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**Estudo da Interação do Hormônio
Tireoideano com o Sistema $\alpha 2$ adrenérgico no
Crescimento Ósseo Endocondral: uma avaliação
em cultura de órgão.**

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RESUMO

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Sabe-se que o hormônio tireoideano (HT) é essencial para o crescimento e desenvolvimento ósseos. No entanto, os mecanismos pelos quais o HT regula esses processos são pouco entendidos. Recentemente, o sistema nervoso simpático (SNS) foi identificado como um potente regulador do metabolismo ósseo. Estudos do nosso grupo mostraram que o HT interage com o SNS para regular a massa e estrutura ósseas, e que essa interação envolve a sinalização adrenoceptora α_2 (α_2 -AR). Também identificamos a presença de todos os subtipos de adrenoceptores α_2 adrenérgicos, o α_2A - , α_2B - e α_2C -AR, na lâmina epifiseal (LE) de camundongos. Além disso, observamos que camundongos com inativação gênica isolada do α_2A -AR e α_2C -AR (α_2A -AR^{-/-} e α_2C -AR^{-/-}) apresentam LEs desorganizadas, ossos longos mais curtos, e atraso na ossificação endocondral. Estudos in vivo revelaram, que as LEs de animais α_2A -AR^{-/-} e α_2C -AR^{-/-} respondem de forma diferente (do que as LEs de animais selvagens) ao excesso e deficiência de HT, o que sugere fortemente que o HT também interage com o SNS para regular o crescimento e o desenvolvimento ósseos. Através de um sistema de cultura de órgãos de ossos longos, o presente estudo teve como objetivo investigar se o HT interage com o SNS diretamente no esqueleto, para regular o crescimento linear ósseo, e se os receptores α_2 adrenérgicos estão envolvidos nessa interação. Assim sendo, avaliamos, in vitro, o crescimento linear ósseo de tíbias derivadas de embriões de camundongos (com 15 dias de vida intrauterina) selvagens (WT) e α_2C -AR^{-/-} (KO) durante 6 dias. Vimos que as tíbias KO apresentam um menor crescimento longitudinal quando comparadas às tíbias WT, e que o tratamento com 10^{-8} M de triiodotironina (T3) diminuiu significativamente o crescimento longitudinal das tíbias WT, o que não foi visto nas tíbias KO. Vimos, ainda, que o tratamento com UK 14,304 (UK), um agonista α_2 não seletivo, induziu o crescimento longitudinal somente nas tíbias KO. A expressão de genes relacionados com a diferenciação terminal de condrócitos (Col X, IGF-1, Wnt-4 e Runx2) mostrou-se aumentada nas tíbias KO (quando comparada à expressão nas tíbias WT). O tratamento com T3, como esperado, estimulou a expressão desses genes nas tíbias WT, porém diminui a expressão nas amostras KO, chamando a atenção para a importância desses receptores na modulação das ações do T3. Observamos, ainda, que a ativação local dos α_2 -ARs com o UK bloqueia a expressão desses genes relacionados à diferenciação dos condrócitos, além de bloquear os efeitos positivos do T3 (UK+T3) na expressão desses genes. Esses achados mostram que os receptores α_2 adrenérgicos atuam diretamente no esqueleto para controlar a diferenciação terminal dos condrócitos e, portanto, o crescimento longitudinal ósseo, além de permitirem uma interação com a via de sinalização do HT para controlar esses processos. Em conclusão, este estudo mostra que HT interage com o SNS, localmente no esqueleto, via sinalização α_2 adrenérgica, para modular o crescimento linear ósseo.

Palavras chave: Crescimento Ósseo. Hormônio Tireoideano. Sistema Nervoso Simpático.

ABSTRACT

Rodrigues MM. Thyroid Hormone interaction with the sympathetic nervous system, via α_2 adrenoceptor signaling, to regulate endochondral bone growth: and in vitro evaluation. [Ph.D. Thesis (Morphofunctional Sciences)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2017.

It is well known that thyroid hormone (TH) is essential for normal bone growth and development. However, the mechanisms by which TH regulates these processes are poorly understood. Recently, the sympathetic nervous system (SNS) was identified as a potent regulator of bone metabolism. In vivo studies by our group have shown that TH interacts with the SNS to regulate bone mass and structure, and that this interaction involves α_2 adrenoceptor (α_2 -AR) signaling. We have also identified the presence of α_{2A} -, α_{2B} -, and α_{2C} -AR subtypes in the epiphyseal growth plate (EGP) of mice. In addition, we have found that mice with isolated gene deletion of α_{2A} -AR and α_{2C} -AR (α_{2A} -AR^{-/-} and α_{2C} -AR^{-/-}) show a disorganized EGP, shorter long bones and a delay in endochondral ossification (EO). In vivo studies revealed that the EGP of α_{2A} -AR^{-/-} and α_{2C} -AR^{-/-} animals respond differently (than those of wild-type animals), to TH excess and deficiency, which strongly suggests that TH also interacts with the SNS to regulate bone growth and development. Through a long bone organ culture system, the present study had the goal of investigating if TH interacts with the SNS directly in the skeleton, to regulate the longitudinal bone growth and if α_2 -AR is involved in this process. Therefore, we evaluated the linear bone growth of tibias derived from 15.5-day-old WT and α_{2C} -AR^{-/-} mouse embryos (E15.5) for 6 days. We have seen that the KO tibias showed a lower longitudinal growth when compared to WT tibias, and that treatment with 10⁻⁸ M triiodothyronine (T3) significantly decreased the longitudinal growth of the WT tibias, which was not seen in the KO tibias. We found that the treatment with UK 14.304 (UK), a non-selective α_2 -agonist, induced the longitudinal growth only of the KO tibias. The expression of genes related to the terminal differentiation of chondrocytes (Col X, IGF-1, Wnt-4 and Runx2) was shown to be increased in the KO tibias (when compared to the expression in WT tibias). Treatment with T3, as expected, stimulated the expression of these genes in WT tibias, but decreased the expression in KO samples, highlighting the importance of these receptors in the modulation of T3 actions. We observed that the local α_2 -AR activation by UK blocked the expression of these chondrocyte differentiation-related genes, in addition to blocking the positive effects of T3 (UK + T3) in the expression of these genes. These findings show that α_2 adrenoceptors act directly in the skeleton, to control the terminal differentiation of chondrocytes and, therefore, the longitudinal bone growth, in addition to allow an interaction with the TH signaling pathway to control these processes. In conclusion, this study shows that TH interacts with the SNS, locally in the skeleton, via α_2 adrenergic signaling, to modulate the longitudinal bone growth.

Keywords: Longitudinal Bone Growth. Thyroid Hormone. Sympathetic Nervous System.

1 INTRODUCTION

1.1 - Skeletal development

Skeletal development stands for a number of skeletal-related processes that follow from the early days of pregnancy until the skeleton has attained complete development in late puberty. These processes include (i) skeletogenesis, which involves recruitment, migration and condensation of precursor cells to the future skeletal sites, tailed by their differentiation to form the skeletal tissues; (ii) skeletal patterning, which determines bone number and shape; (iii) skeletal growth in length and width, which depends on proliferation, survival and differentiation of chondrogenic and/or osteogenic cells; (iv) and maturation of the skeleton, determined mainly by ossification of a primordial skeleton (1, 2).

Skeletogenesis and bone maturation occur through two processes, endochondral and intramembranous ossification. In both processes, groups of mesenchymal cells aggregate (mesenchymal condensation) and initiate a characteristic genetic program that leads to bone formation. In intramembranous ossification, cells in the mesenchymal condensations differentiate directly into osteoblasts and, consequently, there is bone formation. In endochondral ossification, there is an intermediate stage where a cartilaginous template is formed to be replaced by bone. Thus, the cells in the mesenchymal condensations differentiate into chondrocytes, which differs in a population of centrally located proliferating chondrocytes expressing collagen type II (Col II), and in a population of chondrocytes located more peripherally, which express collagen type I (Col I) (2), thus forming a cartilaginous template.

In response to several signals, the chondrocytes in the central region of the cartilaginous template stop proliferating, change their genetic program to suffer hypertrophy and to synthesize collagen type X (Col X) and other proteins and factors characteristic of hypertrophic chondrocytes (HC), composing a centrally located hypertrophic region in the cartilaginous mold (3). These HCs then signals to the adjacent perichondrial cells to differentiate into osteoblasts, inducing the formation of a bone collar and periosteum around the cartilaginous template. At the same time, HCs mediate the mineralization of the surrounding

extracellular matrix (ECM), release vascular endothelial growth factor (VEGF), which induces angiogenesis, and undergo apoptosis leaving gaps in the cartilaginous matrix. These gaps are invaded by blood capillaries, leading to the vascularization of the avascular cartilaginous mold. With this vascularization, pre-osteoblastic cells invade the hypertrophic region, secrete Col I in the ECM and mediate the formation of bone tissue within the cartilaginous mold, composing the primary spongiosa (consisting of osteo-cartilaginous spicules), which constitutes a primary ossification center (POC). HCs also secrete receptor activator of nuclear factor kappa B ligand (RANKL), a factor that induces osteoclastogenesis, contributing to the recruitment of osteoclast cells into the cartilaginous template (4). Osteoclasts, in turn, digest the matrix synthesized by HCs.

As the ossification process proceeds, the ossification center expands toward the bone ends. In the case of the cartilaginous molds of the long bones, the primary ossification center occurs in the diaphysis and expands towards the epiphyses (5). In the epiphyses, rounded chondrocytes continue to proliferate, and those near the primary ossification center flatten and form columns of proliferating chondrocytes (PCs). These cells stop proliferating and differentiate into HCs, which are located even closer to the primary ossification center. Subsequently, secondary ossification centers are formed in the epiphysis.

The rounded chondrocytes in the center of the ends of the cartilaginous mold (future bone epiphyses) stop dividing, differentiate into HCs, and lead the formation of the secondary ossification centers. Cartilage is maintained at the ends of the bone epiphysis (articular cartilage) and between the primary and secondary ossification centers, establishing an epiphyseal growth plate (EGP), which contains the longitudinal bone growth machinery.

Figure 1 – Schematic diagram of endochondral ossification. (Boundless Biology – Types of Skeletal System, page 2).

The mammalian EGP is composed of three well defined main zones that follow an elegant developmental program: reserve (RZ), proliferative (PZ) and hypertrophic (HZ) zones (6). The RZ, the one closest to the epiphysis, consists of undifferentiated progenitor cells, the reserve or resting chondrocytes (RC). These cells are rounded, relatively small and are dispersed in an ECM rich in type II collagen and proteoglycans. These cells differentiate into proliferating chondrocytes (PC), which are disk-shaped (flattened) cells organized in columns parallel to the longitudinal axis of the long bones (7), composing the PZ. The PCs secrete mainly Col II, the main component of the PZ ECM of the PZ, and differentiate into HCs.

It is worth mentioning that the PZ plays a crucial role in endochondral ossification and linear bone growth, since it is an active site of cell replication in the EGP (8). When the PC divides, the two daughter cells align to the longitudinal axis of the bone (9). This spatial organization directs bone growth and is thus responsible for the elongated shape of various endochondral bones. The HCs also assume a columnar organization in the HZ. These cells, as well as in the primary and secondary ossification centers, secrete Col X and VEGF, among other proteins and factors, and command the endochondral ossification process. Thus, the linear bone growth occurs in the EGP by deposition of cartilage in the epiphysis-diaphysis direction and endochondral ossification of this cartilage in the diaphysis-epiphysis direction (7). Therefore, growth is determined by chondrocyte proliferation, ECM synthesis and chondrocyte

hypertrophy in the EGP. In adolescence, ossification overcomes the deposition of cartilage, resulting in the total ossification of the EGP, which ceases linear bone growth by age of 18 years in women and 21 years in men.

Figure 2 – Schematic representation of the growth plate, showing chondrocytes proliferation, differentiation and apoptosis (Modified from Williams GR, 2017 (10)).

The mechanisms of regulation of endochondral ossification and linear bone growth, including regulation of proliferation, organization, differentiation and apoptosis of chondrocytes, as well as subsequent vascular invasion are still poorly understood. It is known, however, that alterations of these processes can result in numerous diseases such as chondrodysplasias and other developmental disorders, demonstrating the need of a better understanding of the mechanisms, pathways and factors that regulate endochondral ossification and linear bone growth.

1.2 Thyroid hormone

The main secretory products of the thyroid gland are iodothyronines, compounds that result from binding of two molecules of iodinated tyrosine. In mammals, about 60-90% of thyroid production is 3,5,3',5'-tetraiodothyronine (thyroxine or T₄), 10-40% is 3,5,3'-triiodothyronine (T₃) and less of which 1% is represented by other biologically inactive iodothyronines. T₄ basically functions as a prohormone and is converted to the active form of TH, T₃, by the action of cellular enzymes, the iodothyronine deiodinases. This conversion occurs in the thyroid gland itself and, mainly, in the target tissues of TH (11). Three types of iodothyronine deiodinases have been identified in mammalian tissue, type I, II and III (D1, D2 and D3, respectively). D1 and D2 convert T₄ to T₃, therefore, they activate TH. D1 also converts T₄ to reverse T₃ (rT₃; 3,3', 5'-T₃), and rT₃ and T₃ to T₂ (3,3'-T₂). Considering that both rT₃ and T₂ are inactive, D1 is capable of both activating and inactivating iodothyronines (12). D3 catalyzes the degradation of T₄ to rT₃, and T₃ to T₂. Studies of our group showed that the three enzymes are expressed in the skeleton of neonates and young mice, which highlights the importance of these enzymes in pre and postnatal skeletal development (13). The actions of TH are primarily the result of T₃ interaction with its nuclear receptors (TRs), which are T₃-inducible transcription factors. These receptors bind to specific regions of the target genes, the TH responsive elements (TREs), and modify gene expression, increasing or decreasing the rate of gene transcription. There are four classical isoforms of TR encoded by two genes (14), THRA and THRB, located in humans on chromosomes 17 and 3, respectively. THRB encodes TRβ1 and TRβ2, while THRA encodes TRα1 and TRα2. This latter isoform does not bind to T₃ and functions, at least in vitro, as an antagonist of TRα1 and TRβ1 (15). TRα1, TRα2 and TRβ1 are expressed in the major skeletal cells, osteoblasts, osteoclasts and chondrocytes (16, 17). A series of studies has shown that TRα1 is the predominant isoform in the bone tissue (18, 19) and the main TR isoform to mediate TH actions in the skeleton. However, a body of studies showed that TRβ1 also mediates important effects of T₃ on osteoblastic cells of rats and mice (20), and especially, in the skeleton of developing rats (21).

1.2.1 Thyroid Hormone and Bone Development.

Classical studies of more than 40 years have identified TH as a potent regulator of skeletal growth and maturation. TH deficiency in the child is often associated with severe delay in bone maturation, short stature, and mechanical failure of the EGP of both hips (22). In animal models of hypothyroidism, T3 deficiency results in delayed intramembranous and endochondral ossification, together with important alterations in EGP, such as reduction in thickness, disorganization of PC columns, and an important deficiency of the differentiation of PCs into HCs. In addition, hypothyroidism during development significantly limits the acquisition of peak bone mass (21). On the other hand, the increase in TH concentrations may result in accelerated skeletal maturation, premature closure of EGPs and a subsequent decrease in linear bone growth (23).

TH, along with growth hormone (GH), insulin-like growth factors, glucocorticoids, estrogens and androgens represent the main systemic factors that influence skeletal growth and maturation. The first four, during childhood and the last two, during adolescence (24). Before adolescence, TH is considered the main prerequisite for normal skeletal maturation. One of the mechanisms through which TH stimulates bone development is indirectly, increasing the synthesis and secretion of GH and IGF-1 (25), factors that have direct effects on bone tissue. GH and T3 interact to promote longitudinal bone growth and bone maturation. The crosstalk between T3 and GH action pathways occurs at various levels, including the effect of T3 on the regulation of GH gene transcription (26), and the stimulation of IGF-1 expression *in vivo* and *in vitro* (27, 28). Thus, in hypothyroidism, there is a reduction in the serum and tissue levels of GH and IGF-1, which promotes important bone changes. However, there is evidence that TH acts directly in EGP chondrocytes through mechanisms independent of GH.

It has been shown, for example, that GH alone is not able to induce the organization of the columns of chondrocytes, neither to promote the differentiation of PCs in HCs in the EGP of hypothyroid rats (29, 30). Robson et al. (31) have shown that, in primary cultures of the rat tibiae, T3 acts directly inhibiting the proliferation of PCs, while promoting their differentiation into HCs. The presence of TRs in chondrocytes of humans (16), rats (32) and mice

support a direct effect of T3 on skeletal development. In rodents, TR α 1, TR α 2 and TR β 1 isoforms were identified in the reserve, proliferative, and prehypertrophic zones and in the primary spongiosa of the EGP, but were not detected in the HZ (31).

In humans, gene and protein expression of TR α 1, TR α 2 and TR β 1 were identified in reserve, proliferating and hypertrophic chondrocytes of endochondral ossification sites, such as osteophytes, heterotopic bone or osteoclastomas (16, 33). Our group showed that treatment of hypothyroid rats with GC-1, a TR β selective agonist, does not normalize serum IGF-1 levels or IGF-1 expression in the EGP, and does not rescue the growth plate columnar organization. However, GC-1 was able to normalize the differentiation of HCs, the expression of type X collagen and the thickness of the EGP in hypothyroid rats (21). In addition, we showed that GC-1 has positive, though modest, effects on bone growth, bone mass acquisition, and endochondral ossification. These findings strongly suggest that TH modulates skeletal development independently of the GH/IGF-1 axis and that TR β mediates some essential effects of T3 on bone.

Some years ago, two signaling proteins, Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP), were identified as crucial for the regulation of growth plate chondrocyte differentiation (34). Ihh, produced by the prehypertrophic zone of the EGP, stimulates PTHrP production in the perichondrium. PTHrP acts on the PZ, inhibiting cell differentiation and thus maintaining a proliferating state. This negative feedback system has the opposite effect of T3. Interestingly, in rats, it was demonstrated an increase in PTHrP mRNA expression in hypothyroidism and a reduction of PTHrP receptor (PTHrP-R) in thyrotoxicosis (35). These effects correlate with the epiphyseal dysgenesis and growth retardation seen in hypothyroidism, and with the accelerated growth rate seen in thyrotoxicosis, suggesting that important physiological actions of T3 on the EGP are mediated through the Ihh/PTHrP-R feedback system.

In the last decades, the identification of mutations in fibroblast growth factor (FGF) receptors has defined essential roles of the FGF signaling pathway in bone development. For example, there is evidence that the FGF pathway negatively regulates proliferation of EGP chondrocytes (5). Studies show that

T3 stimulates the expression of FGF receptors (FGFR-1 and FGFR-3) and increases the effect of FGF2 on the mitogen-activated protein kinase (MAPK) signaling pathway in ATDC5 chondrogenic cells, osteoblasts and osteocytes (36). In addition, FGFR-3 expression has been shown to be normal in TR β KO mice, but markedly reduced in the EGP of TR α KO mice, which have a phenotype similar to that of hypothyroidism (37). Therefore, these studies suggest that T3 inhibitory effects on the proliferation of EGP chondrocytes are mediated by the FGF pathway and via TR α .

More recent evidence shows that TH also regulates the terminal differentiation of epiphyseal chondrocytes by modulating the Wnt/ β -catenin signaling pathway (38). The Wnt (consists of a family of proteins) signaling pathway plays an important role in the development and maintenance of various organs and tissues, including bone. Although Wnt proteins signal through various pathways to regulate cell growth, differentiation, function, and death, the Wnt/ β -catenin signaling pathway, also known as canonical, appears to be the most important for bone biology (39). If Wnt proteins are not expressed or the binding to their receptors is inhibited, β -catenin degradation is facilitated through their interaction with a protein complex consisting of APC (adenomatous polyposis coli), axin and GSK3 (glycogen synthase kinase 3). APC and axin act as supporting proteins, allowing GSK3 to bind and phosphorylate β -catenin, thereby marking it for ubiquitin/proteasome degradation. Activation of the Wnt/ β -catenin pathway occurs by binding of Wnt proteins to their Frizzled (7-transmembrane domain-spanning frizzled receptors) and LRP5/6 (low-density lipoprotein receptor-related protein 5 and 6) receptors. This binding inhibits the activity of GSK3, through mechanisms involving axin, Frat-1 and Dsh (disheveled) proteins, causing accumulation of β -catenin, which in turn is translocated to the nucleus, where it binds to TCF/LEF (T cell transcription factor / lymphoid enhancer factor) and activates transcription of Wnt responsive genes (39).

Recently, members of the Wnt family have been recognized as key regulators of chondrocyte proliferation and differentiation during limb development. Inactivation of β -catenin in chondrocytes of mouse embryos produces a dwarfing phenotype with decreased chondrocyte proliferation, and delayed differentiation of HCs and endochondral ossification (40).

Overexpression of Wnt-8c or β -catenin induces terminal differentiation of chicken chondrocytes (40) and positively regulates gene expression of Runx2 (an essential gene for osteoblastic differentiation), through the activation of the TCF/LEF binding sites in the promoter of Runx2 (41). Overexpression of ICAT (β -catenin and TCF inhibitor) in mouse cartilage impairs long bone growth, with a delay in chondrocyte differentiation and a delay in the appearance of the secondary ossification centers.

Wang et al. (42) have shown that TH stimulates Wnt-4 expression and Wnt/ β -catenin signaling in the EGP of rats. In addition, they have shown that Wnt antagonists, Frzb/sFRP3 and Dkk1, inhibit T3 activation of the Wnt/ β -catenin pathway, besides inhibiting the T3 effects on the terminal maturation of growth plate chondrocytes. These findings strongly suggest that TH regulates terminal differentiation of EGP chondrocytes, at least in part, through the modulation of the Wnt/ β -catenin pathway. More recently, the same group demonstrated that TH interactions with the Wnt/ β -catenin pathway, to regulate differentiation of EGP chondrocytes, are modulated by the IGF-1/IGF-1R signaling pathway (42). Wang et al (2010)(43) showed that IGF-1/IGF-1R induces Wnt-4 expression and β -catenin activation in EGP chondrocytes. Besides, they showed that IGF-1/IGF-1R has prominent effects on chondrocyte proliferation and modest effects on terminal chondrocyte differentiation, both of which were shown to be partially inhibited by the Wnt antagonists sFRP3 and Dkk1 (Wang et al., 2010 (43)). Interestingly, this study also showed that T3 positively regulated proliferation in addition to induce Wnt-4 expression, β -catenin activation, and terminal differentiation of growth plate chondrocytes, but these effects were partially prevented by the IGF-1R inhibitor picropodophyllin (Wang et al., 2010) (43). These data indicate that the interactions between TH and Wnt/ β -catenin signaling pathways to regulate growth plate chondrocyte proliferation and terminal differentiation involve T3 actions on IGF-1/IGF1R pathways (Wang et al., 2010 (43)).

In summary, it is known that TH, independently of the GH/IGF-1 axis, plays an essential role in endochondral ossification, in the columnar organization of PCs, and in the differentiation of HCs. Due to these effects, TH is essential for normal bone growth and maturation. To date, T3 has been shown to promote some of its effects on skeletal development by modulating

the GH/IGF-I axis; the *Ihh*/PTHrP-R pathway, FGFs and Wnt/ β -catenin. However, a number of T3 mechanisms of action in skeletal development remain to be identified and studied. The identification and study of these mechanisms will contribute to the understanding of how T3 promotes bone maturation and linear bone growth.

1.3 The sympathetic nervous system

The Autonomic Nervous System (ANS) is the efferent component of the Visceral Nervous System, and is divided into Sympathetic Nervous System (SNS) and Parasympathetic Nervous System (PNS). The vast majority of post-ganglionic sympathetic fibers have noradrenaline (NE) as the neurotransmitter and are therefore adrenergic (44). The adrenergic system is an essential regulator of neural, endocrine, cardiovascular, vegetative and metabolic functions. Endogenous catecholamines, adrenaline and NE, transmit their biological signals via G protein-coupled receptors to regulate a variety of cellular functions (45). These receptors are called adrenergic receptors, and it is through them that the SNS performs its actions on the target organs. Most of these receptors are in the plasma membrane of neurons and in neural and non-neural target cells. In the target cells of this system, there are α -adrenergic and/or β -adrenergic receptors. There are 9 subtypes of adrenergic receptors, which are subdivided into α 1 (α 1A, α 1B and α 1D) (46), α 2 (α 2A, α 2B and α 2C) and β (β 1, β 2 and β 3) (47). All nine subtypes of adrenergic receptors are activated by adrenaline and NE. All β -adrenergic receptor isoforms stimulate adenylate cyclase (AC) and, thus, induce cyclic 3',5'-adenosine (cAMP) synthesis (48), which is the second messenger of NE actions mediated by β - adrenergic antagonists. On the other hand, α 2 receptors inhibit AC and, therefore, decrease cAMP formation, antagonizing the β -adrenergic receptor-mediated actions.

α 2-adrenergic receptors (α 2-ARs) are located in the presynaptic membranes of adrenergic neurons (sympathetic endings), where they act as autoreceptors (an autoreceptor is a type of receptor present in the presynaptic membranes of nerve cells, which is only sensitive to the neurotransmitters or hormones released by the neuron on which it is situated), negatively regulating the exocytosis of NE from the sympathetic nerves as part of a negative

feedback loop (49). α 2-ARs are also located in non-adrenergic neurons, where they act as heteroreceptors (a heteroreceptor is sensitive to neurotransmitters and hormones that are not released by the cell on which it is located), modulating the release of other neurotransmitters or hormones, such as serotonin (Mongeau et al, 1998 (50)), GABA (Jackisch et al, 1999 (51)), dopamine (Milan et al, 2000 (52)) and GHRH (53). α 2-ARs, acting as autoreceptors and heteroreceptors, have been described in the central and peripheral nervous systems (54)(Gilsbach, 2011). In addition to these presynaptic neuronal receptors, α 2-ARs have been also identified in many non-neuronal cell types, including blood vessels, myometrium, spleen, adipose tissue and pancreatic islets (46), where they have essential roles, for example, regulating blood pressure and insulin release.

The in vivo activation of α 2-ARs by the endogenous (adrenaline and NE) or synthetic agonists, such as brimonidine, clonidine, dexmedetomidine, moxonidine, medetomidine and rilmenidine (Cambridge, 1981; Kallio et al., 1989; Bylund et al., 1994; Fairbanks et al., 2009), causes diverse effects, including hypotension, bradycardia, analgesia, hypothermia, sedation, hypnosis and anesthetic-sparing (54).

The role of each α 2-AR isoform is not clear yet, because of the lack of drugs with sufficient isoform-selectivity. On the other hand, studies in mice with inactivation of individual α 2-AR subtypes have contributed to dissociate the role of each isoform, revealing that only a few functions are mediated by a single subtype. These studies showed that α 2B-AR has an important role in the development of the placenta (55, 56) and the lung (57), besides regulating vascular tone (58) and mediating some analgesic effects (59). α 2C-AR was identified as the major feedback receptor of adrenaline release from chromaffin cells of the adrenal medulla (60), in addition to cooperated with α 2A-AR in the presynaptic inhibition of NE release. On the other hand, α 2A-AR was identified as the main receptor acting to inhibit the release of NE from sympathetic nerves. It is noteworthy that the majority of the physiological and pharmacological actions of α 2-AR activation seems to be mediated by α 2A-AR subtype (54, 61). Some of these α 2A-AR-mediated actions include insulin release by the pancreatic islets (62), the facilitation of working memory by the prefrontal cortex (63), hypotension, bradycardia and modulation of the

baroreflex sensitivity (MacMillan et al., 1996; Lakhani et al., 1997; Niederhoffer et al., 2004), in addition to sedation and hypnosis (63).

The study of a transgenic mouse model that does not express $\alpha 2C$ -AR globally but expresses $\alpha 2A$ -AR only in adrenergic neurons (autoreceptors) (64) confirmed the role of $\alpha 2A$ -AR as the main autoreceptor to control NE release from the sympathetic nerve terminals and to control spontaneous locomotor activity at night-time (64). More importantly, further studies of this mouse model revealed that the hypotensive effects (bradycardia, hypotension and baroreceptor sensitivity) of $\alpha 2$ -AR agonists are primarily mediated by $\alpha 2A$ -heteroreceptors, whereas $\alpha 2A$ -autoreceptor has only a minor role in mediating these effects. In addition, sedation, anesthetic sparing, hypothermia and analgesia were also shown to be primarily mediated by $\alpha 2A$ -heteroreceptors. Therefore, most of the known effects $\alpha 2$ -AR activation are mediated by $\alpha 2A$ -heteroreceptors.

$\alpha 1$ receptors act mainly on the regulation of vascular tone and cardiac development, besides participating in behavior modulation. These $\alpha 1$ receptors are located in the CNS, right atrium, ventricle, liver, spleen, ileum, parotid glands, nasal mucosa, bladder, urethra, cavernous body and prostate glands and veins of various tissues (46, 65).

1.3.1 Sympathetic nervous system actions in the skeleton

An important finding of the recent years is that bone remodeling is also under control of the central nervous system (CNS), with the SNS acting as the peripheral effector via β_2 -adrenoceptors (β_2 -AR) expressed in osteoblasts(66, 67). It was shown that the SNS inhibits bone formation and stimulates bone resorption (68, 69). Therefore, the SNS activation has osteopenic effects.

The role of the SNS in bone remodeling was supported by a high bone mass (HBM) phenotype in mouse models of low sympathetic activity: Ob/Ob mice, which are deficient in leptin; and mice with gene inactivation of dopamine β -hydroxylase ($DBH^{-/-}$), the rate-limiting enzyme responsible for the synthesis of catecholamines (69). These mouse models, however, have endocrine dysfunctions including hypercorticism, hyperinsulinemia and hypogonadism, which can interfere with the SNS effects on bone tissue. A more specific role of β_2 -AR in bone mass was shown by the analyzes of animals with gene

inactivation of this receptor, the β_2 -AR^{-/-} mice, which have no endocrine or metabolic abnormalities. These animals present normal body weight, but also exhibit a HBM phenotype, due to an increase in bone formation and decrease in bone resorption (70). In addition, these mice are resistant to bone loss induced by ovariectomy.

Subsequently, a study of our group analyzed the skeletal phenotype of a mouse model with chronic sympathetic hyperactivity (47), due to the double gene deletion of α_2 A and α_2 C adrenergic receptors (α_2 A/ α_2 C-AR^{-/-}). As discussed before in this introduction (section 3.1), α_2 -ARs are presynaptic autoreceptors that negatively regulate the release of catecholamines, especially norepinephrine (NE). Therefore, activation of these receptors in the brain stem decreases the sympathetic tone, which results in decreased heart rate and blood pressure(48). These effects are enhanced by stimulation of α_2 -ARs in sympathetic nerve terminals. Hein et al (71) showed that simultaneous blockade of α_2 A-AR and α_2 C-AR in mice leads to a chronic elevation of sympathetic tone.

These knockout (KO) mice have high mortality and decreased heart function after 4 months of age (71). As expected, we observed that α_2 A/ α_2 C-AR^{-/-} mice present high plasma levels of NE and cardiac hypertrophy, which confirms the hyperactivity of the SNS. Considering the evidence that the activation of the SNS has negative effects on bone mass, our expectation was that α_2 A/ α_2 C-AR^{-/-} mice would present an osteopenic skeleton. Surprisingly, we found that these animals show a widespread phenotype of HBM, a better quality in bone microarchitecture and greater bone resistance to mechanical stress (46). By immunohistochemistry, we identified α_2 A-AR, α_2 B-AR and α_2 C-AR expression in osteoblasts, osteocytes and osteoclasts. In addition, these receptors were also detected in the EGP, specifically in chondrocytes of the reserve and hypertrophic zones, and in hypertrophic chondrocytes of the secondary ossification centers. The fact that α_2 A/ α_2 C-AR^{-/-} animals present HBM phenotype, even with an increase in sympathetic tone and normal β_2 -AR expression, shows that β_2 -AR is not the only adrenoceptor involved in bone metabolism control, and that α_2 A-AR and/or α_2 C-AR may play an important role in mediating the actions of the SNS in bone mass regulation. Interestingly, we found that mice with single inactivation of α_2 C-AR (α_2 C-AR^{-/-}) present lower

trabecular bone volume (BV/TV) and number (Tb.N), and increased trabecular separation (Tb.Sp) in the femur compared with WT mice; which was accompanied by decreased bone strength in the femur and tibia. The contrary was found in the vertebra, where $\alpha 2C\text{-AR}^{-/-}$ mice show higher BV/TV, Tb.N and trabecular thickness (Tb.Th), and decreased Tb.Sp, compared with WT animals. These findings suggest that $\alpha 2C\text{-AR}$ subtype mediates the effects of the SNS in the bone in a skeletal site-dependent manner (72).

The presence of α_2 adrenergic receptors in the EGP and HC of the secondary ossification centers raised the hypothesis that the SNS also has a role in the longitudinal bone growth and skeleton ossification. Corroborating this hypothesis, recent studies from our laboratory showed that adult mice with isolated $\alpha 2A\text{-AR}$ or $\alpha 2C\text{-AR}$ gene inactivation present shorter femurs and tibias (FAPESP Projects number: 2010/06409-0, 2010/50068-2 and 2010/04911-10). Further studies of our group (FAPESP Project number 2013/02247-3 and 2012/11858-3) showed that the EGP morphology of $\alpha 2A\text{-AR}^{-/-}$ and $\alpha 2C\text{-AR}^{-/-}$ animals present several modifications, when compared with WT mice. The growth plate of $\alpha 2C\text{-AR}^{-/-}$ animals showed a structural disorganization in the columns of PCs and a significant increase (30%) in the number of HCs. The single KO mice for $\alpha 2A\text{-AR}$ ($\alpha 2A\text{-AR}^{-/-}$) showed a more evident disorganization in the PZ than $\alpha 2C\text{-AR}^{-/-}$ animals. On the other hand, $\alpha 2A\text{-AR}^{-/-}$ mice showed a reduction in the number of HCs. Altogether, these findings support the hypothesis that SNS regulates the morphology and physiology of the EGP, as well as the longitudinal bone growth. Also, they suggest that the SNS actions in these processes are, at least partially, modulated by α_2 adrenoceptors. On the other hand, these findings raise an important question: Do these SNS actions take place directly in the skeleton or are they indirect, i.e. depend on the SNS action in other tissues, organs and systems?

1.4 Evidence that thyroid hormone interacts with the SNS, via α_2 adrenergic receptors, to regulate bone mass and bone metabolism.

Thyroid Hormone (TH) modulates bone remodeling and bone mass, besides controlling bone development. Triiodothyronine (T3) stimulates bone formation and resorption by regulating the activity of osteoblasts and

osteoclasts (73). TH excess stimulates more osteoclastic than osteoblastic activity, which results in increased calcemia and decreased bone mass.

An important characteristic of TH, but poorly understood, is its interaction with the SNS. It is well known that a synergism between catecholamine and TH is required for the maximum thermogenesis, lipolysis, glyconeogenesis and glycogenolysis (74). Previous studies have shown that patients with hyperthyroidism have increased sympathetic tone (75).

Considering that TH excess causes bone loss and the SNS activation also has a negative effect on bone mass, we hypothesized that TH also interacts with the SNS to regulate bone metabolism and, consequently, bone mass. One evidence of this possible interaction is the fact that treatment of hyperthyroid patients with propranolol (a β -adrenergic antagonist) corrects the thyrotoxicosis-induced hypercalcemia (76). Furthermore, patients with hypothyroidism treated with propranolol showed a decrease in the urinary excretion of hydroxyproline, a biochemical marker for bone resorption (77).

To investigate this hypothesis, 40 day- old WT and $\alpha 2A/\alpha 2C\text{-AR}^{-/-}$ female mice were treated with approximately 10 times the physiological dose of T3 ($10\times T3 \mu=3.5 \text{ g}/100\text{g BW}/\text{day}$) or saline during 12 weeks (78). As expected, T3 treatment resulted in a generalized decrease in the bone mineral density (BMD) of WT mice (determined by DEXA), followed by deleterious effects on the trabecular and cortical bone microstructural parameters (determined by μCT) of the femur and vertebra. In addition, this treatment also had deleterious effects on the biomechanical properties (maximum load, ultimate load, and stiffness) of the femur. Surprisingly, $\alpha 2A/\alpha 2C\text{-AR}^{-/-}$ mice showed to be resistant to most of these T3-induced negative effects. Interestingly, the mRNA expression of osteoprotegerin (OPG), a protein that limits osteoclast activity, was upregulated and downregulated by T3 in the bone of KO and WT animals, respectively. $\beta 1\text{-AR}$ mRNA expression and IGF-I serum levels, which exert bone anabolic effects, were increased by T3 treatment only in $\alpha 2A/\alpha 2C\text{-AR}^{-/-}$ mice. In vitro, T3 inhibited the cell growth of calvarias-derived osteoblasts isolated from WT mice, but this effect was abolished or reverted in cells isolated from KO mice. Altogether, these findings support the hypothesis of a TH-SNS interaction to control bone mass and structure of young adult mice and suggests that this interaction may involve $\alpha 2\text{-AR}$ signaling. Mice with single inactivation of $\alpha 2C\text{-AR}$

($\alpha 2C\text{-AR}^{-/-}$) also showed resistance to the detrimental effects of thyrotoxicosis on bone mass, architecture, and biomechanical properties in the femur and tibia, suggesting that thyrotoxicosis depends on $\alpha 2C\text{-AR}$ signaling to promote bone loss, which sustains the hypothesis of a TH-SNS interaction to modulate bone remodeling and structure.

As previously mentioned, studies from our laboratory (FAPESP Project numbers: 2010/06409-0, 2010/04911-10 and 2010/50068-2) showed that adult mice with isolated $\alpha 2A\text{-AR}$ or $\alpha 2C\text{-AR}$ inactivation present shorter femurs and tibias. More importantly, these studies have shown that treatment for 30 or 90 days with supraphysiological doses of T3 impairs the linear growth of the femur and tibia of WT mice, but not of $\alpha 2A\text{-AR}^{-/-}$ or $\alpha 2C\text{-AR}^{-/-}$ mice. These findings, along with the fact that the expression of these receptors was detected in the EGP (46), strongly suggest an interaction of TH with the SNS via $\alpha 2A\text{-AR}^{-/-}$ and / or $\alpha 2C\text{-AR}^{-/-}$ to control longitudinal bone growth.

To confirm this hypothesis, we performed a detailed evaluation of the effects of TH deficiency and excess on the EGP of $\alpha 2A\text{-AR}^{-/-}$ and $\alpha 2C\text{-AR}^{-/-}$ mice (FAPESP project numbers 2013/02247-3 and 2012/11858-3). As expected, in WT mice, hypothyroidism (hypo) promoted significant reductions on bone longitudinal length, disorganization of the PZ and a reduced number of HC. Surprisingly, in $\alpha 2C\text{-AR}^{-/-}$ animals, the effects of hypo were quite different than those observed in WT animals. The euthyroid $\alpha 2C\text{-AR}^{-/-}$ mice presented a disorganized PZ and an increase in the number of HC. In $\alpha 2C\text{-AR}^{-/-}$ animals, hypo was able to revert, at least partially, the disorganization of the PZ, and resulted in an increase in the number of HC. Hyperthyroidism (hyper) led to an increase in the number of HC in WT animals and a reduction in $\alpha 2C\text{-AR}^{-/-}$ animals. The EGP responses of $\alpha 2A\text{-AR}^{-/-}$ mice to hypo and hyper status were also surprising. Hypo had no effect on the EGP of $\alpha 2A\text{-AR}^{-/-}$ animals, on the other hand, hyper led to an increase in the RZ thickness, which was not seen in WT animals. These findings demonstrate that the SNS regulates the EGP morphology and the longitudinal bone growth. They also show that the integrity of the SNS, especially of the α_2 adrenergic pathways, is necessary for TH to promote its normal actions in the growth plate.

It is important to consider, however, that the studies described above were conducted *in vivo* with global KO models of α_2 adrenoceptors ($\alpha 2A/\alpha 2C$ -

AR^{-/-}, α_{2A}-AR^{-/-} and α_{2C}-AR^{-/-} mice), which provide important information, but, at the same time, present limitations. An important limitation of *in vivo* studies, using global KOs, is the difficulty of discriminating direct effects (local) or the role of certain genes. Therefore, the findings described above do not allow us to elucidate whether (i) the actions of the SNS are direct (skeletal); (ii) if α₂ adrenergic receptors mediate the SNS actions locally in the skeleton; (ii) if the TH-SNS interaction occurs locally in the skeleton; and finally, (iv) if α₂ adrenergic receptors mediate local interaction of TH with the SNS.

In vitro studies using bone organ cultures and primary cultures of chondrocytes, derived from bone epiphyses, are valuable tools to investigate these questions.

1.5 Clinical relevance of the SNS actions, via α₂adrenergic Receptors, in the Skeleton.

Since the skeletal effects of α₂-AR activation are only now been identified, there is no information in the literature regarding positive or adverse effects of α₂-AR agonists in the skeleton. On the other hand, α₂-AR agonists have been in clinical use for decades, to manage a variety of therapeutic conditions (59, 79). These agonists have been primarily used in the treatment of hypertension, because of the well-known hypotensive effects of α₂-AR activation (bradycardia, hypotension, and baroreceptor sensitivity), however, their side effects, including sedation, drowsiness and dry mouth, have limited their prescription to special hypertension conditions (80, 81).

Other important applications occur particularly in the field of anesthesia and pain management, due to the capacity of α₂-AR agonists to enhance the effects of traditional anesthetics. Thus, these agonists have been used as sedatives and to reduce the need for inhalative anesthetics during surgical or invasive procedures (82). They have also been used to lower the sympathetic tone during cardiac surgery (83). Studies have shown that premedication with α₂-AR agonists prevents postoperative cardiovascular complications (84) and lowers mortality (85). On the other hand, limiting side effects for these indications are the profound bradycardic and hypotensive effects of α₂-AR activation. Another use of α₂-AR agonists is its intravenous administration after

induction of anesthesia to reduce the incidence, severity, and duration of postoperative shivering (86).

Clinical trials have tested the use of moxonidine, one of the α_2 -AR agonists, to treat chronic heart failure. This drug was effective to lower plasma catecholamine in patients with chronic heart failure (87), however, it caused adverse effects and even increased the mortality in chronic heart failure. Some of these side effects are probably triggered by α_2 -heteroreceptor activity, which includes augmentation of vagal tonus, a crucial factor for the hypotensive and bradycardic responses, and for the increased baroreceptor sensitivity (88, 89). Considering these negative results (90), the use of α_2 -AR agonists for the treatment of cardiovascular diseases is very limited.

In the ophthalmic practice, α_2 -AR agonists are used to prevent intra-ocular hypertension during laser surgery of the eye and as a daily medication for the treatment of glaucoma. Of note, the chronical use of these agonists has been proved to be useful for the treatment of chronic pain, including neuropathic pain in cancer patients; and for the treatment of muscle spasticity, opiate and alcohol withdrawal, and for some behavior disorders, such as insomnia, Tourette syndrome, tics, and attention-deficit hyperactivity disorder (ADHD) (59).

It is noteworthy that most of these disorders may be present in childhood and adolescence, and that the chronic treatment of these disorders with α_2 -AR agonists seems to be increasing. ADHD, for example, is one of the most common behavior disorders in children (3-5 % of all children), that persist into adolescence and adulthood in most of the cases. ADHD is marked by a pattern of inattention, hyperactivity, and impulsivity, which may result in learning disabilities and poor school performance. The pharmacological treatment of ADHD normally uses central nervous system stimulants, such as d-amphetamine and methylphenidate. These drugs increase the ability to focus and decrease the hyperactivity in most patients. However, some individuals (10 to 30%) are unresponsive to these drugs or do not stand their side-effects. Alternative treatments for these patients include α_2 -AR agonists, such as clonidine (91). Many trials have supported the effectiveness of clonidine in the treatment of ADHD, as a monotherapy and specially as an adjuvant to stimulant-based therapy (92). Its combined use with methylphenidate has also

been shown to be effective to treat sleep disturbances, aggressive behavior and tics associated to ADHD (93, 94), allowing the doses of stimulant to be reduced. As expected, the safety of this therapy has focused on the known side effects of α 2-AR activation, especially the cardiovascular effects.

Considering the emerging roles of α 2-AR signaling to control bone mass and linear bone growth, skeletal side effects of chronic therapies with α 2-AR agonists should be considered, especially during childhood and adolescence. Therefore, the elucidation of the roles and mechanisms of action of α 2-AR signaling in the skeletal are extremely relevant at this point.

2 CONCLUSION

In conclusion, the present study shows that α 2-AR signaling has local actions in epiphyseal chondrocytes to modulate the growth plate morphology and the linear bone growth. In addition, this study shows that TH interacts with the SNS, locally in the skeleton, via α 2-AR signaling, to regulate bone development.

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