Mini Review

Effect of estrogen on bone cells: what is new?

Efthymia Karlafti, Kalliopi Lampropoulou-Adamidou, Symeon Tournis, George Trovas, Ioannis K. Triantafyllopoulos

Laboratory for Research of the Musculoskeletal System “Th. Garofalidis”, Medical School, National and Kapodistrian University of Athens, General Hospital of Athens KAT, Greece

Abstract
After the observations of Fuller Albright, that the loss of estrogen in women is related to osteoporosis, many studies have proved the principal role of estrogen in the regulation of bone metabolism in both genders. Apart from studies that have proven the protective effect of sex steroids on bone resorption, later studies have focused on the effect of sex steroids on osteoblasts, osteocytes and bone lining cells. These studies came to the conclusion that sex steroids contribute to bone formation directly and regulate bone adaptation to mechanical load. Further studies will have to be made to help us clarify the mechanisms by which sex steroids affect bone cells and their function, especially regarding bone lining cells.

Keywords: Estradiol, Osteoporosis, Osteoclasts, Osteoblasts, Osteocytes

Introduction

Bone tissue consists of four types of bone cells, osteoblasts, osteoclasts, osteocytes, and bone lining cells1,2. These cells regulate the organic and inorganic component of bone tissue which mainly consists of calcium hydroxyapatite, collagen, proteoglycans and other matrix proteins3. The function and activity of these bone cells depend on the effect of local and systemic factors.

Bones undergo development and growth during the first two decades of life, upon which peak bone mass is obtained. Afterwards bone mineral density remains stable until the fourth decade of life. In women, bone mineral density is rapidly reduced during menopause, at approximately the age of 50, and a further more gradual reduction is observed in both men and women after the age of 70.

Osteoporosis is a bone disease that is characterized by low bone density and the disruption of bone microarchitecture, which leads to increased fragility and increased risk of bone fracture. Reifenstein and Albright first commented on the association between declining estrogen levels at menopause and rapid bone loss, osteoporosis, and associated fragility fractures six decades ago1. Estrogen deficiency leads to the increase of bone remodeling, bone loss and osteoporosis because bone resorption surpasses bone formation4,5. Lindsay et al have proved the prevention of bone loss with the administration of estrogen to ovariectomized women6 and rats7,8.

In pre-menopausal women, more than 94% of estradiol and estrone is produced by the ovaries. In post-menopausal women, most estrogen is produced by other tissues, other than the ovaries, such as the adipose tissue. In men, 15% of estradiol is produced by the testicles and 84% by peripheral aromatization. As far as androgens are concerned, 95% of testosterone is produced by the testicles in men, and in women 25% of testosterone is produced by the ovaries, 25% by the adrenal glands and 50% by peripheral tissues. Moreover, the androgenic effect of testosterone on bone tissue is exerted by conversion to 5a dihydrotestosterone, by the enzyme 5a reductase. Furthermore, adrenal glands produce C19 androgens which include dehydroepiandrosterone, sulphate dehydroepiandrosterone and androstenedione. In humans, extragonadal production of sex steroids, such as conversion of C19 androgens to estrone and conversion of testosterone to estradiol and dihydrotestosterone, is very

The authors have no conflict of interest.
Corresponding author: Efthymia Karlafti, Laboratory for Research of the Musculoskeletal System “Th. Garofalidis”, Medical School, National and Kapodistrian University of Athens, General Hospital of Athens KAT, 10 Athinas Str., Kifissia, PC:14561, Athens, Greece
E-mail: kariawi@hotmail.com

Edited by: Konstantinos Stathopoulos

Accepted 17 May 2019
important. The bioavailability of sex steroids depends on their bond with blood proteins such as SHBG (sex hormone binding protein) and albumin. Only 1-5% of testosterone, dihydrotestosterone and estradiol, which is the percentage of sex hormones which does not bind to human albumin, is biologically drastic.

Sex hormone receptors are detected in osteoblasts, osteoclasts, progenitor cells of osteoblasts and mesenchymal stromal cells. The effect of sex steroids on bone tissue is exerted through estrogen and androgen receptors (ERα, ERβ, AR), which are mainly localized in the cytoplasm and cell membrane. Noticeably, the level of expression of these receptors in bone cells is much lower than that of the cells of the reproductive system, but the level of expression in bone cells is similar in both genders. Studies have revealed that ERA and ERβ despite the fact that they are present in all types of bone cells, have a differential expression on human bone, with ERA being expressed dominantly in cortical bone and ERβ in cancellous bone. As far as AR is concerned, studies confirm their presence in osteocytes and osteoblasts and in rat osteoclasts in vitro, but not in human osteoclasts. These receptors comprise of a N-terminal region (NTD), a ligand-binding domain (LBD) and a central DNA-binding domain. After binding with their ligand, all three types of receptors make homodimer forms and translocate in the cellular nucleus where they bind to DNA regions, called ERES (estrogen response elements) and ARES (androgen response elements). These DNA regions are located in the promoter of target genes and their activation leads to gene transcription. Their activation is mediated by transcription factors AF-1 and AF-2, which are localized in the N-terminal region (NTD) and ligand-binding domain (LBD), respectively. This mechanism of activating gene transcription is called genomic pathway and relies on the ligand-receptor translocation and binding of promoters of target genes. Moreover, there is another pathway by which sex steroids affect cells and this is called non-genomic because it doesn’t require ligand-receptor translocation and binding to DNA. Finally, there are results of sex hormone receptors that are ligand-independent such as the effect of mechanical forces on bone via receptor ERα and ERβ, and stimulation of sialoprotein expression by osteoblasts via ligand-independent effect of ERα. The presence of a ligand-independent method of activating androgen receptors has not been proven.

This review focuses on the effect of estrogen on bone cells and describes the mechanisms by which estrogens affect bone cells based on in vitro and in vivo studies with adult women, animal models and cell cultures. Increasingly, more studies reveal new mechanisms by which sex steroids affect bone cells.

**Osteocytes**

In order for the skeleton to maintain its integrity, it has to correct the microfractures that are caused by mechanical forces and strain which are exerted on bone tissue. This goal is achieved by a process called bone remodeling and it takes place in both cortical and trabecular bone. This process of bone remodeling is accomplished by a group of cells called basic multicellular unit which consists of osteocytes, osteoblasts and osteoclasts. Cells that form the basic multicellular unit are covered by a line of cells that have osteoblast phenotype and are strongly connected to capillaries. Under the canopy where bone lining cells form, hemopoietic precursors, supplied by bone marrow, are transformed into pre-osteoclasts and mesenchymal stem cells, supplied by bone marrow, change through the osteoclast precursor stage into functional synthesizing osteoclasts. Lining cells may also change into active osteoclasts. Osteocytes communicate with the surface cells, particularly osteoblasts, through their canaliculi.

Osteocytes play a principal role in bone remodeling since they detect and respond to mechanical forces and hormonal modifications such as estrogen deficiency. Based on the role of osteocytes on bone remodeling and metabolism, many studies in adult women and rodents, were made in regard to the effect of estrogens on the osteocytes’ function. Deletion of ERα and ERβ receptors in the osteocytes of genetic mutant mice, with the use of Cre recombinase, resulted in the reduction of trabecular bone mass. The above was a result of increased apoptosis of osteocytes due to estrogen deficiency, which subsequently increased the rhythm of bone remodeling and resorption. Estrogen deficiency enhances the osteocyte’s apoptosis and inhibits autophagy, the osteocyte’s protective mechanism against apoptosis. Previous studies in ovariectomized rats, ovariectomized mutant female mice, and in vitro studies with osteocyte-like MLO-Y4 cells and human osteoblast cell lines, have shown that estrogens protect osteocytes from apoptosis by the activation of the MAPK/ERK pathway (extracellular signal-regulated kinases) and the suppression of the JNK pathway (c-Jun terminal kinases) that mediates cell apoptosis via a non-genomic way.

In vitro studies with cell cultures of MLO-Y4 cells, MC3-T3 cells and human osteoblast lines have revealed another mechanism by which estrogens protect osteocytes from apoptosis is via the NO/cGMP/PKG pathway. Estrogens activate nitric oxide synthase (NOS), which leads to nitric oxide (NO) production, which promotes cGMP production, which activates cGMP-dependent protein kinases (PKGs) and subsequently extracellular signal-regulated kinases (ERKs).

The mechanism by which the apoptosis of osteocyte, due to estrogen deficiency, leads to the increase of the rhythm of bone remodeling has not been clarified and it needs more investigation. During bone resorption apoptotic osteocytes are engulfed by osteoclasts. Studies in rats, mice and pre-menopause female humans support the theory that the stimulation of bone remodeling is a result of RANKL release after osteocyte apoptosis. RANKL stimulates osteoclasts directly or stimulates neighbouring osteocytes.
to produce RANKL which subsequently stimulates osteoclastogenesis\textsuperscript{52-54}. In vitro studies with the use of MLO-Y4 cells, have shown that another mechanism by which osteocytes modulate osteoclastic activity with the effect of estrogens, is by inducing TGF\textsubscript{B} expression and inhibiting bone resorption\textsuperscript{55}. More mechanisms by which estrogens affect osteocytes are revealed constantly.

Studies in rats and cell cultures of MLO-Y4 osteocyte like cells, have revealed that the osteocytes’ response to strain is improved by estrogens\textsuperscript{56}. Osteocytes’ mechanosensitivity is served by intracellular calcium, the concentration of which is modified depending on mechanical loading. Estrogens release calcium from intracellular stores\textsuperscript{57} and activate Akt kinases and ERK 1/2 proteins\textsuperscript{21}, increasing in this way the osteocytes’ mechanosensitivity\textsuperscript{58}. Connexin 43 is a transmembrane protein that contributes to forming gap junction between bone cells, and inducing in this way signal transduction. Recent studies have shown that besides the fact that estrogen increases the expression of connexin 43, it enhances the mechanosensitivity of MLO-Y4 cells to mechanical strain via estrogen receptors\textsuperscript{59}, Voisin et al proposed that the reduction of b3 integrin expression in ovariectomized rats might be responsible for the impaired mechanosensitivity of osteocytes in the case of estrogen deficiency\textsuperscript{42}.

**Osteoclasts**

Osteoclasts are multinucleated cells that derive from hemopoietic precursors detected in bone marrow. The process of forming mature osteoclasts is called osteoclastogenesis and is controlled by a number of factors and cytokines with paracrine and autocrine effect, that are produced by and is controlled by a number of factors and cytokines of forming mature osteoclasts is called osteoclastogenesis

**Effect of estrogen on bone cells: what is new?**

In vivo studies in female rats and in vitro studies with avian and murine osteoclasts have shown that osteoclasts are direct cell targets of sex steroids\textsuperscript{12,62,63}. Studies have proven that estrogen suppresses the formation of osteoclasts and activity in cultures of both rat and human osteoclasts\textsuperscript{64,65}. Estrogen inhibits bone resorption by directly inducing the apoptosis of murine and rabbit osteoclasts in vitro\textsuperscript{66,67}. It has been noticed that estrogens reduce osteoclast’s lifespan by inducing their apoptosis, through enhancing Fas-ligand (FasL) gene transcription. FasL is a transmembrane protein, which exists in membrane bound and soluble form and induces osteoclast apoptosis, after binding to its receptor, Fas receptor, and subsequently stimulates a transduction pathway that leads to cell apoptosis\textsuperscript{68}. This is the principal theory for the mechanism by which estrogen reduces bone resorption\textsuperscript{66,69}. However, contrarily, latest studies in mice support that estrogen induces osteoclast’s apoptosis indirectly, by upregulating FasL gene in osteoblasts\textsuperscript{70}, a mechanism which will be analyzed in the next chapter, where the effect of estrogen on osteoblasts is referred to. Additionally, new molecules and pathways interfere with the osteoclast’s apoptosis mediated by estrogen, such as receptor-interacting protein 14. Receptor-interacting protein 140 is a regulator of transcription factors and modulates signal transduction\textsuperscript{71}. Latest studies revealed that receptor-interacting protein 140 enhances the osteoclast’s apoptosis, mediated by estrogen, by inducing the transcription of Fasl gene\textsuperscript{72}.

Moreover, another mechanism by which estrogen affects osteoclasts is by inducing the Wnt/b-catenin pathway, an intracellular pathway that inhibits osteoclastogenesis\textsuperscript{71}. Studies in genetic mutant mice and in vitro studies with cultures of murine osteoclast and human monocyte-derived osteoclasts, with the deletion of b-catenin in osteoclasts, have shown a rise in the number of osteoclasts and a reduction of bone mass\textsuperscript{73,74}.

Recent studies in female mice, rats and in vitro studies with cultures of RAW 264.7 cells and murine osteoclasts, have proven other pathways by which estrogen inhibits osteoclastic activity. Estrogen downregulates the expression of genes that are related to the osteoclast’s resorption activity such as tartrate-resistant acid phosphatase and cathepsin K\textsuperscript{75}. Latest discoveries reveal new mechanisms of estrogen’s effect on osteoclasts, such as the suppression of ephA2/ephrin A2 signaling pathway\textsuperscript{76}, the decrease of HIF1a (hypoxia inducible factor 1 alpha)\textsuperscript{77,78}, the increase of the expression of TRPV5 channel (transient receptor potential vaniloid 5)\textsuperscript{79} and the regulation of the expression of other molecules that could be possible targets for the production of antiosteoporotic drugs\textsuperscript{80}.

Another new regulator of estrogen’s effect on bone cells, is the estrogen-response element-binding protein in bone (ERE-BP). This is a protein that competes with Era to occupy ERES (estrogen response elements). Experiments showed that ERE-BP increased the expression of RANKL gene, suppressed the OPG expression in osteoblasts and increased the expression of RANK in osteoclasts. Moreover, estrogen increased the expression of ERE-BP in osteoblasts but not in preosteoclasts, suggesting that there is a different regulation of ERE-BP in bone cells, and that maybe osteoclasts are more sensitive to the effect of estrogen due to the lack of ERE-BP induction by estrogen in osteoclasts. This upregulation of ERE-BP by estrogen may suggest that ERE-BP is an intracellular feedback mechanism of estrogen’s effect on bone cells\textsuperscript{81}.

Finally, studies in genetic mutant mice with the deletion of Era receptors in osteoclasts, have shown a rise in the number of osteoclasts and a reduction of trabecular bone mass. On the other hand, no effect was detected in endocortical bone, proving that the protective effect of estrogen on cortical bone is not a direct effect of estrogen on osteoclasts but is probably mediated indirectly by osteoblasts. It is of great concern, to mention that an important portion of the estrogen’s effect on osteoclasts is mediated by osteoblasts\textsuperscript{54}. The mechanisms by which this is accomplished are analyzed in the next chapter, where the effect of estrogen on osteoblasts is referred.
Osteoblasts

It is known that estrogen deficiency leads to activation of bone remodeling as a result of increased osteoclast function. Furthermore, since bone resorption is connected to bone formation, the increased bone resorption in the case of estrogen deficiency will lead to increased bone deposition. Unfortunately, bone deposition is inadequate, leading to bone mass reduction. Studies in mice and in vitro studies with mouse osteoblast-like MC3T3-E1 cells showed that estrogen preserves bone mass by inducing the proliferation and activity of osteoblasts. Recently, the dose dependent effect of estrogen on the differentiation of osteoblasts to adipocytes has been revealed. Studies have shown considerable mechanisms by which estrogen directly affects osteoblasts.

Estrogen prevents apoptosis of osteoblasts by suppressing the expression of apoptotic genes, thereby extending the life span of osteoblasts. In vitro studies with murine cell lines revealed that the increase of the osteoblast lifespan due to estrogen is a result of the activation of the MAPK/ERK intracellular pathway (extracellular signal-regulated kinases) and the suppression of the JNK pathway (c-Jun terminal kinases) that mediates cell apoptosis. The MAPK/ERK intracellular pathway is involved in the protective effect of estrogen on osteoblasts through autophagy, a protective mechanism of cells against apoptosis. The three basic subgroups of MAPK kinases are ERKs (extracellular signal-regulated kinases), JNKs (c-Jun terminal kinases) and p38 kinases. Estrogen activates the MAPK/ERK pathway, which consists of proteins that communicate chemical and physical signals from the cellular surface to the nucleus. These proteins modify transcription factors and subsequently regulate the expression of genes that are related to the survival of osteoblasts. We need to mention that the same pathway is involved in the effect of estrogens on the reduction of the osteoclasts lifespan.

A study by Thouverey, proved that the p38α MAPK pathway is involved in the increased expression of RANKL and IL-6 by osteoblasts, which leads to bone loss in the case of ovariectomized mice. Moreover, Manolagas et al showed the importance of oxidative stress and its products on the reduction of osteoblastogenesis, the suppressive effect that oxidative stress has on osteocytes, and its role in the survival of osteoclasts and osteoclastogenesis, mainly through the antagonism of the Wnt/b-catenin pathway. Estrogen induces the expression of Wnt protein and subsequently enhances the survival of osteoblasts through the Wnt/b-catenin pathway. In vivo studies in humans and in vitro studies with MC3T3-E1 cells showed that in estrogen deficiency the expression of Wnt 1 6, a protein that is involved in the Wnt/b-catenin pathway, is decreased, and plenty of other Wnt proteins have been proven to mediate estrogen's effect on osteoblastic and osteoclastic activity.

Finally, Chang et al showed that NF-kB inhibition leads to an increased expression of Fos-related antigen 1 (Fra-1), which is a transcription factor that is necessary for the production of bone matrix. NF-kB is a transcription factor that regulates the expression of numerous genes. In the case of osteoclasts the binding of RANKL to RANK receptor leads to activation of NF-kB mediated by TNF receptor-associated factors (TRAFs), and subsequently leads to the increase of osteoclastic activity. The same study concluded that estrogen deficiency leads to an increased activity of NF-kB in osteoblasts and a reduction of JNK activity that leads to the reduction of bone formation. There is another study by Manolagas et al which shows that estrogen regulates the production of osteoclastogenetic cytokines by osteoblasts and other cells and in the case of estrogen deficiency this regulation is disturbed.

In particular, during menopause, an increase in IL-1 and IL-6 has been noticed, and those are the first cytokines to be responsible for bone loss due to estrogen deficiency. IL-6 has been related to inducing osteoclastogenesis. It has been proven that estrogen and androgens inhibit IL-6 expression and IL-6 receptor on mesenchymal cells of human osteoblastic line, and subsequently suppress the osteoclastogenic effect of IL-6. Many other cytokines have been discovered to mediate estrogen's effect on bone cells such as TNF-a. In vivo studies in mice and in vitro studies with human osteoblast-like cells and mouse osteoblasts have shown that estrogen inhibits the expression of TNF-a by osteoblasts and T cells and subsequently inhibits RANKL expression and osteoclastogenesis. The expression of osteoclastogenetic cytokines in estrogen deficiency is a result of down-regulation of the expression of Bmi-1 (B lymphoma Mo-MLV insertion region 1), a gene that regulates cell cycling, in lymphocytes. Finally, estrogen seems to regulate the expression of cytokine’s receptors on osteoclasts. All of the above shows the importance of the immune system in skeletal homeostasis and its role in the effect of estrogen on bone tissue, which will not be further analyzed since it diverges from the purposes of this review.

After the OPG/RANK/RANKL pathway was discovered, and its role in bone remodeling and osteoclastogenesis was clarified, studies showed that estrogen affects this pathway both directly and indirectly through the cells of the immune system, with IL-1, TNF-a and PGE2 being some of the relevant cytokines which are related to the mechanism by which estrogen affects the OPG/RANK/RANKL pathway. In vivo studies in human, rats and in vitro studies with human monocytes and RAW 264.7 cells showed that estrogen inhibits osteoclastogenesis through the inhibition of RANKL expression from osteoblasts and osteocytes. Recently, Martin et al have proven that estrogen and androgens inhibit the association of RANKL with osteoblastic membrane, whilst RANKL mRNA levels maintain stable. The above leads to the conclusion that sex steroids inhibit osteoblastic induced osteoclastogenesis through disassociation of RANKL from osteoblastic membrane. Moreover, studies in humans...
have revealed that specific polymorphisms of RANKL/RANK are related to postmenopausal osteoporosis\(^{129,130}\). Additionally, estrogen stimulates OPG expression, a decoy receptor of RANK, from osteoblasts, and subsequently inhibits osteoclastogenesis\(^{131}\). Moreover, studies in mutant mice have revealed that estrogen enhances osteoclast’s apoptosis though Fas ligand (FasL) and MMP3 (matrix metalloproteinase 3) expression in osteoblasts. FasL is a transmembrane protein, which exists in membrane bound and soluble form and induces osteoclast apoptosis, as mentioned previously. MMP3 is necessary for the generation of the soluble form of FasL that affects osteoclasts, and is upregulated by estrogen\(^{70,132}\).

Additionally, transforming growth factor-beta (TGF-b) mediates the effect of estrogen on osteoblasts. Studies in mice and in vitro studies with human and murine osteoblasts showed that estrogen induces the expression of TGF-b, which subsequently promotes osteoblast’s differentiation and activity\(^{132-135}\). Moreover, TGFb inducible early gene-1 (TIEG1), a transcription factor that regulates TGF-b\(^{136}\), is another factor that mediates estrogen’s effect on osteoblasts and increases their lifespan\(^{137}\). Furthermore, TIEG1 seems to inhibit the expression of sclerostin by osteoblasts\(^{138}\). Sclerostin is produced by osteoblasts and osteocytes and antagonizes the WNT/β catenin pathway, which is necessary for osteoblastic differentiation and activity\(^{139}\). Since TIEG1 is an estrogen regulated gene, more studies are needed to further investigate the mechanisms by which estrogen modulates sclerostin’s expression by osteoblasts and osteocytes.

Finally, studies constantly reveal more molecules and mechanisms by which estrogen affects osteoblasts, such as morphogenetic protein 4 and morphogenetic protein 2\(^{140,141}\). Morphogenetic proteins (BMPs) come under the TGF-b superfamily and have an important role in the differentiation of osteoblasts, which is mediated by estrogen\(^{114,142}\). Recent in vitro studies with human osteoblastic cell lines, revealed that estrogen inhibits the expression of sclerostin by osteoblast, through interaction with BMP2 and the WNT/β catenin pathway\(^{143}\). Sclerostin is produced by osteoblasts and osteocytes and antagonizes the WNT/β catenin pathway, which is necessary for osteoblastic differentiation and activity\(^{139}\).

Despite the fact that estrogen has an important role in regulating bone production through its direct effect on osteoblasts, the phase at which they affect osteoblastic activity and their lifespan is not well understood.

**Bone lining cells**

Few things are known about the mechanisms by which estrogen affects lining cells. Lining cells cover most of the non-remodeling bone surfaces of the adult skeleton and seem to affect the balance between bone formation and resorption\(^{144}\). A study by Bowman and Miller on the effect of estrogen administration in a male Japanese quail showed a rise in the number of lining cells that cover bone surfaces, a change in their shape from thin, flat cells to round, and finally their differentiation to osteogenic cells that produce bone matrix\(^{145}\). The exact mechanism by which this modification happens is not yet clarified. Streicher et al showed that lining cells act as bone defenders against bone resorption. In the case of estrogen deficiency, the authors showed that the increase in bone resorption is a result of lack of Era-mediated suppression of RANKL expression in bone lining cells\(^{146}\).

**Conclusion**

This review is referred to the effect of estrogen on the cells of trabecular and cortical bone but not on the mechanisms by which estrogen contribute to other functions such as bone longitudinal growth and periosteal expansion. Furthermore, this review focuses on the effect of estrogen on bone cells, despite the fact that they have impact on many other different cells that are connected to bone homeostasis. Moreover, we have to keep in mind that the mechanisms by which sex steroids affect bone depends on sex, age, and bone region and we have to take this into consideration when designing a study with regard to the effect of sex steroids on bone.

Finally, we need to pay attention to the use of genetically modified mutant mice and the use of rodent models for the study of the effect of sex steroid hormones on bone cells, and to the adjustment of the conclusions with regards to humans. In conclusion, further studies need to be done with the use of cell cultures and molecular biology, in order to understand the mechanisms by which sex hormones affect bone cells and bone homeostasis.

**References**


Effect of estrogen on bone cells: what is new?


Effect of estrogen on bone cells: what is new?


117. Sunyer T, Lewis J, Collin-Osdoby P, Osdoby P. Estrogen’s bone-protective effects may involve differential IL-1 receptor regulation and IL-1 receptor antagonism in osteoblastic cell line with high levels estrogen receptors. Journal of endocrinology 2010;216(2):243-53.


