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THE OXYTOCIN-BONE AXIS

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Abstract

We recently demonstrated a direct action of oxytocin (OT) on skeletal homeostasis mainly mediated through stimulation of osteoblasts (OBs) formation and through the reciprocal modulation of osteoclast (OCs) formation and function. Thus, mice lacking the hormone or its receptor develop a low turnover osteoporosis that worsens with age in both sexes. The skeleton of OT and OT receptor (Oxtr) null mice display a pronounced decrease in vertebral and femoral trabecular volume. At cellular level OBs from OT^{-/-} and Oxtr^{-/-} mice exhibit lower mineralization activity and, at mRNA level, all master genes for osteoblast differentiation are down regulated. Moreover, OT has dual effects on OCs: it increases osteoclast formation both directly, by activating NF- κ B and MAP kinase signaling, and indirectly, through the up-regulation of RANK-L synthesis by OBs. On the other hand, it inhibits bone resorption by triggering cytosolic Ca²⁺ release and nitric oxide synthesis in mature OCs.

OT is locally produced by osteoblasts acting as paracrine-autocrine regulator of bone formation modulated by estrogens. The estrogen signal involved in this feed forward circuit is non genomic, since it requires an intact MAPK kinase signal transduction pathway, instead of the classical nuclear translocation of estrogen receptor. The ability of estrogen to increase bone mass *in vivo* is to an extent OTR-dependent. Thus Oxtr^{-/-} mice injected 17 β -estradiol did not show any effects on bone formation parameters, while the same treatment increases trabecular and cortical bone in wild type mice. An intact OT autocrine-paracrine circuit seems to be essential for optimal skeletal remodeling.

INTRODUCTION

The neuropeptide oxytocin (OT), synthesized in the hypothalamus and in many reproductive tissues during pregnancy, has been since long been known to be related to parturition and milk ejection,. However, in recent years, several studies have indicated that the distribution, biological significance and regulation of the OT/OT receptor (Oxtr) system is unexpectedly more ubiquitous than previously anticipated (1). In the last decade, research has revealed that OT plays an important role in social interactions that go far beyond the previously

documented actions in the regulation of uterine contractions during labor and milk ejection during lactation.

Oxtrs are expressed in pituitary, kidney, ovary, testis, thymus, heart, vascular endothelium, osteoclasts, osteoblasts, myoblasts, pancreatic islet cells, adipocytes and several types of cancer cells (2–6). The receptors are all functional in that they induce various intracellular signaling pathways in response to OT application. However, their relevance in terms of physiological functions, specifically with respect to people, has not been fully established. Nevertheless, results from animal and in vitro studies suggest roles for OT in pituitary function, male and female fertility, T-cell function, cardiovascular control, muscle formation and the growth control of certain cancer cells (1). The widespread distribution of OT receptors in the brain and the specific behavioral effects of centrally applied OT have firmly established OT as a central neurotransmitter with roles in reproductive and social behaviors, memory, food and drink intake, and modulation of anorexia. Specifically, a role in mediating maternal behavior, sexual receptivity and partnership bonding has been proposed: OT has appropriately been dubbed the ‘love hormone’ (7–8).

We recently investigated OT action on bone cells both on isolated cells or taking advantage of mice models null for *OT* or the *Oxtr*, demonstrating the relevant role of this hormone on skeletal homeostasis.

ANABOLIC ACTIONS OF OT ON THE SKELETON

Physiological Effects

Genetically modified mice, null for *OT* or for its receptor (9–10) enabled us to obtain striking evidence of the profound effects of OT on bone remodeling (11–14). Considering that calcium is mobilized from the maternal skeleton during late pregnancy and lactation, we speculated that the same hormone that regulates parturition and lactation might also control skeletal homeostasis. OT has a direct and dominant action on the skeleton that is mediated mainly through its stimulation of osteoblast formation, but also through a modulation of osteoclast formation and function. Thus, *OT* and *Oxtr* null mice develop low turnover osteoporosis that worsens with age in both genders (11). Histomorphometry and microCT analysis reveal a pronounced decrease in vertebral and femoral trabecular volume, already evident in the heterozygous, accompanied by a significant reduction in bone formation rate (11).

In view of OT’s known central actions, we attempted to determine whether there was a central or a peripheral action of the neuropeptide, and noted that intra-cerebro-ventricular (ICV) OT injections did not affect bone remodeling, indicating that the effect was due to the peripheral OT (11).

Signalling Pathways

At the cellular level, osteoblasts from *OT*^{-/-} mice exhibit a decreased mineralization in ex vivo cultures, and at RNA level, all the master genes for osteoblast differentiation are negatively affected. OT stimulates the differentiation of osteoblasts to a mineralizing

phenotype by causing the up-regulation of BMP-2 through the Schnurri-2 and ATF-4 pathways (11).

The signaling cascades that mediate OT action on bone cells ultimately release Ca^{++} from intracellular stores and trigger ERK signaling. Oxt stimulation induces increases in intracellular calcium concentration, but with a different pattern: a single spike, returning immediately back to the baseline in osteoblasts and a slower but long lasting increase in osteoclasts (11). Receptor endocytosis is observed after OT treatment, but, new receptors appear on the membrane after 24–48 hours, as seen by immunofluorescence and confirmed at the mRNA level by quantitative PCR (11).

In mature osteoblasts, OT treatment reduced the expression of osteoprotegerin (OPG), while increasing RANK-L, thus stimulating osteoclast differentiation. Oxytocin, in fact, has a dual effect on osteoclasts: it increases osteoclast formation both directly, by activating NF- κ B and MAP kinase signaling, and indirectly, through the up-regulation of RANK-L. On the other hand, it inhibits bone resorption by 40% in the 48 hours following OT stimulation by mature osteoclasts by triggering cytosolic Ca^{2+} release and nitric oxide synthesis (11). This apparent paradox of increased osteoclastogenesis coupled to temporary decreases in bone resorption can be explained considering the cyclic activity of the hormone due to the down regulation of receptors. The physiological meaning could be the cyclic availability of higher levels of circulating calcium in periods such as the last phases of pregnancy and after parturition when high levels of this ion are necessary for the mineralization of the fetal skeleton and for lactation. It can also explain at least part of the protective action of estrogens on the skeleton. It has been observed, in fact, and applied in pharmacology for PTH, that alternate phase of bone resorption, can induce a reversal phase of bone formation with evident benefits for bone.

ROLE OF OT IN MATERNAL BONE REMODELING DURING PREGNANCY

One of the physiological effects of OT on the skeleton could be related to the last phases of pregnancy and after parturition where higher levels of circulating calcium are required for the mineralization of the fetal skeleton and for lactation. These periods correspond, respectively, to maximal fetal and post-natal bone growth, because of which, the mother must lose ~120g of calcium from her skeleton (15). Hormonal adaptations, including low estrogen and elevated PTHrP levels, facilitate this maternal hyperresorption and intergenerational calcium transfer (16). However, it is surprising that shortly after these periods of profound bone loss, the mother's skeleton is rapidly replenished: otherwise pregnancy- and lactation-associated osteoporosis occurs. The mechanisms for this dramatic skeletal recovery have been remained for long time poorly understood until the role of OT, as direct player on the skeleton during pregnancy and/or lactation, prompted us to explore its pathophysiology. We noted that osteoclast formation *ex vivo* was diminished in pregnant mothers with genetic OT-deficiency (12). MicroCT measurements of *OT*^{-/-} pup skeleton at day E20 revealed normal bone volume, but increased trabecular numbers, indicating trabecular hypomineralization. This confirmed OT as a facilitator of intergenerational calcium ion transfer from a pregnant mother to the pups, playing a key role in maternal skeletal mobilization during pregnancy (12).

Together, these results are consistent with the hypothesis that oxytocin may be responsible, mainly during pregnancy and lactation, for maintaining a high rate of cell activity in bone, stimulating the proliferation of both forming and resorbing cells, but controlling the amount of bone resorbed. The phenotype observed in null mice can be due to the reduced proliferation/differentiation of bone cells. Together, the complementary genetic and pharmacologic approaches reveal OT as a novel anabolic regulator of bone mass: an estrogen-mediated switch that turns on bone cell activity, stimulating pulses of bone resorption and formation.

REGULATION OF OT AND OXTR SYNTHESIS IS ESTROGEN MEDIATED

The estrogen dependence of the synthesis of the hormone and its receptors (17–18) has further extended the biological implications of the OT/Oxtr interplay: OT and Oxtrs in fact are regulated by sex steroids and by oxytocin itself. Considering that OT is locally produced in several organs (2–4), we investigated if estrogen could stimulate an autocrine OT loop in bone cells and if the well known protective effects of estrogens on the skeleton could be mediated by OT. The effects on the skeleton of estrogen withdrawal have been extensively investigated: no doubts exist that loss of estrogen leads to an increased rate of bone remodeling that tilts the balance between bone resorption and formation to cause net bone loss. The main reason for this has been ascribed to the proapoptotic actions of estrogen and androgen on osteoclasts rather than anti-apoptotic effects on osteoblasts and osteocytes (19). Loss of sex steroids, moreover, accelerates the effects of aging on bone by decreasing defense against oxidative stress (20) and estrogen deficiency stimulates T cells activation, which, in turn, induces osteoclast formation by increasing the production of TNF- α , increasing the number of OC precursors (21–22). Furthermore, low estrogen causes elevated FSH levels, a reduction of which by a specific antibody to FSH, attenuates hypogonadal bone loss by reducing resorption and enabling new bone synthesis (23–24). However, so far, a positive direct anabolic role of estrogen on osteoblasts has been more taken for granted than clearly demonstrated.

We demonstrated with several experimental approaches that osteoblasts are indeed capable of synthesizing and secreting OT as early as 2 hours after estradiol treatment, and that estradiol regulates bone cell activity through OT. (13, 14). In view of this rapid induction of OT mRNA, we tested whether estrogen action was exerted through a non-genomic, cell surface-mediated mechanism. OT expression requires an intact MAPK kinase signal transduction pathway, since MAPK kinase inhibition ablates OT synthesis (13). The estrogen signals involved in this circuit are non genomic since BSA-conjugated estradiol, which is mostly unable to permeate plasma membrane, increases OT-mRNA to levels comparable with free 17- β -estradiol treatment (13). Together these findings suggested a non-genomic, Erk-dependent pathway for the induction of OT by estrogen.

Conversely, Oxtr induction by 17 β -estradiol followed a slower time course than the induction of OT in osteoblasts. To differentiate a genomic from a non-genomic mechanism for estrogen-mediated Oxtr induction, we again utilized the putative cell-impermeant 17 β -estradiol-BSA-conjugate. In contrast to OT, where both 17 β -estradiol and 17 β -estradiol-BSA equally up-regulated OT expression, Oxtr expression was induced only by native 17 β -

estradiol and not by the cell-impermeant 17 β -estradiol-BSA conjugate (13). This meant that OTR induction by estrogen occurred through a traditional genomic mechanism. In agreement with this, we found that *Oxtr* expression was less sensitive to the MAPK kinase inhibition with the PD98059 compound, which blocks ERK phosphorylation (13). (Figure 1)

In pursuing to demonstrate that estradiol regulates bone cell activity through OT, we utilized *Oxtr*-silenced M3T3 osteoblasts or primary cells obtained from *Oxtr*-KO mice. After estrogen treatment in control conditions, there was a up-regulation of many osteoblast differentiation genes, as osteopontin, BSP, osteocalcin, the transcription factors Runx2, Osterix and ATF4 (14); these responses are lacking in *Oxtr*-silenced M3T3 osteoblasts and in primary cells obtained from *Oxtr*-KO mice (14).

Histomorphometric studies show that *Oxtr*^{-/-} mice injected for a month with 17 β -estradiol do not show any change in bone formation parameters, compared to untreated littermates, while the same treatment significantly increased trabecular and cortical bone parameters in wild type mice compared to controls (14). This attested to the ability of estrogen to increase bone mass in wild type mice *in vivo* in an *Oxtr*-dependent manner. Similar results were obtained in a osteoblast specific *Oxtr* knockout mice (Col2.3-Cre⁻/OTR^{fl/fl} mice). Col2.3-Cre⁻/OTR^{fl/fl} mice failed to display increases in bone mass in response to 17 β -estradiol. These findings definitively rule out mediation of OT effects on the skeleton through the central nervous system: the osteopenia of global *Oxtr* deficiency is mimicked in its entirety by osteoblastic *OTR* deficiency (14), as summarized in Table 1.

Exploring whether OT signalling also mediates the effect of estrogen on bone mass in hypogonadal mice, we found that bone mineral density measurements dropped after ovariectomy in wild type and *Oxtr*^{-/-} mice at the lumbar spine and femur. Moreover, when compared to vehicle injected mice, sham-operated and wild type mice treated with 17 β -estradiol showed increases in BMD, whereas the respective *Oxtr*^{-/-} mice did not (14). It is well established that estrogen can attenuate hypogonadal hyper-resorption through its effect on the osteoclast ER α to inhibit JNK phosphorylation (25). Therefore, mice lacking the osteoclast estrogen receptor (ER α) lose their anti-resorptive responses to estrogen, while they continue to display increases in bone formation (26). Our result confirms that the OT/*Oxtr* axis contributes to the action of estrogen in hypogonadism and can thus be used for the treatment of hypogonadal bone loss, wherein the repletion of osteoblastic autocrine OT signaling may represent a non-steroid means of restore bone formation.

Additionally, osteoblasts in bone marrow produce abundant OT, suggesting that locally released OT may be an autocrine regulator of bone formation and bone mass (13). In this local circuit OT produced from osteoblasts in response to estrogen acts upon the *Oxtr* to stimulate further OT release, which amplifies estrogen action (14). Physiologically, in addition of being a downstream mediator of estrogen action on bone, the OT autocrine circuit may serve to coordinate the bone-forming activity of neighboring osteoblasts.

CONCLUSION

Osteoporosis constitutes a major worldwide public health burden characterized by enhanced skeletal fragility. Whereas an increase in bone resorption is considered as the main contributor of bone loss, this loss is accompanied by increased bone marrow adiposity. Osteoblasts and adipocytes share the same precursor cell and an inverse relationship exists between the two lineages. Therefore, identifying signaling pathways that stimulate mesenchymal stem cells osteogenesis at the expense of adipogenesis is of major importance for developing new therapeutic treatments. Elabd et al. (27), by transcriptomic analysis, have indicated the Oxt pathway as a potential regulator of the osteoblast/adipocyte balance in human multipotent adipose-derived stem (hMADS) cells. Both OT and carbetocin (a stable OT analogue) negatively modulated adipogenesis while promoting osteogenesis in both hMADS cells and human bone marrow mesenchymal stromal cells. Consistent with these observations, ovariectomized mice and rats, which become osteoporotic and exhibit disequilibrium of this balance, were found to have significantly decreased OT levels compared to sham-operated controls. Subcutaneous OT injection reversed bone loss in ovariectomized mice and reduced marrow adiposity. Clinically, plasma OT levels were found to be significantly lower in postmenopausal women developing osteoporosis than in their healthy counterparts (28). Taken together, these results suggest that plasma OT levels could represent a novel diagnostic marker for osteoporosis and that OT administration holds promise as a potential therapy for this disease. According to this future perspective is important a more careful evaluation of peripheral levels of OT, since a recent study has highlighted the poor reliability of the diagnostic methods used thus far (29). In summary, we suggest that OT, as a circulating peptide, is responsible for basal skeletal homeostasis in both genders alike, and may play an additional role in the initial mobilization and subsequent restoration of the maternal skeleton during periods of calcium stress in pregnancy and lactation. We speculate that because of its skeletal anabolic action, recombinant OT or its analogs might have potential utility in therapy for human osteoporosis. OT analogues that are long-acting and do not cross-react with AVP receptors could indeed be employed as anabolic stimuli to restore the skeletal loss occurring after pregnancy and lactation or in postmenopausal women.

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Figure 1.

Schematic representation of the osteoblastic autocrine OT/OTR circuit triggered by estrogen. Osteoblastic OTRs and OT are up-regulated by estrogen via genomic and non-genomic mechanisms, respectively. Estrogen-induced OTR and OT production amplifies autocrine OT signalling and enhances osteoblast differentiation. Released OT may also trigger a paracrine signal to coordinate the differentiation of adjacent osteoblasts. (Ref. 13)

Table 1

Fully characterized skeletal phenotype of the mutants are listed in Table 1. The two Cre transgenic lines were crossed initially with OTR^{fl/fl} mice, following which the Cre⁺/OTR^{fl/+} genotypes were crossed with OTR^{fl/fl} mice to yield the respective osteoblasts and osteoclast-specific Cre⁺/OTR^{fl/fl} mice. The absence of the OTR in osteoblasts (but not in osteoclasts) in Col2.3-Cre⁺/OTR^{fl/fl} mice, resulted in a significant reduction in BV/TV and BMD, confirming that osteoblast-specific deletion of OTRs recapitulates global OTR deficiency.

	BV/TV	BMD
Wild Type	Normal	Normal
Oxtr+/-	<i>Low</i>	<i>Low</i>
Oxtr-/-	<i>Low</i>	<i>Low</i>
TRAP-Cre/Oxtr^{fl/fl}	Normal	Normal
Coll2.3-Cre/Oxtr^{fl/fl}	<i>Low</i>	<i>Low</i>