

Sex Steroids and Bone

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ABSTRACT

The adult skeleton is periodically remodeled by temporary anatomic structures that comprise juxtaposed osteoclast and osteoblast teams and replace old bone with new. Estrogens and androgens slow the rate of bone remodeling and protect against bone loss. Conversely, loss of estrogen leads to increased rate of remodeling and tilts the balance between bone resorption and formation in favor of the former. Studies from our group during the last 10 years have elucidated that estrogens and androgens decrease the number of remodeling cycles by attenuating the birth rate of osteoclasts and osteoblasts from their respective progenitors. These effects result, in part, from the transcriptional regulation of genes responsible for osteoclastogenesis and mesenchymal cell replication and/or differentiation and are exerted through interactions of the ligand-activated receptors with other transcription factors. However, increased remodeling alone cannot explain why loss of sex steroids tilts the balance of resorption and formation in favor of the former. Estrogens and androgens also exert effects on the lifespan of mature bone cells: pro-apoptotic effects on osteoclasts but anti-apoptotic effects on osteoblasts and osteocytes. These latter effects stem from a heretofore unexpected function of the classical “nuclear” sex steroid receptors outside the nucleus and result from activation of a Src/Shc/extracellular signal-regulated kinase signal transduction pathway probably within preassembled scaffolds called caveolae. Strikingly, estrogen receptor (ER) alpha or beta or the androgen receptor can transmit anti-apoptotic signals with similar efficiency, irrespective of whether the ligand is an estrogen or an androgen. More importantly, these nongenotropic, sex-nonspecific actions are mediated by the ligand-binding domain of the receptor and can be functionally dissociated from transcriptional activity with synthetic ligands. Taken together, these lines of evidence strongly suggest that, in sex steroid deficiency, loss of transcriptional effects may be responsible for the increased osteoclastogenesis and osteoblastogenesis and thereby the increased rate of bone remodeling. Loss of nongenotropic anti-apoptotic effects on mature osteoblasts and osteocytes, in combination with an opposite effect on the lifespan of mature osteoclasts, may be responsible for the imbalance between formation and resorption and the progressive loss of bone mass and strength. Elucidation of the dual function of sex steroid receptors has important pathophysiologic and pharmacologic implications. Specifically, synthetic ligands of the ER that can evoke the nongenotropic but not the genotropic signal may be bone anabolic agents, as opposed to natural estrogens or selective estrogen receptor modulators that are antiresorptive agents. The same ligands may also circumvent the side effects associated with conventional hormone replacement therapy.

I. Introduction

Loss of ovarian function at menopause represents the most important factor for the development of osteoporosis, the metabolic bone disease that affects millions worldwide and contributes considerably to the morbidity and mortality among elderly women. Loss of androgens in males from chemical or surgical castration or an age-associated decline of androgen levels – albeit not as universal or abrupt as menopause – has the same adverse effect on the skeleton. Appreciation of the adverse effects of estrogen and androgen deficiency on bone in the 1940s by Fuller Albright (Albright and Reisfenstein, 1948) launched a quest for the elucidation of the mechanism of the skeletal actions of sex steroids and the pathogenesis of the bone loss resulting from sex steroid deficiency.

The purpose of this review is to provide a personal perspective of our group's contribution to the advancement of understanding of the cellular and molecular mechanisms by which estrogens and androgens influence adult bone homeostasis and, by extension, the pathogenetic mechanisms responsible for the development of osteoporosis following sex steroid deficiency. This chapter also will point out new pharmacotherapeutic opportunities brought about with the elucidation of the molecular mechanisms of the anti-osteoporotic effects of sex steroids.

A. THE WORKING HYPOTHESIS

The adult skeleton regenerates continuously in the form of a periodic replacement of old bone with new. This process, called remodeling, is accomplished by temporary anatomic structures comprising osteoclasts in the front, which excavate old bone, and osteoblasts in the rear, which fill the fresh cavities with new bone. These so-called basic multicellular units (BMUs) arise from progenitor cells residing in the bone marrow. After the BMUs complete their task, usually in a period of 6 to 9 months in humans, they disappear without trace due to the apoptotic death of the executive cells of the unit. The rate of remodeling depends on the number of cycles but the effects on bone mass depend on the focal balance within each cycle. Sex steroid deficiency, as well as old age and glucocorticoid excess, do not cause loss of bone mass by turning on a completely new process. Instead, they cause a derangement in the normal process of bone regeneration.

Until the 1990s, research on the pathophysiology of osteoporosis had focused on the functions of differentiated cells: the rates and/or total amounts of bone resorbed or formed by individual osteoclasts or osteoblasts. But these cells have relatively short active lifespans that are within the range of lifespans of the other cells that originate in the bone marrow. Such cells must be continually replaced; the number present depends both on the birth rate, which reflects the frequency of cell division of the appropriate precursor cell, and on the lifespan,

which most likely reflects the timing of death by apoptosis. The importance of the distinction between cell number and individual cell function often has been obscured by using the vague term “activity” (Manolagas, 1999). The essential hypothesis tested by our work is that the balance between bone resorption and bone formation depends more on the number of cells carrying out these processes than on their individual capacities. Since these cells originate from precursor cells within the bone marrow, our research has placed particular attention to relationships between the different components of this complex tissue and the birth of osteoblasts and osteoclasts. Recognition of the fact that osteoblasts and osteoclasts disappear without a trace guided us more recently to the study of cell death that, for both cell types, is as important as cell birth in determining overall cell numbers.

B. DISTINCTION BETWEEN GROWING AND NONGROWING SKELETON

In addition to their effects on the maintenance of the homeostasis of the adult skeleton during its continuous regeneration by the process of bone remodeling, estrogens and androgens play a very profound role in skeletal growth and perhaps the sexual dimorphism of the skeleton. Bone mass later in life is a function of the bone accrued during the second decade and the amount of bone lost after menopause. Bone mass accrual during puberty depends more on sexual maturation than chronological age. Hence, the effects of sex steroids on the growing skeleton are relevant to the development of osteoporosis later in life. However, the stimulating effects of estrogens on bone growth during puberty are different from their effects on the maintenance of the mature skeleton and prevention of bone loss. To place the significance of the evidence we will cover later in the appropriate perspective, we will first discuss important distinctions between growing and nongrowing skeleton vis-a-vis estrogen action.

Gender is a major determinant of the size and shape of the skeleton, reflecting the need of the female pelvis to accommodate gestation and delivery of the offspring. Sexual skeletal dimorphism is most likely determined by genetic programs, which have yet to be elucidated. Nonetheless, estrogens or androgens may contribute to such dimorphism by actions exerted during fetal development (Couse and Korach, 1999). Evidence that short-term exposure to exogenous estrogen during fetal life affects bone growth and development in postnatal life suggests the existence of an imprinting mechanism that acts on bone cell programming early in skeletal development (Migliaccio *et al.*, 1996). Sexual skeletal dimorphism is also influenced by the well-known effects of sex steroids on the initiation of the pubertal growth spurt and the closure of the epiphyses at the end of puberty (Frank, 2000; Parfitt, in press). The cellular and molecular mechanisms responsible for these latter effects are also unknown. Linear skeletal

growth is governed by the chondrocytes of the growth plate. Therefore, the effects of sex steroids on pubertal growth and epiphyseal closure result from direct actions of these hormones on chondrocytes. Consistent with this contention, chondrocytes are direct targets for sex steroid action, as they express classical receptors for estrogens and androgens. Thus, the target cells of the effects of sex steroids on bone growth are different from the target cells of their effects on the maintenance of the mature skeleton.

True bone mineral density (i.e., the amount of mineral per volume of bone (gr/cm^3)) does not appear to increase with size or age. The apparent changes in areal bone mineral density, the popular measurement performed with dual-energy x-ray absorptiometry (DEXA) – the so-called bone mineral density (BMD) that is expressed in gr/cm^2 – is a reflection of growth and an increase in size rather than an increase in bone mineral per unit volume. Hence, the purported changes of BMD during growth are predominantly due to changes on bone volume, not to changes in true bone density. As we reviewed recently elsewhere (Manolagas *et al.*, in press), most of the 40- to 50-fold increase in bone mass from birth to maturity is the result of growth. During growth, bone mass and density may change in opposite directions. In contrast, in the mature skeleton, the change in bone size with time is trivial and mass and density always change in the same direction. Furthermore, the growing and nongrowing skeleton differ profoundly in the spatial and temporal organization of bone cell function. These differences were encapsulated several years ago by Frost in his definitions of modeling as a more-or-less continuous process of bone redistribution and remodeling as a relatively infrequent process of bone replacement (Frost, 1973; Parfitt, 1997). Very importantly, the changes in cortical bone surface location due to modeling can, in some bones, amount to more than 1 cm from birth to maturity or about $2 \mu\text{m}/\text{day}$. By contrast, changes in cancellous bone surface location due to remodeling are expansion of about $2 \mu\text{m}/\text{year}$ during growth (Parfitt *et al.*, 2000) and contraction of about $1 \mu\text{m}/\text{year}$ after age 50 (Parfitt, 2000). During cortical bone growth, large numbers of new cells are needed over an extended area every day for many years. In this situation, cell recruitment is not rate limiting and the lifespan of an individual cell is relatively unimportant. But, during adult cancellous bone remodeling, a $5\text{-}\mu\text{m}$ increase in the depth of a resorption cavity (about 10%), due to corresponding prolongation of osteoclast lifespan, or a $5\text{-}\mu\text{m}$ decrease in the thickness of new bone deposited, due to corresponding shortening of osteoblast lifespan, can have a large effect on the rate of bone loss, which in a few years may determine whether or not a vertebral fracture occurs.

Unfortunately, lack of appreciation of these facts – and, even more seriously, the incorrect use of BMD measurements by DEXA in growing children – has led to the mistaken conclusion that genetic disorders of the estrogen receptor (ER) or the aromatase gene (Smith *et al.*, 1994; Carani *et al.*, 1997; Bilezikian *et al.*, 1998) cause osteoporosis. Lack of estrogen action in the growing skeleton

prolongs the duration of growth, leading to increases in height and in long bone length (Simpson, 1998; Grumbach and Auchus, 1999). Hence, in contrast to osteoporosis, the longer (eunuchoid) bones of these individuals suggest that, if anything, they accrued more bone than normal. Furthermore, the increased rate of remodeling in these subjects is due to the fact that they did not reach skeletal maturity. Indeed, increased rate of remodeling is physiologic and appropriate for the rapid growth and sculpting of bones during puberty. On the other hand, increased remodeling of the mature skeleton caused by loss of sex steroids – or, for this matter, other conditions – is always pathologic and inappropriate.

II. Effects of Sex Steroids on Osteoclastogenesis: The IL-6 Paradigm Shift

Classical receptors for estrogens (ER α and ER β) or androgens (AR) are present in chondrocytes, bone marrow stromal cells, osteoblasts, and osteoclasts and their progenitors (Eriksen *et al.*, 1988; Komm *et al.*, 1988; Benz *et al.*, 1991a; Bellido *et al.*, 1995; Couse and Korach, 1999), indicating that the effects of sex steroids on bone are mediated, at least in part, directly. The level of receptor expression in osteoblastic and osteoclastic cells is low (at least 10-fold), compared to reproductive sites of sex steroid action. The distribution of these receptors in bone cells does not vary by gender, as similar levels of ER and AR have been found in bone cells from males and females (Benz *et al.*, 1991a,b; Braidman *et al.*, 2000). However, in spite of the demonstration of receptors, convincing evidence that estrogens or androgens modulate the biosynthetic activity of osteoblasts and osteoclasts in a manner that can explain the well-documented skeletal effects of estrogen loss *in vivo* remained elusive until the critical role of the bone marrow in bone remodeling was appreciated about a decade ago (Scheven *et al.*, 1986; Kurihara *et al.*, 1989).

Osteoclasts are derived from hematopoietic progenitors of the myeloid lineage, colony-forming unit-granulocyte/macrophage (CFU-GM) and CFU-M. Osteoblasts, as well as the hematopoiesis-supporting stromal cells and adipocytes of the bone marrow, are derived from mesenchymal stem cells. More important, the development of osteoclasts depends on a network of autocrine and paracrine factors produced by stromal and osteoblastic cells. With this knowledge in hand, we proposed the hypothesis that estrogens act to regulate the production of osteoclastogenic cytokines by bone marrow stromal cells and osteoblasts (Manolagas and Jilka, 1992).

Interleukin-6 (IL-6) is a paradigm of a cytokine important for osteoclastogenesis (Manolagas *et al.*, 1996). IL-6 is a member of a family of structurally related cytokines that use the gp130 signal transducer in their receptor complex. Besides IL-6, the family includes IL-11, oncostatin M (OSM), leukemia inhibitory factor (LIF), and cardiotropin 1. Binding of IL-6 to its specific cell surface

receptor (gp80) causes recruitment and dimerization of gp130, which is then tyrosine phosphorylated by members of the JAK family of tyrosine kinases. This event results in tyrosine phosphorylation of several downstream signaling molecules, including members of the signal transducers and activators of transcription (STAT) family of transcription factors. Phosphorylated STATs, in turn, undergo homo- and hetero-dimerization and translocate to the nucleus, where they activate cytokine-responsive gene transcription. The gp80 subunit of the IL-6 receptor also exists in a soluble form (sIL-6R) but, unlike most soluble cytokine receptors, it functions as an agonist by binding to IL-6 and then interacting with membrane-associated gp130 to stimulate JAK/STAT signaling.

We found that both estrogens and androgens suppress the production of IL-6 as well as the expression of the two subunits of the IL-6 receptor, IL-6R α and gp130, in cells of the bone marrow stromal/osteoblastic lineage (Girasole *et al.*, 1992; Passeri *et al.*, 1993; Manolagas *et al.*, 1994; Bellido *et al.*, 1995; Jilka *et al.*, 1995; Manolagas and Jilka, 1995; Lin *et al.*, 1997; Manolagas, 1998). Similar results were obtained subsequently by others in rats as well as in humans, in the bone marrow and in the peripheral blood (Manolagas, 2000a; Scheidt-Nave *et al.*, 2001). Importantly, we and others showed that neutralization of IL-6 with antibodies or knockout of the IL-6 gene in mice prevents the upregulation of CFU-GM in the marrow, and the expected increase of osteoclast numbers in trabecular bone sections; and also protects the loss of bone following loss of sex steroids (Jilka *et al.*, 1992; Poli *et al.*, 1994; Bellido *et al.*, 1995). In support of the pathogenetic role of IL-6 in the bone loss caused by loss of sex steroids, IL-6 seems to play a similar role in several other conditions associated with increased bone resorption, as evidenced by increased local or systemic production of IL-6 and the IL-6 receptor in patients with multiple myeloma, Paget's disease, rheumatoid arthritis, Gorham-Stout or disappearing bone disease, hyperthyroidism, primary and secondary hyperparathyroidism, as well as McCune Albright syndrome (Papanicolaou *et al.*, 1998; Manolagas, 2000a). Nonetheless, IL-6 does not seem to be required for osteoclastogenesis *in vivo* under normal physiologic conditions. In fact, osteoclast formation is unaffected in sex steroid-replete mice treated with a neutralizing anti-IL-6 antibody or in IL-6-deficient mice (Jilka *et al.*, 1992; Poli *et al.*, 1994). This situation probably reflects the fact that the expression of the gp80 subunit of the IL-6 receptor in bone is a limiting factor for the effects of the cytokine.

At the molecular level, we determined that the suppressive effects of estrogens or androgens on IL-6 production were mediated via the classical receptors and resulted from an indirect effect of the receptor protein on the transcriptional activity on the proximal 225-bp sequence of the human IL-6 gene promoter (Pottratz *et al.*, 1994; Bellido *et al.*, 1995). Moreover, whereas both androgens and estrogens could inhibit IL-6 transcription, their effects were strictly dependent on the expression of their respective receptor and could not be

due to actions of androgens mediated by the ER, or vice versa (Figure 1). In support of these findings, others established that suppression of IL-6 production by estrogen, or selective estrogen receptor modulators (SERMs) such as raloxifene, is due to protein-protein interaction between the ER and transcription factors such as nuclear factor kappa beta ($\text{NF-}\kappa\beta$) and CCAAT/enhancer binding protein (C/EBP) (McDonnell and Norris, 1997).

We also found that receptors for IL-6-type cytokines are expressed by stromal/osteoblastic cells and that binding of the ligands to these receptors induces progression toward a more-mature osteoblast phenotype (Bellido *et al.*, 1996,1997; Taguchi *et al.*, 1998) and upregulation of the gp130 promoter (O'Brien and Manolagas, 1997). As in other cell types, the actions of IL-6-type cytokines on osteoblastic cells involve activation of both the JAK/STAT and the mitogen-activated protein kinase (MAP-K) pathway (Bellido *et al.*, 1997) and probably are mediated by the cyclin-dependent kinase (CDK) inhibitor p21^{WAF1,CIP1,SD11} – a downstream effector of gp130/Stat3 activation (Bellido *et al.*, 1998).

The paradigm of IL-6 as a target gene for sex steroid action in bone – and, in particular, the suppressive effects of these hormones on osteoclastogenesis –

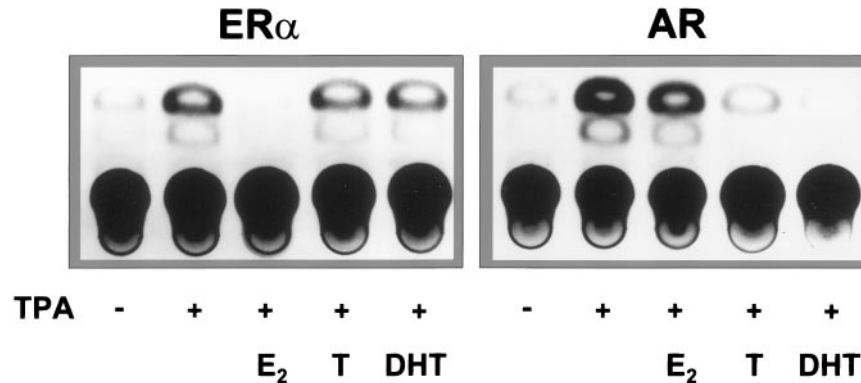


FIG. 1. Sex-specific inhibition of interleukin-6 (IL-6) transcription by estrogens or androgens. HeLa cells transiently transfected with either estrogen receptor-alpha (ER α) or androgen receptor (AR) and a proximal 225-bp sequence of the human IL-6 gene promoter driving the expression of chloramphenicol acetyl transferase (CAT). Cells were maintained for 24 hours in medium without or with tetradecanoyl phorbol acetate (TPA) in the presence or absence of estrogen (E2), testosterone (T), or dihydrotestosterone (DHT). Cells were extracted and the CAT products visualized after thin-layer chromatography. [Adapted with permission from Pottratz ST, Bellido T, Mocharla H, Crabb D, Manolagas SC 1994 17 β -estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. *J Clin Invest* 93:944–950; and Bellido T, Jilka RL, Boyce BF, Girasole G, Broxmeyer H, Dalrymple SA, Murray R, Manolagas SC 1995 Regulation of interleukin-6, osteoclastogenesis and bone mass by androgens: the role of the androgen receptor. *J Clin Invest* 95:2886–2895.]

has been since extended to include tumor necrosis factor (TNF) and macrophage-colony-stimulating factor (M-CSF) (Srivastava *et al.*, 1998,1999). In addition, there has been evidence that estrogens stimulate the production of osteoprotegerin (OPG), a potent anti-osteoclastogenic factor. OPG acts as a decoy, blocking the binding of the receptor activator of NF- κ B (RANK), expressed in osteoclast progenitors, to RANK-ligand (RANKL) which is expressed in committed pre-osteoblastic cells (Hofbauer *et al.*, 1999). The high-affinity binding of RANKL to RANK is evidently essential and, together with M-CSF, sufficient for osteoclastogenesis. IL-6, IL-11, IL-1, and TNF as well as parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D3 seem to exert at least part of their osteoclastogenic effects by stimulating the expression of RANKL. PTH and 1,25-dihydroxyvitamin D3 also inhibit the production of OPG (Hofbauer *et al.*, 2000). IL-6 and IL-11 may influence osteoclastogenesis by stimulating the self-renewal and inhibiting the apoptosis of osteoclast progenitors (Girasole *et al.*, 1994; Jilka, 1998). Estrogen loss also may increase the sensitivity of osteoclasts to IL-1 by increasing the ratio of the IL-1RI over the IL-1 decoy receptor (IL-RII) (Sunyer *et al.*, 1999) or increasing the number of TNF-synthesizing lymphocytes (Cenci *et al.*, 2000). As in the case of IL-6, the effects of estrogen on TNF and M-CSF are mediated via protein-protein interactions between the ER and other transcription factors (Table I). Because of the interdependent nature of the production of IL-1, IL-6, and TNF, a significant increase in one of them may amplify, in a cascade fashion, the effect of the others (Jilka, 1998).

TABLE I
Estrogens Regulate Cytokines via Estrogen Response Element (ERE)-independent Mechanisms

Cytokine	Mechanism of action
Interleukin (IL)-6	Interference with nuclear factor κ B binding to promoter
Tumor necrosis factor	Interference with jun kinase, jun, AP-1 actions on the promoter
IL-1RI/IL-1RII	?
Macrophage-colony-stimulating factor	Interference with cKII, egr-1, and Sp1 activity
Transforming growth factor β	?
Osteoprotegerin	?

III. Effects of Sex Steroids on Osteoblastogenesis

Loss of sex steroids increases not only the resorption but also the formation of bone. Based on evidence that increased bone resorption after loss of sex steroids increases the number of osteoclast progenitors in the murine bone marrow, we hypothesized that estrogen loss also stimulates osteoblastogenesis. In support of this hypothesis, we found that, indeed, the number of osteoblast progenitors, CFU-OB, was increased after ovariectomy, in parallel with an increase in the circulating levels of the bone formation marker osteocalcin (Jilka *et al.*, 1998a). The temporal pattern of these changes was very similar to the increase in osteoclastogenesis and the rate of bone loss. In a unique mouse model of osteopenia due to defective osteoblastogenesis, we also found defective osteoclastogenesis (Jilka *et al.*, 1996). The osteoclastogenic defect was secondary to impaired osteoblast formation, as evidenced by the fact that osteoclastogenesis in *ex vivo* cultures of the bone marrow of these mice could be restored by addition of osteoblastic cells from normal mice. Moreover, unlike control mice, ovariectomy or orchidectomy in these mice failed to increase osteoclastogenesis. These lines of evidence led us to the idea that stimulation of mesenchymal cell differentiation towards the osteoblastic lineage following sex steroid loss may be the first event that ensues following the hormonal change. Increased osteoclastogenesis and bone loss might be downstream consequences of this change. In support of this contention, we found that the rate of remodeling and the expected bone loss following gonadectomy were attenuated in the mice with the defective osteoblastogenesis (Weinstein *et al.*, 1997).

The defining property of stem cell progenitors in regenerating tissues is their ability to self-renew (Loeffler and Potten, 1997). Indeed, such progenitors may divide to produce two identical daughter cells (self-renewal with amplification), or one identical daughter cell and one cell with more differentiated characteristics (self-renewal without amplification), or two cells with a more differentiated phenotype. The decision to self-renew or to differentiate may be stochastic or may be governed by factors in the local microenvironment. Rapid increases in executive cell production can thus be achieved by stimulating the self-renewal of early transit amplifying progenitors. Based on this understanding and our earlier results showing that loss of estrogens upregulates the production of CFU-OB, we examined the mechanism by which estrogen may regulate CFU-OB production. We found that most CFU-OB are dividing early transit amplifying cells and that 17β -estradiol suppresses their self-renewal via the α form of the estrogen receptor (Di Gregorio *et al.*, 2001) (Figure 2). A similar inhibitory effect of estrogens has been demonstrated in self-renewing progenitors that give rise to cells of the inner root sheath and the mature hair fiber (Chanda *et al.*, 2000). Although not shown directly, it is very likely that a similar mechanism might be operating in the actions of estrogen on the closure of the growth plate at the end

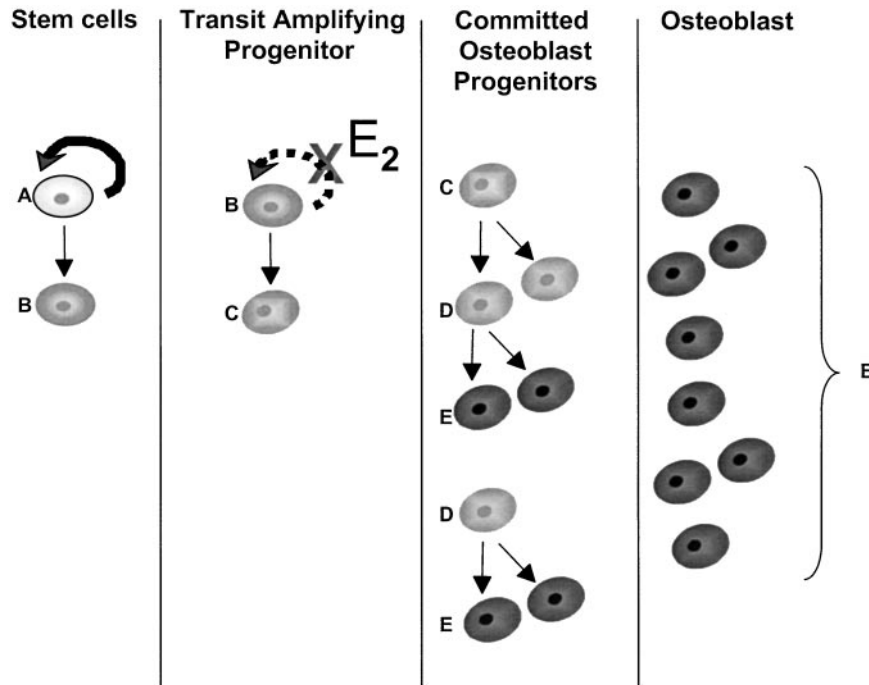


FIG. 2. Attenuation of early mesenchymal osteoblast progenitor (CFU-OB) self-renewal by estrogens via ER α . Mesenchymal stem cells (A) with high self-renewal capacity (solid arrow) give rise to early-transit amplifying osteoblast progenitors (B) with limited self-renewal capacity (dashed arrow). The latter differentiate into late-transit amplifying progenitors lacking self-renewal capability (C). Subsequently, late-transit amplifying cells develop into committed osteoblast progenitors (D) that eventually give rise to fully differentiated osteoblasts (E). Estrogens (E₂) suppress the self-renewal of the early-transit amplifying progenitors. [Adapted with permission from Di Gregorio GB, Yamamoto M, Ali AA, Abe E, Roberson P, Manolagas SC, Jilka RL 2001 Attenuation of the self-renewal of transit-amplifying osteoblast progenitors in the murine bone marrow by 17 β -estradiol. *J Clin Invest* 107:803–812.]

of puberty, as estrogens have been shown to inhibit prehypertrophic chondroblast proliferation (Tajima *et al.*, 1998). Unpublished studies of ours show that the inhibitory effect of estrogen on CFU-OB self-renewal may be critical for their bone-protective effects. Thus, the mouse model of osteopenia displays blunted osteoblastogenic and osteoclastogenic response, blunted bone loss following loss of estrogens, as well as low CFU-OB self-renewal, compared to control mice (DiGregorio-Taguchi *et al.*, 2001).

Attenuation of CFU-OB self-renewal and osteoclastogenic cytokine production by estrogens, together with the evidence for the molecular dependency of osteoclastogenesis on mesenchymal cell differentiation (through RANK/RANKL

interaction), provides a strong mechanistic basis for explaining the antiremodeling properties of these hormones. Moreover, in view of the fact that both osteoblasts and the stromal/osteoblastic cells that are required for osteoclast development are derived from CFU-OB, suppression of the self-renewal of this common progenitor may, in fact, represent the key mechanism of the antiremodeling effects of estrogens.

IV. Effects of Sex Steroids on Osteoblast and Osteocyte Apoptosis

Increased remodeling, resulting from upregulation of osteoblastogenesis and osteoclastogenesis, alone can cause a transient loss of bone because, during remodeling-based regeneration, resorption always precedes (and is faster) than bone formation, creating a temporary deficit, otherwise referred to as expanded remodeling space. However, whereas turnover depends on the number of cycles of remodeling, in the long-term, bone mass depends on the focal balance between formation and resorption within each cycle.

Following loss of sex steroids, osteoclasts erode deeper than normal cavities, leading to the removal of entire cancellous elements and loss of connection between the remaining ones (Parfitt *et al.*, 1996; Eriksen *et al.*, 1999). This phenomenon is most likely due to the removal of pro-apoptotic effects of estrogens on osteoclasts (Hughes *et al.*, 1996). Based on our studies indicating that the majority of osteoblasts die by apoptosis (Jilka *et al.*, 1998b), we have turned our attention to the effects of estrogens as well as androgens on osteoblast and osteocyte apoptosis.

We have found that ovariectomy or orchidectomy causes a dramatic increase in the prevalence of osteoblast and osteocyte apoptosis in mice. Addition of estrogens or androgens suppresses osteoblast and osteocyte apoptosis induced by a variety of pro-apoptotic stimuli *in vitro* (Kousteni *et al.*, 2001) (Figure 3). We undertook an extensive effort to investigate the molecular mechanism of these anti-apoptotic effects, using primary cultures of murine osteoblastic cells as well as an osteocytic cell line, mouse embryonic fibroblasts, and HeLa cells. We determined that both estrogens and androgens increase the phosphorylation of extracellular signal-regulated kinases (ERKs) in osteoblasts and osteocytes, with a peak at 5 minutes and return to baseline by 15 minutes (Kousteni *et al.*, 2001). ERKs are one of the three subfamilies of MAPK. The other two subfamilies are the jun N-terminal kinases (JNKs), also called stress-activated protein kinases (SAPK), and the p38 kinases. MAPKs are serine/threonine kinases that transduce chemical and physical signals from the cell surface to the nucleus, thereby controlling proliferation, differentiation, and survival (Chang and Karin, 2001). The transduction of the signal from the cell surface to the MAPKs begins with phosphorylation and recruitment of accessory proteins such as Ras, son of sevenless (SOS), Src, or Shc (Figure 4). This event is followed by a three-step

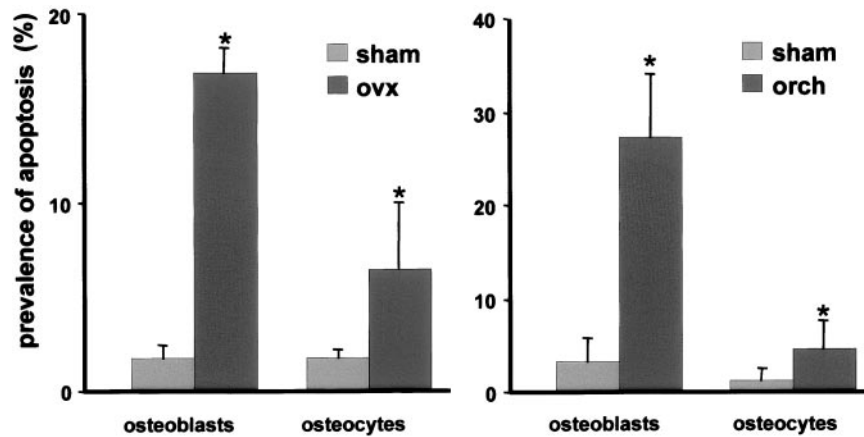


FIG. 3. Control of osteoblast and osteocyte apoptosis by sex steroids *in vivo*. Prevalence of osteoblast and osteocyte apoptosis in cancellous bone of mice at 4 and 3 weeks after ovariectomy or orchidectomy, respectively. Bars are means \pm SD of four to five animals per group. * $p < 0.05$ vs. sham by Student's t-test. [Adapted from Cell 104, Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, Di Gregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC, Non-genotropic, sex non-specific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity, 719–730, Copyright 2001, with permission from Elsevier Science.]

cascade leading to MAPK activation by two intermediate kinases: a MAPK kinase (e.g., MAPKK, MKK, or MEK) and a MAPKK kinase or MEK kinase (e.g., MAPKKK or MEKK). Activation of ERKs has been associated with cell survival in a variety of cell types and can result in phosphorylation – and thereby inactivation – of the pro-apoptotic protein Bad, a member of the Bcl-2 family of proteins that controls release of caspase-activating factors from the mitochondria. Pro-apoptotic members of this family form heterodimers with anti-apoptotic members like Bcl-2 and Bcl-^{XL} (Chao and Korsmeyer, 1998). Phosphorylation and inactivation of Bad frees Bcl-2 to form homodimers that prevent the release of caspase activators.

We have found that ERK activation and the protective effect of sex steroids on apoptosis can be blocked by specific inhibitors of Src and MEKK. Moreover, the effects of sex steroids on ERK activation and anti-apoptosis are mediated via the classical ERs (ER α or ER β) or the androgen receptor (AR) and require the activation of a Src/Shc/ERK signaling cascade. This effect is absent in cells deficient in Src or in cells overexpressing a kinase-deficient Src mutant. Further, the anti-apoptotic effect of either class of sex steroids can be abrogated with Src mutants lacking the SH2 and SH3 domains or with Shc mutants in which the primary sites of phosphorylation by Src kinases are substituted by alanine.

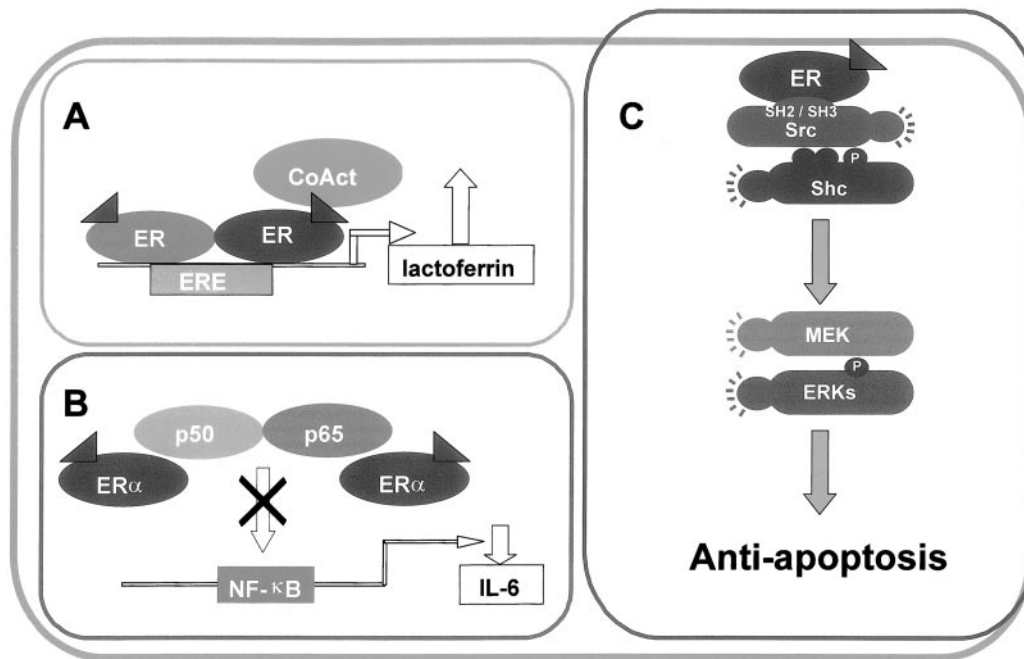


FIG. 4. Paradigms of genotropic and nongenotropic actions of the ER. Cartoon A depicts the protein-DNA interaction of the ER in the case of the transcriptional regulation of an ERE-containing gene (e.g., lactoferrin). Cartoon B depicts the protein-protein interaction responsible for the transcriptional regulation of a gene that does not contain an ERE (e.g., IL-6). Cartoon C depicts a nongenotropic action involving the interaction of a membrane-associated form of the ER with the Src/Shc/ERK signaling pathway. Although in this third model, the ligand-activated receptor protein is depicted as a monomer, it may well be that, in fact, the membrane-associated receptor, as it is the case with the one localized in the nucleus, interacts with components of the signal transduction pathway as a dimer. [Reprinted with permission from Manolagas SC, Kousteni S 2001 Perspective: nonreproductive sites of action of reproductive hormones. *Endocrinology* 142:2200–2204. Copyright The Endocrine Society.]

Using the ER α as a paradigm, we also found that activation of the Src/Shc/ERK pathway by estrogens or androgens is nongenotropic and mediated via a region of the classical ER that is distinct from the one responsible for the genotropic actions of the ligand-activated protein (Kousteni *et al.*, 2001). Indeed, this effect requires only the ligand-binding domain. Furthermore, targeting this domain to the plasma membrane reproduces fully the ERK-mediated anti-apoptotic function of the full-length ER α . In contrast, targeting this domain exclusively to the cell nucleus results in complete loss of its anti-apoptotic activity. The Src kinase physically associates with the ER or AR proteins (Migliaccio *et al.*, 2000). As in the case of the Src/Shc/ERK pathway, estrogens can activate the PI3 kinase (PI3K). Activation of PI3K results from direct interaction of the ER with the p85 regulatory subunit of the PI3K and is independent of the transcriptional activity of the receptor (Simoncini *et al.*, 2000).

Several members of the MAPK signaling pathway, including Src, Shc and ERKs, are clustered in caveolae – specialized membrane invaginations that are enriched in the scaffolding protein caveolin-1 and compartmentalize signal transduction (Okamoto *et al.*, 1998). Caveolae are found in a variety of cell types, including neuronal, endothelial, and osteoblastic cells (Toran-Allerand *et al.*, 1999; Solomon *et al.*, 2000). ER α co-immunoprecipitates with caveolin-1 (Schlegel *et al.*, 1999). Furthermore, a subpopulation of ER α has been co-localized in caveolae with both caveolin and endogenous nitric oxide synthase (eNOS) in human endothelial cells (Chambliss *et al.*, 2000). These lines of evidence, taken together with the finding that the anti-apoptotic signal of the ligand-binding domain of the ER is preserved when targeting this protein to the membrane but is lost when targeting it to the nucleus, suggest strongly that nongenotropic activation of signaling pathways by sex steroids is mediated via the classical receptors, or perhaps a shortened spliced variant that is localized in the membrane and, in particular, within caveolae. In support of this notion, both ER α and ER β can be detected in the cell membrane. The membrane receptors appear to be derived from the same transcripts as the nuclear ones (Razandi *et al.*, 1999). A membrane-impermeable form of estrogen rapidly activates the p38 MAPK and protects endothelial cells from hypoxia-induced apoptosis (Razandi *et al.*, 1999,2000). Membrane-impermeable forms of estrogen or testosterone activate ERKs in osteoblasts and osteocytes and protect them from apoptosis (Manolagas, 2000a).

In contrast to reproductive tissues where development, growth, maintenance, and function depend on estrogens in females and androgens in males, in nonreproductive tissues like bone and the cardiovascular and central nervous systems, this specificity is greatly relaxed (Manolagas and Kousteni, 2001). For example, closure of the epiphyses of long bones is estrogen dependent in both sexes (Grumbach and Auchus, 1999; Frank, 2000). On the other hand, estrogens or nonaromatizable androgens prevent osteoblast and promote osteoclast apop-

tosis or activate eNOS in endothelial cells *in vitro*, irrespective of the gender of the cell donor (Chen *et al.*, 2001) (P. Shaul, personal communication). Furthermore, estrogens restore bone mass in males with aromatase deficiency (Bilezikian *et al.*, 1998), while nonaromatizable androgens can protect the female skeleton against the adverse effects of estrogen deficiency (Tobias *et al.*, 1994; Coxam *et al.*, 1996). Some of these paradoxical effects may be explained by the fact that the distribution of the receptors for sex steroids does not vary by gender. In other words, similar levels of ER and AR have been found in bone, endothelial, and neuronal cells from males and females. Hence, estrogens could very well exert some of their effects in males through ERs residing in male cells and vice versa for androgens.

We have determined that the nongenotropic Src/Shc/ERK-mediated anti-apoptotic effect of estrogens or androgens on osteoblasts and osteocytes is transmitted by the ER α , ER β , or the AR with similar efficiency, irrespective of whether the ligand is an estrogen or an androgen (Kousteni *et al.*, 2001). Gender-independent signaling through the ERs or ARs may be operating in the neuroprotective effects of estrogen in males and androgen in females (Roof and Hall, 2000). A great deal more research will be required to establish the significance of the phenomenon *in vivo*. But it is theoretically plausible that it may provide an additional mechanistic basis for the relaxed gender specificity of the effects described above (Gee and Katzenellenbogen, 2001; Kousteni *et al.*, 2001).

One of the most striking and unexpected results of the elucidation of the nongenotropic regulation of kinases by the classical sex steroid receptors was the discovery that ERK activation – and the resulting prevention of apoptosis – can be dissociated from the transcriptional activity of the nuclear receptors with synthetic ligands (Kousteni *et al.*, 2001). Indeed, by comparing the effects of various ligands on the transcription of the Complement 3 gene, which contains an ERE in its promoter and is regulated by estrogen in a classical manner (or on the transcriptional activation of an ERE-driven promoter or the IL-6 promoter), with their anti-apoptotic effect on primary calvaria cells, we have demonstrated that an estrogen, with no transcriptional activity, exhibits potent anti-apoptotic efficacy. On the other hand, a pyrazole with potent transcriptional activity has minimal anti-apoptotic efficacy. The relative anti-apoptotic potency of these two compounds correlates with their ability to induce ERK phosphorylation.

Extension of the working life of the bone-resorbing cells and simultaneous shortening of the working life of the bone-forming cells can explain the imbalance between bone resorption and formation that ensues following loss of sex steroids. In agreement with our findings, others have demonstrated an increase in osteocyte apoptosis following loss of estrogen in rats as well as in humans (Tomkinson *et al.*, 1997,1998). The increase in osteocyte apoptosis could further weaken the skeleton by impairment of the osteocyte-canalicular

mechanosensory network. The increase in bone remodeling that occurs with estrogen deficiency would partly replace some of the nonviable osteocytes in cancellous bone. However, cortical apoptotic osteocytes might accumulate because of their anatomic isolation from scavenger cells and the need for extensive degradation to small molecules to dispose of the osteocytes through the narrow canaliculi. Hence, the accumulation of apoptotic osteocytes caused by loss of estrogen could increase bone fragility even before significant loss of bone mass because of the impaired detection of microdamage and repair of substandard bone.

V. Pharmacotherapeutic Implications of the Anti-apoptotic Effects of Sex Steroids: the ANGELS Concept

Besides the critical role of sex steroid-controlled osteoblast and osteocyte apoptosis for the pathogenesis of osteoporosis caused by loss of sex steroids, several additional studies from our group have demonstrated that osteoblast and osteocyte apoptosis are key pathogenetic mechanisms in other forms of osteoporosis. Moreover, control of osteoblast and osteocyte apoptosis may explain the antiosteoporotic efficacy of the most commonly used drugs.

Specifically, we have found that in rodents as well as in humans increased apoptosis of osteoblast and osteocytes is a key pathogenetic mechanism of the adverse effects of glucocorticoid excess on bone (Weinstein *et al.*, 1998; Manolagas and Weinstein, 1999). Osteocyte apoptosis also might be the cause of the so-called osteonecrosis of the hip (Weinstein *et al.*, 2000). Moreover, osteocyte apoptosis might explain the results of a large epidemiological study showing a rapid onset of fracture incidence following the initiation of glucocorticosteroid treatment and, most unexpectedly, a rapid offset of the incidence of fractures following discontinuation of steroid treatment (Manolagas, 2000b).

It has long been known that intermittent administration of PTH exerts an anabolic effect on the skeleton, which is opposite from the better-known catabolic effect of chronic elevation of PTH in primary hyperparathyroidism. To address the mechanism of this anabolic effect, we examined the effect of daily PTH injections in mice over a 1-month period (Jilka *et al.*, 1999). We found a dramatic increase in the number of osteoblasts, bone formation rate, and BMD. All these changes could be accounted for by a dramatic decrease in the prevalence of osteoblast apoptosis, not by increasing the generation of new osteoblasts or the proliferation of existing ones. Importantly, in other species, including humans, the increased bone formation caused by daily PTH injections is also associated with increased osteoblast numbers without change in osteoblast proliferation. The only other agent known to increase bone mass by rebuilding bone, prostaglandin E, also increases the lifespan of mature osteoblasts by reducing the prevalence of their apoptosis (Machwate *et al.*, 1998). Our findings

provided for the first time proof of principle of the concept that increased work performed by a cell population by suppressing apoptosis can augment bone mass. They also offered a rational explanation for the efficacy of daily PTH injections in various forms of osteoporosis, including the one induced by glucocorticoid excess (Manolagas *et al.*, 1998).

All currently approved drugs for the prevention and treatment of osteoporosis – estrogens, bisphosphonates, the SERM raloxifene (McDonnell, 1999), and calcitonin – are “anti-resorptive” agents. The first two stimulate osteoclast apoptosis. Through their pro-apoptotic mechanism of action alone (as in the case of bisphosphonates) or together with the ability to decrease the development of osteoclast progenitors (estrogens and SERMS) or the recruitment and function of osteoclasts (calcitonin), all these agents slow the rate of bone remodeling (Manolagas, 2000a). In most instances, this is sufficient for preventing or slowing the rate of bone loss that is due to the decrease of estrogens after the menopause.

We have demonstrated that like sex steroids, bisphosphonates and calcitonin attenuate osteoblast and osteocyte apoptosis *in vitro* and *in vivo* (Plotkin *et al.*, 1999). In the case of bisphosphonates, attenuation of apoptosis is mediated via ERK activation. The anti-apoptotic effect of these two drugs on osteocyte apoptosis provides a potential explanation for their disproportional effects on BMD and antifracture efficacy. Hence, estrogens, PTH, and bisphosphonates exert their anti-apoptotic effects on osteoblasts/osteocytes by stimulating signaling pathways at sites upstream from the common executioners of the apoptosis program (Figure 5). As discussed above, the effect of estrogens (and androgens for this matter) is mediated by a novel paradigm of sex steroid action that requires only the ligand-binding domain of the receptor, extranuclear localization of the protein, and sex nonspecificity (Kousteni *et al.*, 2001). Importantly, the effect of bisphosphonates is exerted via connexin43 hemichannels – a novel gap junction – independent action of connexin (Plotkin *et al.*, 2001). PTH, on the other hand, inhibits apoptosis via cyclic AMP (cAMP)-dependent activation of several survival pathways mediated by protein kinase A (Bellido *et al.*, 2001). Taken together, these discoveries and the demonstration of the principle that increased work output of a cell population by suppressing apoptosis can augment tissue mass, are pointing to an entirely new approach for the treatment of osteoporosis: one that could lead to cure rather than prevention or slowing of the disease process. The results of a recent clinical trial with daily injections of PTH for 18 months support strongly this contention by showing that the anabolic property of PTH restores BMD in the normal range and prevents bone fractures to levels much greater than those seen with antiresorptive agents (Neer *et al.*, 2001).

In the United States, fewer than 7% of estrogen-deficient women with osteoporosis are receiving therapy. Of those who receive therapy, only 30% stay on it long enough to enjoy its benefits during their late 60s and 70s when the incidence of the most serious bone fractures (e.g., hip) reaches its zenith

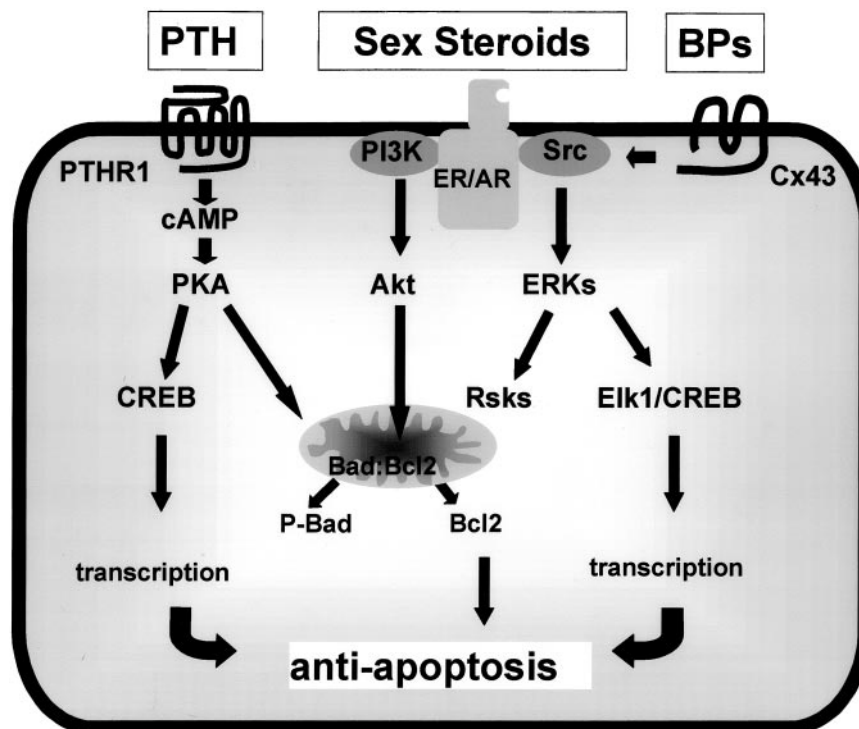


FIG. 5. Survival signaling by PTH, sex steroids, and bisphosphonates. The G protein-coupled receptor for PTH (PTH1R), the membrane-associated ERs or ARs depicted in physical contact with the phosphatidylinositol 3 kinase (PI3K) and the Src kinase, and the connexin43 (Cx43) hemichannels can all trigger anti-apoptotic signals. The principal downstream kinases of these signaling pathways – protein kinase A, Akt, and ERKs – phosphorylate and inactivate the pro-apoptotic Bad and/or activate transcription of genes that suppress apoptosis through effects on transcription factors, such as Elk-1 and CREB. [Reprinted with permission from Manolagas SC 2001 Manipulating programmed cell death for better living! Science's STKE http://www.stke.org/cgi/content/full/OC_sigtrans;2001/87/pe1. Copyright The American Association for the Advancement of Science.]

(Salamone *et al.*, 1996; Centers for Disease Control and Prevention, 1999). Consequently, the optimal therapeutic modality for osteoporosis, especially in patients who have already suffered significant bone loss, is obviously an anabolic agent that can restore bone mass by rebuilding bone within a short period of time. Bisphosphonates, and to a lesser extent estrogens, cause a small increase in apparent BMD, owing to the contraction of the remodeling space (i.e., the temporary deficit of bone created by the fact that in each site undergoing a cycle of remodeling, resorption precedes formation by several weeks).

Since daily PTH administration or prostaglandins increase bone mass by preventing osteoblast apoptosis without slowing remodeling, we have reasoned that ER ligands with anti-apoptotic but not antiresorptive/antiremodeling properties will expand the pool of mature osteoblasts at sites of new bone formation and allow these cells more time to make bone, to a much greater degree than the antiresorptive agents that also slow remodeling (Manolagas, 2000a). Based on our findings that the nongenotropic activity of the ER can be dissociated from its transcriptional activity with synthetic ligands (Kousteni *et al.*, 2001), we have coined the acronym ANGELS for ER ligands that function as “activators of nongenotropic estrogen-like signaling.” These ligands lack, completely or partially, the ability to induce the transcriptional activity of the ER. Several reasons made us suspect that this class of compounds may be a more rational and advantageous approach to the classical hormone replacement therapy or even SERMS, during postreproductive life. First, nonreproductive tissues express lower levels of receptors, compared to reproductive tissues (10- to 50-fold), which may be sufficient for accommodating nongenotropic actions of sex steroids but is insufficient for genotropic actions (Manolagas and Kousteni, 2001). Second, nonreproductive tissues exhibit a lack of variation of the levels of the ERs or ARs in cells from males versus females, which may account, at least in part, for the relaxed gender specificity in the responsiveness of females and males to estrogens or androgens. Hence, nonreproductive tissues might be ideal targets for gender-neutral ligands. Third, and most important, unlike reproductive tissues in which the majority of estrogenic effects are mediated via transcriptional activation, in nonreproductive tissues, the predominant mechanism of ER action is seemingly nongenotropic. In support of these ideas, we have now obtained evidence that synthetic ligands with potent anti-apoptotic but no genotropic activity increase BMD and bone strength significantly more than estrogens, in estrogen-deficient mice, without affecting the breast or the uterus. Future studies will, of course, be required to establish the validity of the ANGELS concept on nonreproductive tissues other than bone. But, if nongenotropic effects of estrogen are as important in other nonreproductive tissues as the evidence suggests, we are optimistic that ANGELS may also retain at least some of the beneficial effects of estrogens on the vasomotor, cardiovascular system, and CNS.

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