3-Ketosteroid Reductase Activity and Expression by Fetal Rat Osteoblasts*

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In addition to reproductive tissue, sex hormones induce transcriptional events in many connective tissue cells, including osteoblasts. Some sex hormone receptor modulators with bone sparing effects selectively target estrogen or androgen receptors, whereas others appear more promiscuous, in part through enzymatic metabolism. Rat osteoblasts express significant oxidative 3α-hydroxysteroid dehydrogenase activity, which can convert precursor substrates to potent androgen receptor agonists. Here we show that they also express 3-ketosteroid reductase activity, exemplified by 7-methyl-17-ethynyl-19-norandrostan-5(10)en-3-one (tibolone) conversion to potent estrogen receptor α agonists. Conversion was rapid and quantitative, with 3α-hydroxytibolone as the primary metabolite. Consistently, tibolone induced estrogen receptor α-dependent gene promoter activity through cis-acting estrogen response elements, increased the stimulatory effect of TGF-β on Smad-dependent gene promoter activity, and enhanced prostaglandin E2-induced activity of transcription factor Runx2. Rat osteoblasts express the 3-ketosteroid reductase AKR1C9, an allo-keto reductase gene family member. Exposure to prostaglandin E2 increased AKR1C9 gene promoter activity and mRNA expression. AKR1C9 promoter activity was also enhanced by overexpression of protein kinase A catalytic subunit or transcription factor C/EBPβ, and the effect of PGE2 was reduced by dominant negative C/EBPβ competition or C/EBPβ antisense expression. Moreover, prostaglandin E2 increased the amount of functional endogenous nuclear C/EBPβ that could bind specifically to a distinct domain ~1.8-kb upstream from the start site of AKR1C9 transcription. In summary, in addition to 3α-hydroxysteroid dehydrogenase, rat osteoblasts express significant and regulatable 3-ketosteroid reductase activity. Through these enzymes, they may selectively metabolize precursor compounds into potent steroid receptor agonists locally within bone.

Several enzyme groups are known to modify sex steroids or their precursors in specific ways, and consequently control their abilities to bind to and drive gene expression by discrete sex steroid receptors (SSRs).3 Of these, some members of the aldo-keto reductase (AKR) gene family can reduce 3-ketosteroids to 3α- and/or 3β-hydroxysteroids, or in some instances perform the reverse reaction. Certain AKR family members exhibit distinct preferences for individual substrates or reaction direction, related to structural aspects of the substrate itself, to the redox state, or to specific cofactor concentrations (1–6).

Earlier studies showed that osteoblasts express various enzymes involved in steroid metabolism (7–11), including more recent evidence for 3α-hydroxysteroid dehydrogenase (12). In this regard, fetal rat osteoblasts rapidly oxidize the synthetic compound estren (4-estren-3α,17β-diol), which has very low affinity for SSRs, to the potent androgen 19-nortestosterone. This effect was replicated in vitro with cell free extract or with a recombinant protein preparation of the AKR family member AKR1C9, a potent 3-ketosteroid reductase when supplemented with cofactor concentrations that preferentially favor substrate oxidation (12). Other studies suggested that within the context of the intact cell, however, oxidation of 3α- and/or 3β-hydroxysteroids may be driven principally if not exclusively by members of the short chain dehydrogenase reductase/short chain oxidoreductase gene family (5). Even so, fetal rat osteoblasts exhibit abundant mRNA levels of AKR1C9 (12), the predominant rat AKR with 3-ketosteroid reductase potential (3). This predicted that in addition to steroid oxidation, osteoblasts can synthesize bioactive reduced 3-hydroxysteroids with conformations analogous to some estrogen receptor (ER) agonists.

Mechanisms that regulate 3-ketosteroid levels locally within the skeleton could have important implications for native steroid metabolism, as well as for exposure to synthetic compounds used for sex steroid hormone replacement therapy (HRT) to prevent osteoporosis. The AKR1C9 gene promoter has been cloned, and sequence analysis identified a variety of

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3 The abbreviations used are: SSR, sex steroid receptor; AKR, aldo-keto reductase; AR, androgen receptor; ARE, androgen response element; C/EBP, CCAAT enhancer-binding protein; DHT, dihydrotestosterone; EMSA, electrophoretic mobility shift analysis; estren, 4-estren-3α,17β-diol; ERα, estrogen receptor α; ERE, estrogen response element; HRT, hormone replacement therapy; PG, prostaglandin; PK, protein kinase; PMA, phorbol 12-myristate 13-acetate; PR, progesterone receptor; promogestone, 17,21-dimethyl-19-norpregn-4,9-diene-3,20-dione; Runx, Runx homology domain transcription factor; 17βE, 17β-estradiol; tibolone, 7-methyl-17-ethynyl-19-norandrostan-5(10)en-3-one; TGF-β, transforming growth factor β.
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possible regulatory cis-acting elements. To date, only few of these elements have been addressed, and essentially all that is known derives from studies in liver cells where the enzyme was initially thought to have its primary activity (13–15).

Tibolone (7-methyl-17-ethynyl-19-norandrostane-5 (10-en-3-one), a 3-ketosteroid androgen receptor (AR) agonist with potential HRT efficacy, has complex effects in vivo through AR, ER, and progesterone receptor (PR), in part through rapid conversion to other metabolites in the organism. Reduction of tibolone at position 3 in steroid ring A by AKR1C family members produces the ER agonists tibolone and 3α-hydroxytibolone and 3β-hydroxytibolone (16–18). In this study we assessed the activation potential of tibolone on ERα-dependent gene induction in rat osteoblasts, using it as a model for endogenous 3-ketosteroid reductase activity in bone. Based on previously unrecognized hormone interactions and on sequence analyses, we also assessed AKR1C9 expression and gene promoter activity in these cells to define molecular mechanisms that drive its expression. Our results show that osteoblasts, through inherent 3-ketosteroid reductase activity, can metabolize steroid substrates into potent estrogens or possibly to limit their androgenic activity. They further predict that hormone-dependent changes in 3-ketosteroid reductase expression by osteoblasts could oppose their endogenous 3α-/3β-hydroxysteroid dehydrogenase oxidation potential, ultimately regulating the levels of ER or AR agonists within the skeletal tissue environment.

EXPERIMENTAL PROCEDURES

Cells—Primary osteoblast-enriched cultures were isolated from parietal bones of 22-day-old Sprague-Dawley rat fetuses (Charles River Breeding Laboratories), as approved by the Yale Institutional Animal Care and Use Committee. Sutures were dissected, and cells were released by five sequential collagenase digestions. Cells pooled from the last three digestions express features of differentiating osteoblasts, including high levels of runt homology domain nuclear factor 2 (Runx2), parathyroid hormone receptor, type I collagen synthesis, and alkaline phosphatase. They also increase osteocalcin expression in response to vitamin D3, exhibit differential sensitivity to transforming growth factor β (TGF-β), bone morphogenetic protein 2, and various prostaglandins (PGs), and form mineralized nodules under conditions promoting long term differentiation in vitro. Cells were plated at 4,000/cm² in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 μg/ml ascorbic acid and grown for 6 days before transfection or treatment, which was performed in serum-free medium (19–26).

Transfection Plasmids—AR-dependent gene expression was assessed with a firefly luciferase reporter plasmid driven by four consensus androgen response elements (ARE) cloned upstream of a minimal RSV promoter, and ERα-dependent gene expression was assessed with a luciferase reporter plasmid driven by two progesterone response elements cloned upstream of a minimal TATA box in cells cotransfected with PR-A or PR-B (27, 28). Runx activity was assessed with luciferase reporter plasmid 5XGal4 driven by five Gal4 response elements in cells co-transfected with an expression plasmid encoding a Runx2-Gal4 DNA binding domain fusion protein (M1Runx2) (12, 29). Smad-dependent gene expression was assessed with luciferase reporter plasmid SBE4 driven by four Smad response elements (29). AKR1C9 gene promoter activity was assessed with a 2.0-kb fragment of the rat promoter (GenBankTM AF180326) (13) subcloned into luciferase reporter plasmid pGL2-Basic. CCAAT enhancer-binding protein (C/EBP) δ and β overexpression were induced with expression plasmids encoding the rat mRNAs. Endogenous C/EBP activity was suppressed by transfection with an expression plasmid encoding a dominant negative rat C/EBPβ (dn-C/EBPβ) fragment encompassing its leucine zipper dimerization and DNA-binding domains but no gene transactivation domain (30, 31), or rat C/EBPβ in the reversed, antisense orientation (as-C/EBPβ). To assess the role of PKA, cells were co-transfected with expression plasmids encoding catalytic, regulatory, or dominant negative subunits of PKA (32–37).

Transfections—Promoter-reporter fusion plasmids, gene expression plasmids, or empty parental vectors, were pre-titrated for optimal expression efficiency and transfected with reagent LT1 (Mirus). Cultures at 70% confluence were exposed to an optimal amount of reporter plasmid (75 ng per cm²) or expression plasmid (10–75 ng per cm²) in medium supplemented with 4% serum. Cells were cultured for 6–72 h and treated in serum-free medium as indicated in the figures. The cells were rinsed and lysed, and supernatants were analyzed for reporter gene activity and corrected for protein content. To account for competition among plasmids for limiting transcriptional components, control cultures were transfected with a compensating amount of empty vector. Transfection efficiency was assessed in parallel in cells transfected with positive and negative reporter plasmids as previously described (23, 38).

HPLC—To assess tibolone metabolites, an elution profile for purified H-labeled tibolone and its known conversion products (17, 18) was established by HPLC. Briefly, [3H]tibolone, [3H]Δ4-tibolone, [3H]3α-hydroxytibolone, and [3H]3β-hydroxytibolone were chromatographed individually on a C18 Bondapak. Samples were applied in 60% methanol and eluted at 1 ml/min for 5 min, followed by a 20-min linear gradient to achieve 65% methanol, and continued at 65% methanol for 15 additional minutes. Elution was monitored by liquid scintillation counting. A combined elution profile of the four readily separated compounds is shown in the upper panel of Fig. 2A, where tibolone migrates at 25 min, Δ4-tibolone at 17 min, 3α-hydroxytibolone at 20 min, and 3β-hydroxytibolone at 28 min. Osteoblasts were then incubated with the 0.5 μCi of [3H]tibolone or its derivatives for 0, 2, 6, or 24 h. Each culture medium was combined with a methanol extract from its cell layer and extracted with hexane. The aqueous fraction was ether extracted and steroids in the ether extract were analyzed by HPLC as described above. Extraction efficiency and recovery was determined with reference to 2 μg of unlabeled Δ4-tibolone as an internal UV light (240 nm) absorbent recovery standard.
All tibolone and tibolone derivatives used in these studies were generously supplied by Organon NV, Oss, The Netherlands.

**mRNA Analysis**—Total RNA was extracted with acid-guanidine-monothiocyanate, precipitated with isopropyl alcohol, and dissolved in sterile water. AKR1C9 mRNA was assessed by fractionation on a 1.5% agarose/2.2 M formaldehyde gel, blotting on charged nylon, and hybridization with 32P-labeled cDNA encompassing a 0.6-kb HindIII/EcoRI restriction fragment (bp 251–854) of the coding region of rat AKR1C9 (12). rRNA was assessed by ethidium staining of a parallel gel. Radiolabeled products were examined by autoradiography and densitometry (39).

**Electrophoretic Mobility Shift Analysis (EMSA)**—Double-strand oligonucleotide probes comprising AKR1C9 promoter fragments that define three separate C/EBP binding sites (Table 1) were labeled with [32P]dCTP and Klenow fragment of *E. coli* DNA polymerase I, and gel-purified. Nuclear protein extracts (3 μg) from control or PGE2 induced cells were preincubated with either no addition, a 100-fold excess of unlabeled oligonucleotide (Table 1), nonimmune IgG, or antisem to C/EBPα or C/EBPβ (Santa Cruz Biotechnology, Inc.), and then supplemented with 32P-labeled probe. Protein-bound DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel and examined by autoradiography (39, 40).

**Statistical Analysis**—Differences were assessed by one-way analysis of variance with Tukey post hoc analysis using SigmaStat (Jandel Corporation) from nine or more replicate samples and two or more independent cell preparations. Results from mRNA and gel shift analyses were from at least two studies. A significant difference was assumed by a p value of <0.05.

**RESULTS**

**Sex Steroid Receptor-dependent Gene Expression by Tibolone in Osteoblasts**—SSR agonists induce direct and indirect effects on gene expression in osteoblasts in relatively receptor restricted ways (12, 29). Unlike the focused effects of dihydrotestosterone (DHT) or 17β-estradiol (17βE), exposure to tibolone produced direct, dose-dependent effects through both AR and ERα in osteoblasts. The effect of tibolone closely paralleled the AR-specific response to DHT through ARE (Fig. 1A, upper panel) and the ERα-specific response to 17βE through ERE (Fig. 1A, lower panel). In addition, like 17βE (12, 29), tibolone enhanced the activity of the essential osteoblast transcription factor Runx2 in combination with ERα but not AR (Fig. 1B). These results revealed that tibolone, itself an AR selective agonist (16), achieved ERα agonist potential in the presence of metabolically active osteoblasts.

**Osteoblasts Rapidly Convert Tibolone to 3α-Hydroxytibolone and 3β-Hydroxytibolone**—Preliminary studies with purified [3H]tibolone or its known derivatives [3H]Δ4-tibolone, [3H]3α-hydroxytibolone, or [3H]3β-hydroxytibolone, established an elution profile from C18 μBondapak by standard reversed phase HPLC methods (upper panel, Fig. 2A). Within 2–6 h of incubation with osteoblasts, virtually all of the [3H]tibolone was metabolized. At least 25% of [3H]tibolone was lost to polar, water soluble metabolites, and the remainder was recovered primarily as [3H]3α-hydroxytibolone, and to a much lesser extent, [3H]3β-hydroxytibolone (lower panels, Fig. 2A).

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**FIGURE 1. Tibolone enhances ERα-dependent gene promoter activity in osteoblasts.** In A, fetal rat osteoblasts were co-transfected for 24 h to express AR (15 ng/cm²) and reporter plasmid ARE (75 ng/cm²), or ERα (22.5 ng/cm²) and reporter plasmid ERE (75 ng/cm²), and then treated with vehicle (0) or the amounts of DHT, 17βE, or tibolone (Tib) shown. In B, the cells were co-transfected to express AR or ERα in combination with M1Runx2 (25 ng/cm²) and reporter plasmid 5XGAL4 (75 ng/cm²), and treated with 10 nM DHT, or 17βE as indicated. Reporter activity was measured after 24 h of treatment. Tibolone and DHT significantly enhanced gene expression through ARE in AR-transfected cells, and tibolone and 17βE significantly enhanced gene expression through ERE and M1Runx2 activity in ERα-transfected cells (p < 0.05).

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studies with the [3H]tibolone derivatives showed that [3H]Δ4-tibolone was stable throughout 24 h of incubation, whereas a large fraction of [3H]3α-hydroxytibolone was converted to [3H]3α-hydroxytibolone, presumably by way of 3α-hydroxysteroid dehydrogenase-dependent oxidation and subsequent 3-ketosteroid reductase re-reduction. A significant portion of [3H]3β-hydroxytibolone was also lost to water soluble metabolites, and no more than 1% of either [3H]3α-hydroxytibolone or [3H]3β-hydroxytibolone appeared as [3H]Δ4-tibolone (data not shown). Thus, through conversion or inter-conversion, 3α-hydroxytibolone accounted for the primary tibolone metabolite, albeit some was lost to other polar, but presently unknown breakdown products. Moreover, these results establish with certainty that osteoblasts express significant 3-ketosteroid reductase activity in addition to their oxidative 3α/β-hydroxysteroid dehydrogenase activity (12).

As expected from chemical structure and receptor binding studies (16), tibolone and Δ4-tibolone induced AR-dependent gene expression through ARE comparable to the effect of DHT. 3α-Hydroxytibolone had no detectable effect, and 3β-hydroxytibolone had more modest activity, presumably because of its oxidized intermediate status during interconversion to 3α-hydroxytibolone (Fig. 2B, upper panel). Analogously, tibolone, 3α-hydroxytibolone and 3β-hydroxytibolone all potently induced ERα-dependent gene expression through ERE comparable to the effect of 17βE (Fig. 2B, lower panel). Also, like 17βE (29), tibolone enhanced the stimulatory effect of TGF-β on Smad-dependent gene promoter activity in ERα-expressing osteoblasts (Fig. 2C). Thus tibolone acted as a potent agonist for ERα.
in osteoblasts resulting from its rapid reduction to 3-hydroxyti- 
bolone metabolites. When osteoblasts were treated with tibo- 
lone in the presence of two different oxidoreeductase inhibitors, 
quercetin or phenolphthalein (41, 42), its ERα activation poten- 
tial was severely reduced. For presently unexplained reasons, 
each inhibitor enhanced basal ERα-dependent gene expression 
in the absence of ligand, but the significant stimulatory effect of 
tibolone was nonetheless reduced by ~70% (Fig. 2D).

Consistent with in vitro receptor binding (16), tibolone and 
Δ4-tibolone also potently enhanced progesterone response ele- 
ment-driven gene expression through PR-B in osteoblasts, 
3β-hydroxytibolone had more modest activity, and 3α-hy- 
droxytibolone had no significant effect. By contrast to results 
with PR-B, highly attenuated effects occurred through PR-A 
(supplemental Fig. S1). This pattern of agonist-dependent 
activity through PR-B was analogous to their AR-dependent 
effects through ARE in osteoblasts, and to their biochemical 
effects in human endometrial cancer-derived cells (43).

PGE2 Enhances Tibolone-dependent Runx2 Activity through 
ERα—PGE2 differentially regulates osteoblast activity in com- 
plex ways (21, 34, 44, 45). This can occur in part through an 
increase in Runx2 activity (32), which may be augmented fur- 
ther in complex with hormone-activated ERα (29). Tibolone 
also superenhanced Runx2 activity in PGE2-activated osteo-
blasts analogous to the effects of 17βE or the 3αβ-hydroxyti-
bolones, as predicted from its ERα-activating potential after 
3-ketosteroid reductase-dependent reduction (Fig. 3, left 
panel). There was no significant increase in PGE2 activity by 
any AR agonist including tibolone (Fig. 3, right panel) or its 
metabolites (data not shown).

PGE2 Increases AKR1C9 Gene Expression in Osteoblasts— 
Sequence analysis with MatInspector (Genomatix Software, 
GmbH), revealed that the 2.0-kb 3′-region of the AKR1C9 gene 
promoter contains several possible domains associated with 
PGE2-induced transcription factors. Accordingly, PGE2 
potently enhanced AKR1C9 gene promoter activity in osteo-
blasts. The stimulatory effect of PGE2 was dose- and time-re- 
lated, achieving an approximate 4-fold increase within 6 h with 
1 μM PGE2 (Fig. 4A, left panel), and declined significantly by 
24 h (Fig. 4A, right panel). The effect of PGE2 was mimicked 
by forskolin but not by the phorbol ester PMA (Fig. 4B), predicting 
gene activation by a protein kinase A (PKA)-sensitive pathway.

Osteoblasts expressed a single species of AKR1C9 mRNA of 
~2.7 kb, consistent with the size calculated by genomic analysis 
(46). In agreement with gene promoter activation, densitomet- 
ric analysis indicated that AKR1C9 mRNA levels increased by 
3.4 ± 0.1-fold within 6 to 12 h, and decreased to 1.7-fold after 
15 h of PGE2 treatment (Fig. 4C).

Overexpression of the catalytic subunit of PKA also 
increased AKR1C9 gene promoter activity in a time-dependent
FIGURE 4. PGE2 enhances AKR1C9 gene expression in osteoblasts. Fetal rat osteoblasts were transfected for 24 h with a 2.0-kb fragment of the rat AKR1C9 gene promoter cloned upstream of firefly luciferase (75 ng/cm²). In A, the cells were treated for 6 h with vehicle (0) or the amounts of PGE2 shown (left panel) or for 6 or 24 h with vehicle (0) or 1 µM PGE2 as indicated. In B, the cells were treated for 6 h with vehicle (0), 1 µM PGE2, 10 µM forskolin (Fsk), or 1 µM phorbol ester (PMA), and reporter activity was measured. PGE2 (at 0.1 and 1 µM) and forskolin significantly enhanced AKR1C9 gene promoter activity (p < 0.05). In C, cells were treated with PGE2 for the times indicated and AKR1C9 mRNA was assessed by Northern analysis. The single panel on the right shows a DNA ladder to estimate the size of AKR1C9 mRNA, and the lower panel shows rRNA levels, visualized by staining with ethidium.

FIGURE 5. PKA-dependent effect on AKR1C9 gene expression in osteoblasts. Fetal rat osteoblasts were transfected for 24 h with a 2.0-kb fragment of the AKR1C9 promoter reporter plasmid in combination with expression plasmids encoding the catalytic subunit of PKA (PKAcatalytic), the regulatory subunit of PKA (PKAregulatory), or mutated dominant negative PKAregulatory (PKAregulatorymut) (each at 25 ng/cm²) for the times indicated in the left panel, or for 44 h and then treated for 6 h with vehicle (control) or 1 µM PGE2 in the right panel, and reporter activity was measured. In B, cells were transfected with PKAcatalytic in the left panel, or PKAregulatorymut in the right two panels for the times indicated without or with PGE2, and AKR1C9 mRNA was assessed by Northern analysis. The lower panels show rRNA levels, visualized by staining with ethidium. PKAcatalytic significantly enhanced basal AKR1C9 gene promoter activity within the first 2 days of transfection, and PKAregulatorymut significantly suppressed the stimulatory effect of PGE2 (p < 0.05).

C/EBPδ Regulates the AKR1C9 Gene Promoter in Osteoblasts—Several PKA-sensitive transcription factors occur in fetal rat osteoblasts, including CREB (47), the C/EBPs (31, 48), Runx2 (32, 49), and Fra2 (33). Of these, CREB, C/EBP, and Runx2 binding sites occur within the 3’ 2.0-kb AKR1C9 promoter region. CREB and Runx2 are constitutively expressed at high levels in differentiated osteoblasts, but within several contexts their abilities to bind DNA do not appear to be PGE2-sensitive (32, 47). However, Runx2 drives the expression of C/EBPδ (32), which then accumulates in the nucleus in osteoblasts and induces gene expression after exposure to PKA activating hormones like PGE2 (31, 50). The possibility for involvement by C/EBPδ was therefore assessed within the context of the 2.0 kb AKR1C9 promoter region. Transgenic overexpression of C/EBPδ significantly enhanced AKR1C9 gene promoter activity, and the stimulatory effect of PGE2 was also greater in C/EBPδ-overexpressing cells (Fig. 6A, left panel). Furthermore, overexpression of a dominant negative C/EBPδ fragment encoding leucine zipper dimerization and DNA binding domains but no gene transactivation domain (31, 39) significantly limited the effect of endogenous C/EBPδ on AKR1C9 gene promoter activity and reduced the effect of PGE2 by 80–90% (Fig. 6A, middle panel). Co-expression with a vector containing C/EBPδ in antisense orientation to limit C/EBPδ mRNA levels also significantly limited AKR1C9 gene promoter activity in PGE2 induced cells, by ~75% (Fig. 6A, right panel). This was only slightly less effective than the inhibitory effect of the dominant negative C/EBPδ construct, which directly targets and suppresses stimulation by the pre-existing pool of functional C/EBPδ protein that occurs in differentiating osteoblasts (31, 32, 47), perhaps due to differences between C/EBPδ mRNA and protein stability. In agreement with an increase in AKR1C9 expression and conversion of tibolone to its reduced derivatives, the stimulatory effect of tibolone on ERα-dependent transcription was also enhanced with C/EBPδ overexpression (Fig. 6B), whereas no significant effect was evident with 17βE, which is endogenously reduced at its 3α position (Fig. 6C).
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C/EBPδ Binds Consensus C/EBP Response Elements in the AKR1C9 Gene Promoter in Osteoblasts—Initial characterization studies revealed multiple C/EBP binding sites in the 2.0-kb 3′-region of the rat AKR1C9 gene promoter (15). Recent re-inspection of this region with MatInspector (Genomatix Software, GmbH) suggested several highly probable C/EBP response elements, as defined by significant degrees of core and matrix similarity. EMSA with oligonucleotide probes comprising three of these putative C/EBP response elements in the AKR1C9 gene promoter (Table 1) exhibited multiple nuclear factor binding complexes. A significant increase in binding by nuclear factor from PGE2-induced cells was limited to slowly migrating complexes in the upper portion of the binding profiles. The increase in nuclear factor binding seen with radiolabeled probe C1/2, which contains 2 C/EBP response elements at nucleotides −1728 to −1720 (designated as site C/C/EBP site C1) and −1704 to −1696 (site C2), was sensitive to competition with unlabeled oligonucleotide C1/2 or C1/2μ in which only binding site C2 was mutated (Fig. 7A, left panel). The increase in binding seen with radiolabeled probe C3, containing a C/EBP response element at nucleotides −1380 to −1372 (site C3) was sensitive to competition with unlabeled oligonucleotide C3 (Fig. 7A, right panel). With both radiolabeled probes C1/2 and C3, the PGE2-induced nuclear protein complexes were also sensitive to competition by unlabeled probe HS3D, the C/EBP response element that occurs in exon 1 in the IGF-I gene promoter (34) (Fig. 7A) and to anti-C/EBPδ antibody (Fig. 7B). The presence of a doublet complex is consistent with C/EBPδ binding in other promoter DNA contexts (31, 39), although the reason for more than a single band remains unclear. The lack of C/EBPδ binding to site C2 may relate to sequence variations within the response element itself or its flanking domains, or to the possibility that site C2 may be more readily sensitive to other C/EBP isoforms not present in

![Figure 6](image)

**FIGURE 6.** C/EBPδ enhances AKR1C9 gene promoter expression and tibolone-dependent activation of ERα in osteoblasts. In A, fetal rat osteoblasts were co-transfected for 24 h with a 2.0-kb fragment of the rat AKR1C9 gene promoter cloned upstream of firefly luciferase as in Fig. 4, in combination with vector (con) or 1 μM PGE2 as indicated. In B, cells were co-transfected for 24 h with ERα and reporter plasmid ERE as in Fig. 1, in combination with vector (vec) or C/EBPδ expression plasmid at 37.5 ng/cm² in the right panel, or at the amounts shown in the left panel, and then treated with control vehicle (con) or tibolone as indicated. In C, cells were co-transfected with PGE2 and reporter plasmid ERE in combination with vector or C/EBPδ expression plasmid at 37.5 ng/cm², and then treated with vehicle, tibolone, or 17βE as indicated. Co-transfection with all concentrations of dn-C/EBPδ or C/EBPδ significantly suppressed the stimulatory effect of PGE2 on AKR1C9 gene promoter activity (p < 0.05). Co-transfection with C/EBPδ significantly enhanced ERα-dependent gene expression in response to tibolone (p < 0.05).

![Figure 7](image)

**FIGURE 7.** C/EBPδ binds to select sites in the AKR1C9 gene promoter. Fetal rat osteoblasts were treated for 4 h with vehicle (0) or 1 μM PGE2 as indicated, and nuclear extracts were tested by EMSA with 32P-labeled oligonucleotide probes C1/2 and C3 from the AKR1C9 gene promoter defined by C/EBPδ binding domains as indicated in Table 1. In A, nuclear extracts were preincubated with non immune IgG, antibody (α) to C/EBPδ, or irrelevant control antibody (AB), and then supplemented with 32P-labeled probes. In B, nuclear extracts were preincubated with non immune IgG, antibody (α) to C/EBPδ, or irrelevant control antibody (AB), and then supplemented with 32P-labeled probes. Identical results were obtained with nuclear extracts from two separate studies.

**TABLE 1**

| Name     | Sequence                                          |
|----------|---------------------------------------------------|
| C1/2     | 5′-GCGCTACTAATACATACCTTTACTAGAGAAGATATTTTCAACTCTTCAT-3′ |
| C1/2μ    | 5′-GCGCTACTAATACATACCTTTACTAGAGAAGATATTTTCAACTCTTCAT-3′ |
| C1μ/2    | 5′-GCGCTACTAATACATACCTTTACTAGAGAAGATATTTTCAACTCTTCAT-3′ |
| C3       | 5′-CTGATCTGATCGAGAATGAAAGATGACCACAAATAT-3′         |
| HS3D     | 5′-TTCCGACGAGATACTGGCCTTCAATAGGAGAA-3′             |
Sex steroids have important targets beyond reproductive tissues. For instance, bone integrity decreases when endogenous sex steroid levels fall during aging, and can be maintained in large part by sex steroid HRT. In bone forming osteoblasts, sex steroids drive gene expression directly through cis-acting DNA response elements that bind hormone activated SSRs, and indirectly through trans-acting interactions between activated SSRs and other transcriptional components or other signaling events (51, 52). Sex steroids also regulate gene expression in neural, cardiovascular, skin, and other connective tissue cells, and more recent evidence reveals an increase in breast and vascular disease with long term sex HRT (51, 53–55). Therefore, there is great interest to identify SSR agonists with more function restricted or tissue selective effects (56, 57).

Several compounds, while structurally similar to sex steroids, contain modifications that could enhance their stability or vary their interactions with SSRs in highly focused ways (58, 59) and appear particularly sensitive to enzymatic modification. This occurs through several enzymes, first identified in liver, which can metabolize precursor compounds as well as active SSR agonists. For example, 3α-hydroxysteroid dehydrogenases can convert the synthetic compound estrone, a weak ERα or AR agonist, to the potent AR agonist 19-nortestosterone (12), whereas individual AKR1C family members can convert the synthetic compound tibolone, itself an AR agonist, to the potent ERα agonists 3α-hydroxytibolone and/or 3β-hydroxytibolone (17, 18). Both enzyme families modify native compounds during the course of steroid synthesis and inactivation, and are also involved in bile acid, retinoid, and PG metabolism, and the carcinogenic activation of some aromatic hydrocarbons (1–3, 60, 61).

Most evidence suggests that several AKR1C gene family members act to reduce specific ketosteroids to 3α-, 3β-, or 20α-hydroxysteroids. In some cases, however, they can also perform the reverse, oxidative reaction. Rat AKR1C9 is one of the best studied members of this gene family. Recombinant AKR1C9 exhibits potent, bi-directional activity when supplemented with appropriate substrates and cofactors in vitro. Nonetheless, AKR1C9 is primarily reductive when overexpressed within the cellular context (1–6), predicting that oxidation principally relies on other, perhaps short chain dehydrogenase reductase/short chain oxidoreductase type enzymes. AKR1C9 expression has mostly been studied in liver where its levels are constitutively high and may be sensitive to control by sex steroids and glucocorticoids (62–64). Original analysis of the AKR1C9 gene promoter revealed many response elements in addition to those for steroid hormones (13–15), suggesting complex levels of regulation.

We previously reported that osteoblasts possess potent oxidative 3α-hydroxysteroid dehydrogenase activity (12), and show here that they also exhibit significant reductive 3-ketosteroid reductase activity by which they rapidly convert the AR agonist tibolone to the ERα agonists 3α- and 3β-hydroxytibolone. 3α-hydroxytibolone is the sole tibolone metabolite produced by recombinant rat AKR1C9 in vitro (18), unlike the appearance of both 3α- and 3β-hydroxytibolone in intact rat osteoblasts. Thus other enzymes with 3-ketosteroid reductase activity could account for the appearance of 3β-hydroxytibolone in rat osteoblasts, or substrate metabolism may differ in subtle but still unknown ways between in vitro assay conditions and intact cells. Even so, tibolone, by way of metabolism, can directly drive gene expression through cis-acting EREs and indirectly activate the essential osteoblast transcription factor Runx2. Little is yet known regarding native genes driven directly through ERE in osteoblasts. However, we found that the ERE-driven oxytocin gene promoter is also sensitive to agonist-dependent ERα activation in osteoblasts,4 consistent with

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4 M. Centrella and T. L. McCarthy, unpublished studies.
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effects on native estrogen-sensitive genes. Even so, indirect transcriptional effects that result from ERα activation may be equally if not more important physiologically. In this regard, transgenic mice generated to express a mutated ERα that fails to drive gene expression through ERE have multiple reproduc-
tive related tissue defects, but retain an increase in uterine cell
proliferation and a decrease in luteinizing hormone secretion in
response to steroid (65–67). Mice expressing a single copy of
the mutated ERα gene also exhibit a focused deficit in cortical
bone. However, they display a seemingly paradoxical increase
in bone mass after ovariectomy that is suppressed by HRT (68).
These and other studies clearly show that osteoblasts possess
the molecular capacity to respond directly and indirectly to
estrogens, and rely on a balance in both response systems.

We also found that the gene promoter for AKR1C9 is
induced in osteoblasts by PKA activation and is driven by tran-
scription factor C/EBPδ. This finding is analogous to those
from analyses of insulin-like growth factor I and transforming
growth factor β receptor type III gene promoters, which also
respond rapidly to PKA-dependent activation and transloca-
tion of pre-existing C/EBPδ in rat and human osteoblasts (31,
39, 50). Original inspection of the AKR1C9 gene promoter
identified multiple C/EBP response elements. However, initial
analysis with a single oligonucleotide probe derived from a dis-
tal upstream region between nucleotides −4349 to −4277 of
the AKR1C9 promoter with a near consensus C/EBP site at
−4285 to −4277, showed no binding by recombinant C/EBPα
or C/EBPβ, and no competition by a consensus C/EBP response
element in combination with nuclear extract from human hep-
ATOM derived HepG2 cells (15). Those results may differ from
ours which focused on more downstream response elements, and
suggest the possibility that not all consensus C/EBP sites are
competent to bind this transcription factor, as we found here
and in previous studies (39). Alternately, they may relate to
preferential binding by isoform C/EBPδ, which may not have been
present in the HepG2 cell nuclei, to distinct elements, or
to the absence of sufficient 3′-terminal flanking sequences in
the probe used in those initial studies (15). Curiously, glucocor-
ticoid increases C/EBPδ and C/EBPα expression in rat hepatocytes
(69), where it also induces AKR1C9 gene promoter activity
(14). In this instance however, the stimulatory effect of
glucocorticoid relies at least in part on imperfect glucocorticoid
response elements that may counteract the effect of constitut-
ine nuclear factor occupancy at inhibitory Oct binding sites
(14). In contrast, glucocorticoid increases the expression of
C/EBPδ and C/EBPδ in osteoblasts (31), but by itself directly
suppresses rather than enhances AKR1C9 gene promoter activ-
ity in these cells. However, consistent with analysis of IGF-I
gene expression, transient exposure to glucocorticoid enhances
C/EBPδ expression and has a synergistic stimulatory effect on
subsequent exposure to PGE2 (31) (supplemental Fig. S2).
Therefore, C/EBPδ may account for PKA-dependent expres-
sion of AKR1C9 in osteoblasts, but not in liver cells, and the
direct inhibitory effect of glucocorticoid in both cell types
appears unrelated to changes in C/EBP expression or activity.

Our evidence for the stimulatory effect of PGE2 on AKR1C9
expression adds the possibility for autoregulation of PG synthe-
sis, inasmuch as members of the AKR1C gene family control

![Diagram](image-url)

**FIGURE 9. Control of steroid precursor metabolism and AKR1C9 expression in osteoblasts.** In rat osteoblasts, the 3-ketosteroid reductase AKR1C9 can reduce facile 3-ketosteroids like tibolone to 3-hydroxysteroids with strong ERα activating potential. In these cells, AKR1C9 gene expression can be induced by hormones like PGE2 that increase cAMP, release the active PKA catalytic subunit, and enhance C/EBPδ activation and nuclear transport. Ligand-activated ERα may then induce direct transcriptional effects through ERE, and indirect effects through the osteoblast-enriched transcription factor Runx2 and through Smads in response to TGF-β stimulation.

The reduction of either PGH2 or PGE2 itself to PGF2α, which is a potent inducer of cGMP and PKC in osteoblasts and regulates PGE2 synthesis (1, 70, 71). High levels of PGF2α might then limit PGE2 activity (21), perhaps accounting in part for its biphasic effects on gene expression. Furthermore, since PGE2 favors the possibility of higher local estrogen levels in the skel-
etal tissue environment by way of C/EBPδ activation and AKR1C9 gene expression, the system may also be self-limiting to the extent that activated ERα can complex with and limit C/EBP-dependent gene expression in osteoblasts (40, 72). Finally, as earlier reported, osteoblasts also express potent 3α-hydroxysteroid dehydrogenase activity (12), which pro-
motes substrate oxidation. Therefore, an eventual restriction in
AKR1C9 expression could enhance the androgenic potential of
certain oxidoreductase-sensitive substrates. In this regard we
found that antisense suppression of AKR1C9 expression by
osteoblasts significantly enhanced AR-dependent gene expres-
sion by tibolone, DHT, and estren (supplemental Fig. S3), con-
sistent with possible counteracting effects by these enzyme
families.

In summary, our studies show that osteoblasts possess
endoogenous 3-ketosteroid reductase activity that allows them
to reduce facile substrates like tibolone to potent ERα agonists,
and perhaps to limit endogenous local androgen levels in the skeleton. We found that the 3-ketosteroid reductase activity can be accounted for in large part by the AKR gene family member AKR1C9 in rat osteoblasts, where its expression is induced by PGE2 in a PKA-dependent way through activation of C/EBPα. Ligand-activated ERα may then induce direct transcriptional effects through ERE, and indirect effects through Runx2 and Smads in response to TGF-β, as modeled in Fig. 9. Importantly, the balance between steroid and steroid precursor reduction and oxidation, through differences in the local redox state or changes in the relative expression of endogenous AKR and short chain reductase dehydrogenase/short chain oxidoreductase enzyme family members, could significantly affect specific SSR agonists and impact bone integrity, and may be more readily apparent with selective HRT. Further studies will help to define the importance of C/EBPα on AKR expression and activity during bone remodeling, mechanical load, trauma, and inflammatory disease, where activation of PKA drives changes in C/EBPα expression and activity in osteoblasts and in this way favors ketosteroid substrate reduction and an increase in the level of ER agonists.

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