Bone 43 (2008) 440–451

Contents lists available at ScienceDirect

Bone

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

Targeting of androgen receptor in bone reveals a lack of androgen anabolic action and inhibition of osteogenesis
A model for compartment-specific androgen action in the skeleton

Kristine M. Wiren [a,b,c,*], Anthony A. Semirale [a,c], Xiao-Wei Zhang [a,c], Adrian Woo [d], Steven M. Tommasini [d], Christopher Price [d], Mitchell B. Schaffler [d], Karl J. Jepsen [d]

[a] Bone and Mineral Research Unit, Portland Veterans Affairs Medical Center, Portland, Oregon, USA
[b] Department of Medicine, Oregon Health and Science University, Portland, Oregon, USA
[c] Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, Oregon, USA
[d] Department of Orthopaedics, Mt. Sinai School of Medicine, New York, New York, USA

ARTICLE INFO

Article history:
Received 12 January 2008
Revised 16 April 2008
Accepted 23 April 2008
Available online 16 May 2008

Edited by: J. Aubin

Keywords:
Androgen receptor
Osteocyte
Bone histomorphometry
Biomechanics
Dual energy X-ray absorptiometry

ABSTRACT

Androgens are anabolic hormones that affect many tissues, including bone. However, an anabolic effect of androgen treatment on bone in eugonadal subjects has not been observed and clinical trials have been disappointing. The androgen receptor (AR) mediates biological responses to androgens. In bone tissue, both AR and the estrogen receptor (ER) are expressed. Since androgens can be converted into estrogen, the specific role of the AR in maintenance of skeletal homoeostasis remains controversial. The goal of this study was to use skeletally targeted overexpression of AR in differentiated osteoblasts as a means of elucidating the specific role(s) for AR transactivation in the mature bone compartment. Transgenic mice overexpressing AR under the control of the 2.3-kb α1(I)-collagen promoter fragment showed no difference in body composition, testosterone, or 17ß-estradiol levels. However, transgenic males have reduced serum osteocalcin, CTx and TRAPC5b levels, and a bone phenotype was observed. In cortical bone, high-resolution micro-computed tomography revealed no difference in periosteal perimeter but a significant reduction in cortical bone area due to an enlarged marrow cavity. Endocortical bone formation rate was also significantly inhibited. Biomechanical analyses showed decreased whole bone strength and quality, with significant reductions in all parameters tested. Trabecular morphology was altered, with increased bone volume comprised of more trabeculae that were closer together but not thicker. Expression of genes involved in bone formation and bone resorption was significantly reduced. The consequences of androgen action are compartment-specific; anabolic effects are exhibited exclusively at periosteal surfaces, but in mature osteoblasts androgens inhibited osteogenesis with detrimental effects on matrix quality, bone fragility and whole bone strength. Thus, the present data demonstrate that enhanced androgen signaling targeted to bone results in low bone turnover and inhibition of bone formation by differentiated osteoblasts. These results indicate that direct androgen action in mature osteoblasts is not anabolic, and raise concerns regarding anabolic steroid abuse in the developing skeleton or high-dose treatment in eugonadal adults.

Published by Elsevier Inc.

Introduction

Androgens are steroids that are generally characterized as anabolic hormones, with effects on many tissues including the brain, the immune system, the cardiovasculature, muscle, adipose tissue, liver and bone. Given the large increase in drug sales for testosterone (the major androgen metabolite), estimated at over 500% in the last fifteen years, analysis of the biological consequences of androgen signaling should receive considerable research attention. However, the specific effects of androgen on the skeleton remain poorly characterized and understudied. Since osteoporosis is often coupled with a hypogonadal state in both men and women, sex steroids are implicated in the maintenance of skeletal health. Although both estrogen and androgen circulate in men and women, albeit at different levels, the influence of each on the remodeling skeleton is distinct [34,55]. Consistent with this, combination therapy with both estrogen and androgen provides an improved response in post-menopausal women compared to estrogen alone [2,47]. Estrogens are thought to act to maintain adult bone mass predominantly through an inhibition of bone resorption by the osteoclast, *i.e.* as anti-resorptive agents, which protects the skeleton from further loss of bone. Non-aromatizable androgens such as 5α-dihydrotestosterone (DHT), on the other hand, have been proposed as possible bone anabolic agents that increase bone formation and bone mass [30,35].

In support of an anabolic effect of androgen on the skeleton, free testosterone concentrations have been shown to correlate with bone mineral density (BMD) in elderly men [56], however testosterone levels

* Corresponding author. Portland VA Medical Center P3-R&D39, 3710 SW Veterans Hospital Road, Portland, Oregon 97239, USA. Fax: +1 503 273 5351.
E-mail address: wirenk@ohsu.edu (K.M. Wiren).

8756-3282/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.bone.2008.04.026

also correlated with muscle mass and strength. Testosterone treatment is effective at ameliorating bone loss during aging, but only in men with low testosterone levels [8,61]. Conversely, men undergoing androgen deprivation therapy for prostate cancer show significantly decreased BMD [49] and an increase in clinical fractures [32]. During growth, there are gender differences in skeletal morphology that develop with puberty particularly in cortical bone, with radial expansion that is predominantly observed in boys [31]. Combined, these findings suggest that androgens serve important functions to both maintain bone mass in the adult and to influence the growing, modeling skeleton (see[63]).

Nevertheless, a controversy exists regarding the consequences and/or importance of androgen signaling on skeletal homeostasis. Whether the observed effects of circulating testosterone are due to direct effects on bone is complicated by the fact that androgens influence a variety of tissues known to be associated with bone health, most importantly muscle. Nonetheless, bone is a direct target tissue with respect to androgen action. AR is expressed in the cell types required for skeletal formation and homeostasis, including mesenchymal stromal precursors [4], osteoblasts [1], osteocytes [1,64] and osteoclasts [57]. An additional complication for interpretation of the direct effects of testosterone results from the consequences of its metabolism. Since testosterone serves as the substrate for estradiol synthesis through the action of the enzyme aromatase, systemic testosterone may have effects mediated predominantly or exclusively through activation of estrogen receptor (ER) signaling. Therefore, a specific role for AR signaling cannot be inferred with simple testosterone therapy.

In addition, not all of the studies examining the association of testosterone levels with BMD in adults have actually shown a positive correlation. In general, correlations between bone mass and serum androgen concentrations in adult men have been either weak or insignificant [17,42,48]. Furthermore, many of the various clinical trials examining androgen therapy have been unable to demonstrate robust effects on bone mass, including treatment with anabolic steroids [11]. In most studies that do show an increase in BMD, the most marked improvement is observed only in men with the lowest testosterone levels [61]. Notably, an anabolic effect of androgen treatment on bone in eugonadal men (or in women) has not been observed, in contrast to known anabolic dose-dependent effects to increase muscle mass [5]. For these reasons and because of concerns about safety, androgen replacement even in hypogonadal men remains a controversial issue [20]. Given the modest therapeutic benefit observed with androgen therapy [33], speculation has arisen that a portion of the positive effect of androgens on bone mass may be mediated indirectly through known effects to increase muscle mass and strength. An increase in lean mass would have beneficial effects on BMD though biomechanical linkage and skeletal adaptation. Consistent with this suggestion, Murphy et al [39] have shown that administration of the synthetic anabolic androgen oxandrolone to severely burned children increases lean body mass three to six months before an increase in bone mineral content is observed.

Not surprisingly given the complex nature of bone tissue, systemic androgen administration has shown distinct responses in different skeletal compartments, *i.e.*, cortical, trabecular (cancellous) or intramembranous bone. In hypogonadal settings, a beneficial response to androgen therapy is observed in the trabecular compartment, the more active surface in bone, with an increase in bone mass. However, this relative increase in bone mass occurs with suppression of bone resorption, with micro-architectural changes demonstrating an increase in trabecular number but not thickness. For example, histomorphometric analysis of androgen replacement in hypogonadal male mice has shown that AR activation preserves the number of trabeculae but does not maintain thickness, volumetric density or mechanical strength [38]. Notably, these studies also demonstrated that the bone-sparing effect of AR activation is distinct from the bone-sparing effect of ERα. In addition, androgen appears to play an important role in intramembranous bone formation [16]. Finally, there are reports of increased cortical bone mass, as a consequence of increased bone width and surface periosteal expansion (see[59,63]). High-dose testosterone therapy over 2 years in (genetic female) female-to-male transsexuals resulted in increased areal BMD at the femoral neck, in a setting where estradiol declined to post-menopausal levels [54]. In men with constitutional delay of puberty, impaired periosteal expansion is observed [68]. Taken together, these reports indicate that androgens *in vivo* act to maintain trabecular bone mass through inhibition of osteoclast activity and to expand cortical bone at the periosteal surface. While these findings argue that androgen positively affects cortical bone at the periosteal surface (see[67]), what is lacking is clear documentation of an anabolic effect to increase bone formation in mature osteoblasts and osteocytes. Thus, the direct consequences of androgen action on differentiated osteoblasts *in vivo* remain unclear, and mechanisms underlying potential positive outcomes on bone formation and bone mass remain uncharacterized.

Concentrations of estrogen and androgen receptors vary during osteoblast differentiation, with AR levels highest in mature osteoblasts and osteocytes [64]. Since osteocytes are the most abundant cell type in bone [50], these cells are likely an important target cell for androgen action, and may represent a central mediator for skeletal responses to testosterone therapy *in vivo*. The goal of this study was to use skeletally targeted overexpression of AR as a means of elucidating the specific role(s) for AR transactivation in the mature differentiated osteoblast. AR overexpression was targeted by the col2.3 promoter and was chosen for several reasons: the skeletal expression patterns for this promoter are bone-selective and well-characterized (see[24–26,36,37]); with strong col2.3 promoter activity in differentiated osteoblasts/osteocytes and mineralizing nodules [25,26,36] but not in osteoclasts [7]. Promoter activity varies in different bone compartments. In intramembraneous bone, strong expression was seen in cells at osteogenic fronts of parietal bones but not in the suture [36]. In long bones, strong transgene expression was observed in most osteoblasts on endocortical surfaces, and in a large proportion of osteocytes in femurs throughout cortical bone but with no expression in periosteal fibroblasts [25,26]. In the trabecular area of metaphyseal bone, strong expression was observed at all developmental stages [26].

A distinct advantage of employing a transgenic model is the enhancement of androgen signaling in a specific target *in vivo*, *e.g.*, mature osteoblasts and osteocytes, as a consequence of increased AR abundance. The AR2.3-transgenic model allows for characterization of skeletal responses in the absence of changes in circulating hormone (testosterone or 17b-estradiol) that occurs with global genetic manipulations, to take advantage of increased sensitivity to androgen in distinct skeletal sites for analysis of compartment-specific effects. At the same time, manipulation of androgen action through AR overexpression, rather than systemic administration, gonadectomy or global knockout, excludes effects that would occur at other androgen target tissues *in vivo* including muscle and fat. Here we describe the skeletal consequences of enhanced androgen signaling that is restricted to mature osteoblasts and osteocytes, employing the 2.3-kb type I collagen promoter to control AR overexpression.

Materials and methods

Cloning of expression plasmids

The pBR327-based plasmid col2.3-ßgal-ClaPa contains the basic rat collagen Iα1 promoter sequence − 2293 to + 115 (provided by Dr. David Rowe, University of Connecticut Health Center), which served as the starting vector. BamHI sites were added to the rat AR cDNA (provided by Dr. Shutsung Liao, University of Chicago) with PCR primers. The PCR product was T/A cloned in pCR 2.1-TOPO vector (Invitrogen Corp., Carlsbad, CA, USA). Finally the BamHI-rAR fragment was cloned into the BamHI site in the col2.3-ßgal-ClaPa (after removal of the ßgal cDNA sequences), to give the expression construct referred to as the AR2.3-transgene. The correct sequence and orientation of the AR insert was verified by direct DNA sequencing.

Generation of AR2.3-transgenic mice

AR2.3-transgenic mice were produced using standard technology by the Oregon Health and Science University (OHSU) Transgenic Mouse Facility, following methodology previously described [67]. Embryos were obtained from matings of C57BL/6 males × DBA/2J