Bone 43 (2008) 921–930

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Deiodinase-mediated thyroid hormone inactivation minimizes thyroid hormone signaling in the early development of fetal skeleton

Luciane P. Capelo [a], Eduardo H. Beber [b], Stephen A. Huang [c], Telma M.T. Zorn [a], Antonio C. Bianco [d], Cecília H.A. Gouveia [a,b,*]

[a] Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, 05508-000, Brazil
[b] Department of Anatomy, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, 05508-000, Brazil
[c] Division of Endocrinology, Children's Hospital Boston, Boston, Massachusetts 02115, USA
[d] Thyroid Section, Division of Endocrinology, Diabetes and Hypertension, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA

ARTICLE INFO

Article history:
Received 1 April 2008
Revised 24 June 2008
Accepted 28 June 2008
Available online 17 July 2008

Edited by: T. Jack Martin

Keywords:
Skeletal development
Congenital hypothyroidism
Deiodinase type 3
Deiodinase type 2
Thyroid hormone

ABSTRACT

Thyroid hormone (TH) plays a key role on post-natal bone development and metabolism, while its relevance during fetal bone development is uncertain. To study this, pregnant mice were made hypothyroid and fetuses harvested at embryonic days (E) 12.5, 14.5, 16.5 and 18.5. Despite a marked reduction in fetal tissue concentration of both T4 and T3, bone development, as assessed at the distal epiphyseal growth plate of the femur and vertebra, was largely preserved up to E16.5. Only at E18.5, the hypothyroid fetuses exhibited a reduction in femoral type I and type X collagen and osteocalcin mRNA levels, in the length and area of the proliferative and hypertrophic zones, in the number of chondrocytes per proliferative column, and in the number of hypertrophic chondrocytes, in addition to a slight delay in endochondral and intramembranous ossification. This suggests that up to E16.5, thyroid hormone signaling in bone is kept to a minimum. In fact, measuring the expression level of the activating and inactivating iodothyronine deiodinases (D2 and D3) helped understand how this is achieved. D3 mRNA was readily detected as early as E14.5 and its expression decreased markedly (~10-fold) at E18.5, and even more at 14 days after birth (P14). In contrast, D2 mRNA expression increased significantly by E18.5 and markedly (~2.5-fold) by P14. The reciprocal expression levels of D2 and D3 genes during early bone development along with the absence of a hypothyroidism-induced bone phenotype at this time suggest that coordinated reciprocal deiodinase expression keeps thyroid hormone signaling in bone to very low levels at this early stage of bone development.

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Introduction

Thyroid hormone (TH) is essential for a number of metabolic and developmental processes. Its actions occur through binding of triiodothyronine (T3), the main active form of TH, to its nuclear receptors (TRs), which function as hormone inducible transcriptional factors [1]. Inappropriate levels or premature exposure of embryos to TH are deleterious, resulting in abnormal development or death [2–5]. TH is classically known to be important for the development of the central nervous system, where its deficiency promotes severe neurological deficits [6]. In similar ways, bone development, maturation, growth and metabolism have been shown as fundamentally dependent on TH action. The identification of TRα1, TRα2 and TRβ1 in osteoblasts [7–10], osteoclasts [11,12] and chondrocytes [12,13], as well as the responsiveness of bone cells to TH when isolated in cell culture systems are evidence of a direct effect of T3 on bone tissue. Decreased availability of TH during post-natal development results in severe abnormalities in the epiphyseal growth plates (EGP), such as disorganization of the proliferative zone, impaired differentiation of hypertrophic chondrocytes and abnormal synthesis of extra cellular matrix [14–17], resulting in growth arrest and other abnormalities [16,18]. In addition, a generalized delay in endochondral and intramembranous ossification is observed [5,17,19].

The importance of TH to the pre-natal development of the skeleton is less clear. It is well established that during development serum thyroid hormone levels are low and tissue concentration of thyroid hormone can be modified by the iodothyronine deiodinases [20–22]. While the type II deiodinase (D2) activates T4 to T3, the type III deiodinase (D3) inactivates T4 to rT3 and T3 to T2 in a tissue- and time-specific fashion. In various developing structures such as the brain, cochlea, retina, and during tadpole metamorphosis, D2- and D3-controlled thyroid hormone signaling was shown to play an important role [6,20,22,23]. In bone related structures, D2 expression has been shown in multiple bone-derived cell lines such as primary calvaria osteoblastic cells, murine bone marrow-derived stromal ST2 cell line that has the phenotypes of osteoblast- and osteoclast-

* Corresponding author. Department of Anatomy, Institute of Biomedical Sciences, Av. Professor Lineu Prestes, 2415, Sao Paulo, SP 05508-0, Brazil. Fax: +55 11 3091 7366.
E-mail address: cgouveia@usp.br (C.H.A. Gouveia).

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doi:10.1016/j.bone.2008.06.020

supporting cells [24], in the MC3T3-E1 mouse osteoblastic cell line, in organ cultures of fetal mouse tibias and ATDC5 cells, a mouse chondrogenic cell line [25,26]. In the developing chicken growth plate, Sonic Hedgehog (Shh)-induced ubiquitin-mediated D2 inactivation along with Dio3 induction creates a relative state of local hypothyroidism and PTHrP secretion that favors chondrocyte proliferation and delays chondrocyte differentiation [27]. A similar scenario was recently described in the skin, with Shh and the deiodinases playing a role in the balance between proliferation and differentiation of keratinocytes [28].

In the present study, we report that marked TH deficiency only minimally affects bone development until 16.5 day of embryonic life (E), indicating that TH signaling in skeleton is minimal up to this developmental stage. Accordingly, D3 expression is high at E14.5 and decreases markedly (~10-fold) subsequently while D2 expression profile is the opposite. The higher expression of D3 and the modest effects of TH deficiency in the skeleton until E16.5 suggest that maintaining low levels of TH is critical for early skeleton development.

Materials and methods

Animals and treatment

All experiments were performed under a protocol approved by the Committee of Animal Ethics of the Institute of Biomedical Sciences of the University of Sao Paulo (protocol number 018-29/02). Two-month old mice were obtained from our breeding colony and maintained under controlled conditions of light and temperature (12 h/12 h dark/light cycle at 25 °C), and access to food and water was *ad libitum*. For all experiments, mice were bred in-house for 3 h at night and the presence of a vaginal plug was considered as day zero of embryonic development (E0). A total of 65 dams were randomly divided into 2 groups: euthyroid (EUT) and hypothyroid (HYPO). Hypothyroidism was induced by maintaining the pregnant animals on methimazole (MMI; 0,05%) from E1 to E5 and MMI (0,1%) and sodium perchlorate (P; 1%) from E6 to the day of sacrifice. Both drugs were added to the drinking water. The pregnant mice were anesthetized, bled and the fetuses were obtained at E12.5, E14.5, E16.5 and E18.5. Fetuses were dissected out from the uterus, washed in sterile PBS and placed on ice. Length and weight of the fetuses were measured using a digital calliper and an analytic balance, respectively. Some fetuses were frozen in liquid nitrogen and kept at −80 °C. Others were fixed in 95% ethanol or 10% formalin for morphological analysis. Care was taken to avoid the risk of contamination by maternal blood. The post-natal studies were performed at 2, 4, 7, 14, 21 and 35 post-natal days (P). After birth, the litter was maintained with the mother in the same cage until the day of sacrifice. The neonatal hypothyroidism was also induced by adding MMI (0,1%) and P (1%) in the drinking water, as both drugs are secreted in the maternal milk.

Determination of T4 and T3 in plasma and tissues

We measured the fetal concentration of T4 and T3 before and after the onset of fetal thyroid activity in E12.5, E14.5, E16.5 and E18.5 fetuses from EUT and HYPO dams by radioimmunoassay (RIA). At the day of sacrifice, maternal blood was collected and the serum was separated by centrifugation and immediately frozen. The protocol used to access total T3 and T4 on fetal tissues was modified from Obregón et al. [29]. Briefly, the whole fetuses were crushed in a steel mortar and pestle set (Fisher Scientific International, Inc, Hampton, NH) precooled in dry ice. The crushed fetuses were transferred to pre-weighted microfuge tubes precooled in ice and 0.15 M NaCl/10^{-3} M PTU was added in a volume that doubled the sample weight. 10^{-3} M PTU was added to avoid artifactual deiodinations. The samples were homogenized, kept on ice (15 min) and centrifuged (30 min 2000 rpm, 4 °C). Supernatant aliquots were dried in a Speedvac® at 45 °C (Eppendorf, Hamburg, Germany) and resuspended in 80 μl (E12.5 and E14.5) or 200 μl (E16.5 and E18.5) of 0.15 M NaCl (fetus suspension). In a second set of experiments, animals on P2, P4, P7, P14, P21 and P35 from euthyroid and hypothyroid groups were killed, and the blood was collected and centrifuged for serum separation. The serum and fetus suspension was assayed for total T3 and T4 quantification. Total T4 and T3 levels were measured by commercial RIA kits (RIA-gnost T4 and RIA-gnost T3; CIS bio international, Schering AG, Germany).

Skeletal preparations and histology

Fetuses on E12.5, E14.5, E16.5 and E18.5 and mice on P2, P4, P7, P14, P21 and P35 from EUT and HYPO groups were measured and weighted immediately after euthanasia. For histological and whole mount preparations, mice were eviscerated and had the skin removed. After a 48 h fixation in 95% ethanol, specimens were stained with alcian blue for cartilage and alizarin red for calcified tissue as previously described [16]. For histological analysis, the samples were fixed in 10% formalin for 24 h–72 h at 4 °C. Fetuses on E18.5 and all the newborns were demineralized in 10% formalin and 10% formic acid solution at room temperature for 48 h. Samples were dehydrated and embedded in Paraplast (Oxford, St Louis, MO, USA). The fetuses of all ages studied were sectioned sagittally for analysis of vertebral column morphology. The vertebral column and right femur of five animals of each group were serially sectioned (5 μm) onto Poly-L-Lysine (EMS, Hatfield, PA, USA) coated slides, deparaffinized in xylene, rehydrated, and stained with alcian blue 8GX (pH 2,5), floxin and hematoxylin. The sections were photographed under light microscopy for analyses. Femurs were analyzed morphometrically. Morphometric measurements were performed using the image analyzer system Image-pro Plus (Media Cybernetics, Silver Spring, MD, USA).

Semi-quantitative real-time PCR

Under a stereomicroscope, femurs were dissected and then crushed in a steel mortar and pestle set (Fisher Scientific International, Inc, Hampton, NH) precooled in dry ice. The crushed bones were transferred to microfuge tubes precooled in ice and total RNA was extracted using Trizol (Invitrogen, Calbard, CA, USA) following the manufacturer's instructions. Reverse transcription was performed with RevertAid-H-Minus M-MuLV Reverse Transcriptase (Fermentas, Hanover, MD, USA) and 1 μg of total RNA. The mRNA expression of TRα1, TRβ1, type X collagen (Col10), type I collagen (Col1), osteocalcin (OC), D1, D2 and D3 were determined by semi-quantitative real-time PCR as described previously [30,31]. mRNA levels of 18SrRNA (18S) and cyclophilin A (Cyclo A) were used as internal controls. The relative levels of mRNA of the tested genes were estimated by real-time PCR by measuring the fluorescence quantified with the ABI Prism 7500 sequence detector (Applied Biosystems) comparing all samples and controls in duplicates. The real-time PCR reactions were performed in a total volume of 25 μl containing different amounts of template and primers, depending on the gene evaluated. D2 and D3 relative expression analysis was performed using 50 ng of template and 300 nM of specific primers. For all the other genes evaluated in this study 20 ng of template and 450 nM of specific primers were used. All primers were designed with the aim of the Primer Express software (Invitrogen) and were synthesized (Integrated DNA Technologies, Coralville, IA, USA) with annealing temperature between 58 and 60 °C, 18 and 30 base pairs and keeping a percentage of 40–60% of G and C bases. The PCR amplifications were performed in duplicates with cDNA diluted in a reaction buffer containing SYBR Green PCR master mix (Applied Biosystems) and specific primers (forward and reverse) in the following cycling conditions: 1 cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All C_t values were normalized using an internal control (18S or Cyclo A mRNA) and the results were expressed as fold-induction relative to

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