogen complex, 17β-estradiol-carboxymethyloxime-bovine serum albumin (BSA)-fluorescein isothiocyanate was found to bind to osteoclasts and to stimulate shape changes [Brubaker and Gay, 1994].

In the present study, estrogen stimulated immediate and transient tyrosine phosphorylation of several proteins in osteoclasts, along sites of attachment as detected by immunofluorescent staining with an anti-phosphotyrosine antibody, an effect that occurs much earlier than known transcriptional activation by steroids. The rapid stimulation by 17β-estradiol suggests an interaction of the hormone receptor complex and a tyrosine kinase, as has been reported in other types of cells. For example, Migliaccio and colleagues [1993] demonstrated an immediate increase in phosphotyrosine levels in MCF-7 breast cancer cells after 10 s exposure to 17β-estradiol and determined that one of the substrates was Src kinase. These investigators also determined that 17β-estra-

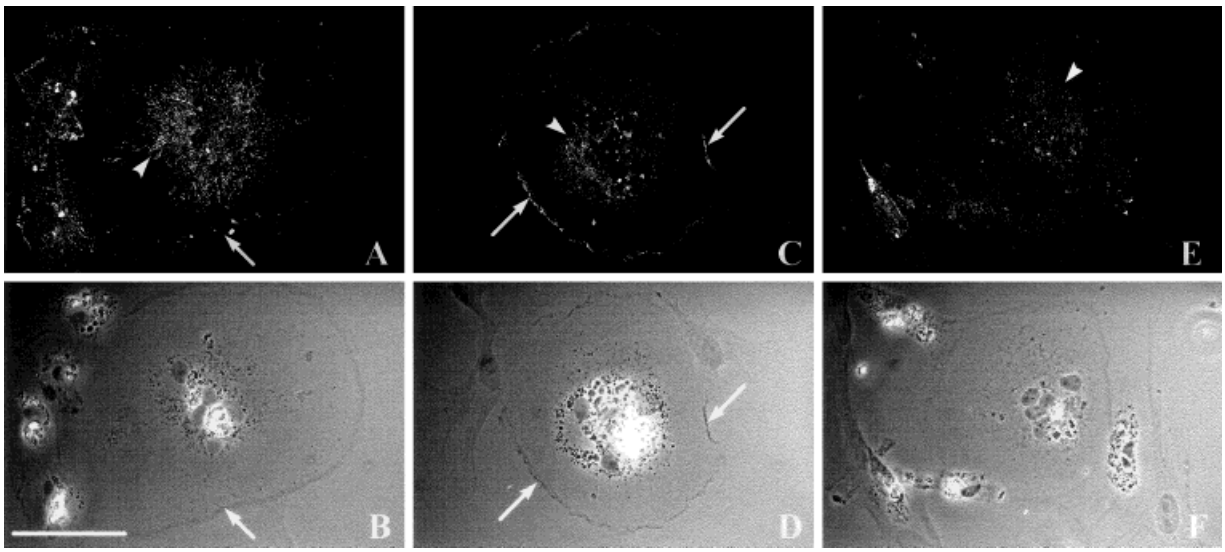

Fig. 5. Src kinase localization in 17β-estradiol-treated osteoclasts. Fluorescent **(A,C,E)** and corresponding phase-contrast **(B,D,F)** images of osteoclasts treated with 10 nM 17β-estradiol for 0 **(A,B)**, 1 **(C,D)**, and 10 **(E,F)** min. Src kinase appeared to translocate to the plasma membrane at 1 min treatment with 17β-estradiol, arrows **(C,D)**. By 10 min, peripheral staining returned to basal levels **(E,F)**. Staining for Src kinase was also observed in the center of the cell associated with vesicles (arrowhead). Scale bar = 50 μm.

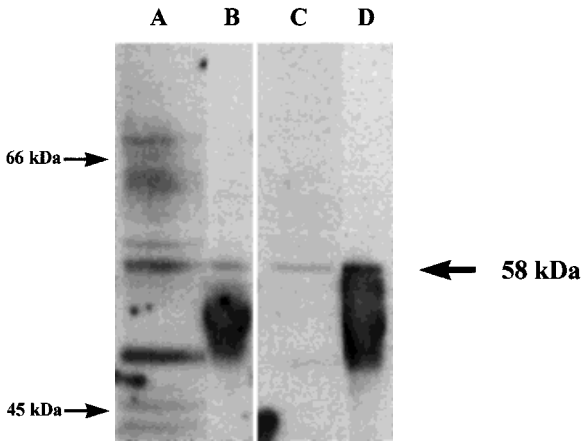

Fig. 6. Immunoprecipitation of Src kinase from osteoclast whole cell lysates. Src kinase was immunoprecipitated from whole cell lysates, using a mouse anti-Src-rabbit anti-mouse-Protein A-Sepharose complex. Duplicate samples were run adjacent to samples of the whole cell lysate on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. **Lanes A,B**, probed with anti-phosphotyrosine antibody; **lanes C,D**, probed with an anti-chicken Src kinase antibody. **Lanes A,C**, whole cell lysates; **lanes B,D**, Src kinase immunoprecipitated from whole cell lysates. A single band (58 kDa) reacted with both anti-chicken Src kinase and anti-phosphotyrosine antibodies. Molecular-weight markers are indicated (arrows). **Lanes B,D**, the smear beneath the Src kinase band is due to cross-reactivity of the secondary antibody with denatured fragments of the original antibodies used in the immunoprecipitation procedure.

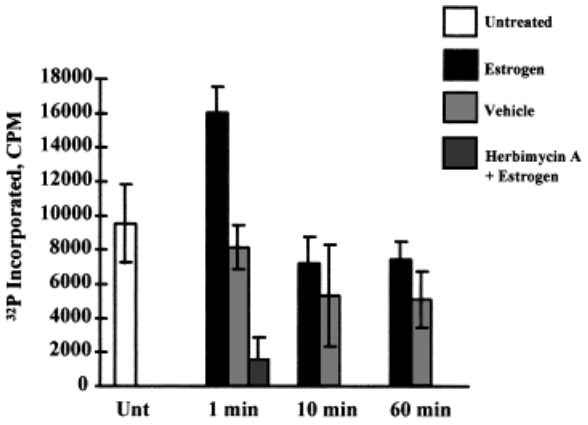

Fig. 7. Src kinase activity in osteoclasts treated with 17β-estradiol or vehicle. Osteoclasts were either untreated or treated with 10 nM 17β-estradiol or vehicle for 1, 10, or 60 min before lysis and immunoprecipitation of Src kinase. Src kinase phosphotransferase activity was subsequently measured by the incorporation of ^{32}P into a peptide corresponding to amino acids 6–20 of p34cdc2. Each bar represents the average of triplicate samples ±SD for two experiments. The 1 min 17β-estradiol treatment was significantly different ($P < 0.001$) from all other treatments.

diol increased the phosphotransferase activity of Src kinase. We observed a similar increase in phosphotyrosine levels and activity of Src kinase in osteoclasts after 1 min exposure to 17β-estradiol. We believe that this increase in activity is solely a response of the osteoclasts in the preparations, because we did not observe

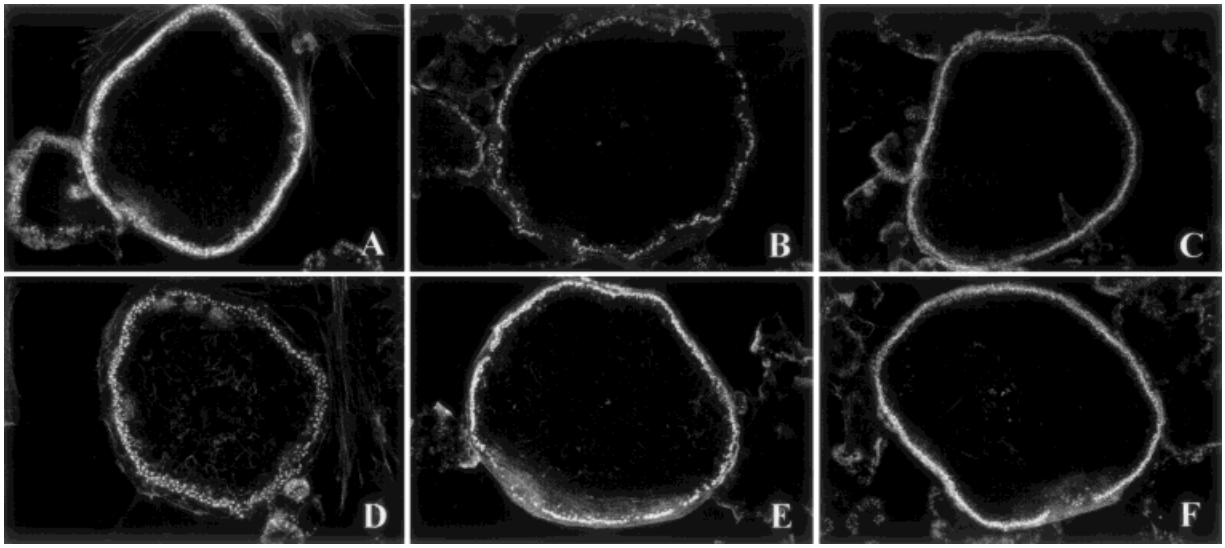

Fig. 8. Actin staining in 17β-estradiol-treated osteoclasts. Osteoclasts were either untreated or treated with 10 nM 17β-estradiol for 1, 5, 10, 30, and 60 min before fixation with 2% paraformaldehyde. Actin was visualized by staining with Texas red-phalloidin. **A:** Untreated cell; **B–F**, cells treated with 17β-estradiol for 1, 5, 10, 30, and 60 min, respectively.

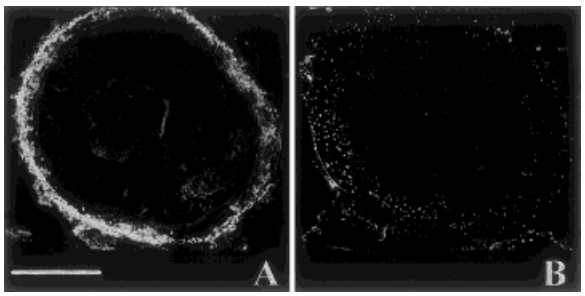

Fig. 9. Distribution of actin in osteoclasts. **A:** Untreated osteoclast stained with Texas Red-phalloidin. **B:** Osteoclast treated with 1 μg/ml cytochalasin B, an inhibitor of actin polymerization, for 20 min before fixation and staining. Scale bar = 50 μm.

an increase in phosphotyrosine levels in fibroblast-like cells, assumed to be stromal cells, through immunofluorescent staining with the anti-phosphotyrosine antibody. Src kinase appeared to translocate to the periphery of the osteoclasts at 1 min of exposure to hormone and it subsequently disappeared from the membrane at longer exposure times. We also observed a discrete ring of phosphotyrosine staining at the outermost edge of the cells that correlated with respect to time and location to the translocation of Src kinase to the plasma membrane. Inhibition of actin polymerization delayed the phosphotyrosine response to 17β-estradiol from 1 to 10 min. Fincham et al. [1996] demonstrated that Src kinase translocation to the periphery of chicken embryo and NIH 3T3 fibroblasts is mediated by actin and is under the control of the Rho family of small G proteins. Therefore, the delayed phosphotyrosine signal we detected could be attributable to interference with Src kinase translocation to the cell periphery.

Src kinase has many substrates, including actin and integrin binding proteins, such as tensin, vinculin, cortactin, talin, paxillin, and $p130^{cas}$; p190, the GTPase activator of Rho; Shc, an adaptor protein; RasGAP, the GTPase activator for Ras; β1 integrin; p85, the noncatalytic subunit of PI3 kinase; and focal adhesion kinase [Brown and Cooper, 1996]. Phosphorylation of these proteins by Src kinase is associated with attachment and activation through integrin binding [Brown and Cooper, 1996]. Activation of Src kinase is known to elicit responses through many pathways, including the MAP kinase pathway.

Migliaccio and colleagues [1996] demonstrated that estrogen stimulated the MAP kinases, erk-1 and erk-2, and increased the active form of $p21^{ras}$ in MCF-7 cells. Estrogen treatment also induced tyrosine phosphorylation of Shc and p190 and association of p190 to $p21^{ras}$ and the p120 GTPase activating protein ($p21^{ras}$-GAP). Estrogen appeared to activate MAP-kinases in MCF-7 cells through the following scheme. Shc, an adaptor protein, is phosphorylated by Src kinase and associates with the Grb2/mSos complex which plays a role in the

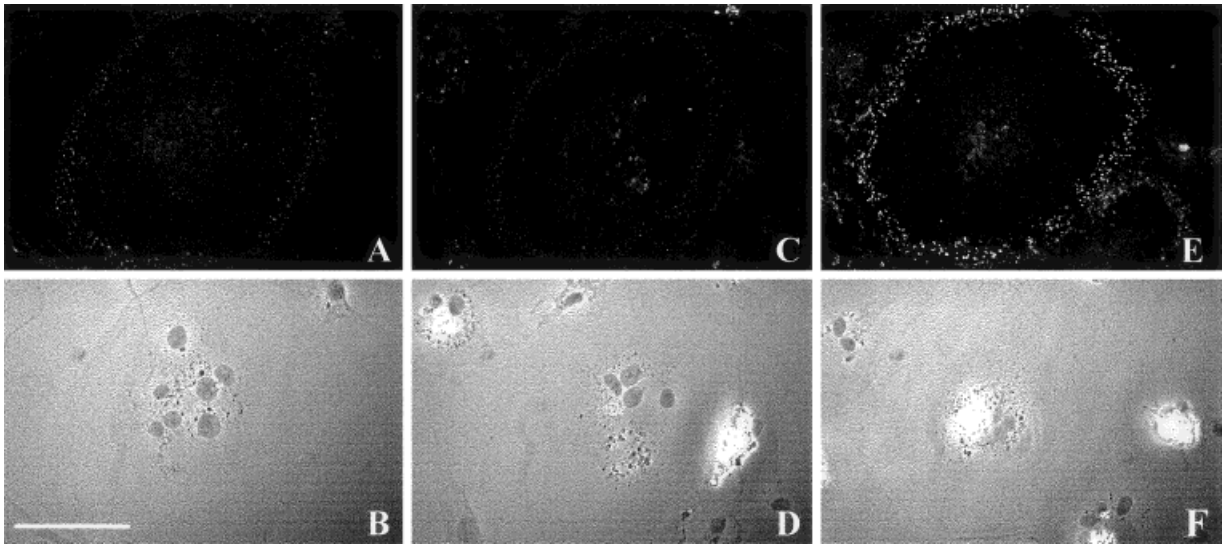

Fig. 10. Phosphotyrosine immunostaining in osteoclasts in which actin filaments had been disrupted. Osteoclasts were pretreated with 1 μg/ml cytochalasin B for 20 min before exposure to 10 nM 17β-estradiol or vehicle for 0, 1, and 10 min. Cells were then fixed with 2% paraformaldehyde and immunostained for phosphotyrosine using a triple antibody labeling procedure. **A,B:** Fluorescent and phase-contrast images of a cell treated with cytochalasin B only. **C,D:** Cytochalasin B treatment, followed by 1 min 17β-estradiol treatment. **E,F:** Cytochalasin B treatment, followed by 10 min 17β-estradiol treatment. No changes were apparent in vehicle controls (not shown). Compare with Fig. 1C for a typical response to 17β-estradiol without cytochalasin B pretreatment. Scale bar = 50 μm.

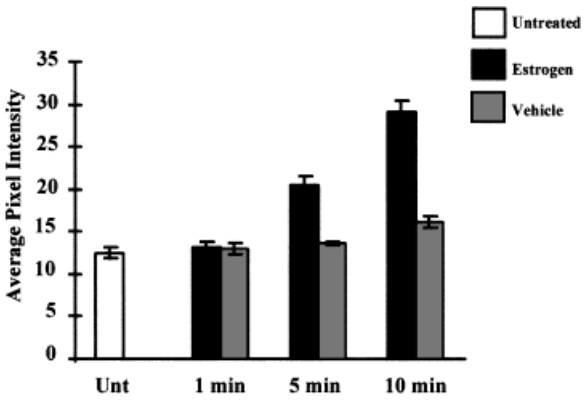

Fig. 11. Average intensity of phosphotyrosine immunostaining in osteoclasts treated with cytochalasin B before estrogen treatment. Osteoclasts were pretreated with 1 μg/ml cytochalasin B for 20 min before exposure to 10 nM 17β-estradiol or vehicle for 0, 1, 5, or 10 min. Cells were then fixed with 2% paraformaldehyde and immunostained for phosphotyrosine. Each bar represents the average pixel intensity of confocal microscope images (±SEM) for a minimum of 45 cells from each of three experiments. The 5 and 10 min 17β-estradiol values were significantly different from the other treatments by $P < 0.001$ and 0.0001, respectively.

activation of $p21^{ras}$ by aiding in the release of GDP from inactive $p21^{ras}$ and switching it to its active GTP-bound state. Phosphorylated p190 can then interact with $p21^{ras}$-GAP, reducing its GTPase activity, thus increasing GTP bound $p21^{ras}$. Activated $p21^{ras}$ then stimulates the MAP kinase pathway.

Several studies indicate that estrogen activates the MAP-kinase pathway in osteoclasts through Src kinase. Pascoe and colleagues [1997] recently reported that Src kinase association with Shc and $p21^{ras}$ decreased after a 30-min incubation with 17β-estradiol in osteoclasts, whereas its association with Csk, a negative regulator of Src kinase activity, increased. They found that a constitutively active Src kinase expression resulted in decreased lysosomal enzyme secretion, whereas expression of dominant negative Src, Ras, or Raf increased secretion of lysosomal enzymes. Estrogen was also shown to stimulate MAP kinase activation in ROS 17/2.8 osteoblastic cells [Endoh et al., 1997]. Endoh and colleagues demonstrated an increase in the phosphotyrosine levels and activity of MAP kinase within 5 min of 10^{-11} M 17β-estradiol treatment.

MAP kinase has been shown to be essential for growth factor signaling pathways [Huang and Erikson, 1996]. MAP kinase activation by estrogen implies that there is crosstalk between these types of cellular regulation. Several genes that lack estrogen response elements (ERE) on their promoters have been reported to be induced by estrogen treatment

[Umayahara et al., 1994; Sukovich et al., 1994]. MAP kinase activation could play a role in the activation of genes important for regulating osteoclast functions through an estrogen receptor independent manner. Our observation that 17β-estradiol stimulates rapid phosphotyrosine and Src kinase activation implies that estrogen can initiate signaling pathways normally associated with growth factors or peptide hormones, a process which requires functional plasma membrane receptors.

Because the rapid effects of estrogen in bone cells have only recently received attention, the pathways involved in this kind of regulation of bone cells, in particular osteoclasts, are still being elucidated. The primary function of osteoclasts is to resorb bone by secreting acid and enzymes that cause the breakdown of bone, but an osteoclast can only function if it is polarized and tightly attached to bone. It can therefore be postulated that rapid regulation of bone resorption can occur through events such as alterations in attachment or cytoskeletal rearrangements [Lakkakorpi and Väänänen, 1996], ion channel activities [Brubaker and Gay, 1995], enzyme activities, such as Src kinase [Soriano et al., 1991], as well as induction of apoptosis [Hughes et al., 1996]. Our study provides evidence for a role of 17β-estradiol in the induction of pathways normally associated with growth factors, namely Src kinase activation. Signaling downstream of Src kinase, such as MAP kinase activation, could be involved in the regulation of enzymes needed for resorption, such as lysosomal proteinases [Oursler et al., 1993] or other factors critical for bone resorption.

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