Regulators of growth plate maturation

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Evidence for genomic and nongenomic actions of estrogen in growth plate regulation in female and male rats at the onset of sexual maturation

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Abstract

Recently, both estrogen receptor (ER) α and β were detected in growth plate chondrocytes of rats before sexual maturation, implying a role for estrogen at this stage.
In this study, therefore, we investigated the effects of ovariectomy (OVX) or estrogen supplementation on parameters of longitudinal growth in 26-day-old rats, which were sexually immature at the start of the experiment. OVX caused an increase in body weight gain, tibial length and growth plate width due to an increased proliferating zone. This increase correlated with an increase in cell number, with a decrease in cell diameter and with increased proliferating cell nuclear antigen (PCNA) immunostaining compared with sham. Interestingly, the increase in proliferation was not caused by an increase in insulin-like growth factor-I (IGF-I) mRNA expression in the growth plate as assessed by real-time PCR. In contrast to OVX, 17β-estradiol (E2) supplementation (0.5 mg/21 days) of 26-day-old female rats caused a strong decrease in body weight gain, tibial length and growth plate width. The latter was explained by a reduction of the proliferating zone width, which correlated with a reduced number of PCNA-positive cells (not significant) and by a reduction of the hypertrophic zone width. In male rats supplemented with E2, similar effects were observed compared with the females. ER α and β immunostaining was found predominantly in late proliferating and early hypertrophic chondrocytes. OVX did not affect ER expression but E2 supplementation strongly decreased immunostaining for both ER α and β in both sexes. Besides E2, desoxyestrone (DE), an activator of nongenomic estrogen-like signaling (ANGEL) and 2-methoxyestradiol (2-MeO-E2), a tissue-selective naturally occurring metabolite of E2, were administered to female and male rats of the same age. Compared with E2, these compounds had less pronounced, though significant, effects on some parameters of longitudinal growth in both sexes, especially on growth plate characteristics. In conclusion, E2 may exert effects on longitudinal growth before and at the onset of sexual maturation, despite very low endogenous serum levels at these stages. There may be a role for nongenomic signaling in body weight gain, tibial length and growth plate width but genomic signaling prevails.

Introduction

Recent findings in three male patients, one with an inactivating mutation in the estrogen receptor α (ERα) and β (ER β) and two with an aromatase p450 enzyme deficiency, have
established that estrogen modulates essential steps regarding growth during puberty, both in boys and girls (1-3). Estrogen can have both direct and indirect effects on the growth plate. It was shown that anti-estrogen lowers growth hormone (GH) secretion, while estrogen augments GH secretion, especially by increasing the secretory pulse mass (4-6). However, patients with Laron syndrome, who are resistant to GH due to a mutation in its receptor, do have a pubertal growth spurt, suggesting GH independent effects of estrogen as well (7). These effects may be mediated in the growth plate itself, since ER α and β have been demonstrated in growth plate chondrocytes of several species, including rabbit, rat and human (8-12).
In various mouse and rat models, effects of 17 β-estradiol (E2) on longitudinal growth have been described. These studies have, however, been mainly performed in animals that were sexually maturing or mature at the start of the experiment and are limited to a few reports. In 7-week-old sexually maturing female rats, ovariectomy (OVX) enhanced longitudinal growth rate (13). In contrast, high-dose E2 (4 mg/kg per day) strongly reduced tibial growth rate of 2-month-old

intact sexually mature male rats (14). In contrast to humans, rats do not fuse their growth plates at the end of sexual maturation, resulting in a continuation of longitudinal growth, albeit at a very low rate. At these stages, E2 still inhibited longitudinal growth as has been observed in 3-month-old intact female rats (15).

Concomitant with longitudinal growth, growth plate thickness was increased after OVX, which was primarily explained by induced chondrocyte proliferation (13). Likewise, OVX enhanced proliferating cell nuclear antigen (PCNA) protein expression, a marker for cell proliferation, in growth plates from 14-week-old sexually immature rabbits (16). In the same study, serum IGF-I levels were elevated in the OVX animals compared with sham, suggesting an involvement of insulin-like growth factor-I (IGF-I) in the observed increase in chondrocyte proliferation (16). No studies had previously been performed in rats before and at the onset of sexual maturation.

Estrogen actions can be exerted through either a genomic (transcription activation) or a nongenomic pathway, which involves putative membrane-bound receptors and activation of the Src/Shc/ERK signal transduction route. Until now, it has been unclear to what extent the two pathways contribute to the regulation of longitudinal growth by E2. Furthermore, specific estrogen receptor modulators (SERMs) have been described which are known for their tissue selectivity. One of the best-studied SERMs is raloxifene, which prevents postmenopausal bone loss but has minor effects on other tissues, like breast and uterus (17).

One example enabling distinction between genomic and nongenomic effects of estrogen is the synthetic compound desoxyestrone (DE), which has less than 1% binding affinity compared with E2 (18) and is believed to act exclusively through the nongenomic pathway. It has anti-apoptotic effects in cultured osteoblasts and osteocytes and is able to increase bone mineral density (BMD) and bone strength in both estrogenreplete and estrogen-deficient mice (19;20).

Turner & Evans have recently described a naturally occurring metabolite of E2, 2-methoxyestradiol (2-MeOE2), which may well act as a SERM in rats. This compound strongly reduced growth plate thickness in 3-month-old intact female rats due to a reduced chondrocytes proliferation and hypertrophy but no effects were observed on various bone parameters, suggesting a tissue-selective effect of 2-MeO-E2 (21).

Recently, we demonstrated both ERs in tibial growth plates of female and male rats well before and during sexual maturation (1, 4 and 7 weeks of age), with no apparent regulation of expression by sexual maturation (12). Since endogenous levels of estrogen are very low before sexual maturation (22;23), we wondered whether the ERs in the growth plate are functional at this stage.

Furthermore, we wanted to know to what extent genomic and nongenomic actions of E2 might contribute to longitudinal growth. To achieve this, 26-day-old rats were either ovariectomized or intact female and male rats of the same age were supplemented with E2 (0.5 mg/21 days), DE or 2-MeO-E2 (both 5 mg/21 days). Three weeks later, halfway to sexual maturation, the effects on longitudinal growth were examined. Moreover, we investigated PCNA and ERα and β and the relative mRNA expression of IGF-I in the growth plate after OVX, using immunohistochemistry and real-time PCR respectively.

Materials and Methods

Animals

Female and male Wistar rats were obtained from Harlan (Broekman Instituut, Someren, The Netherlands). They were kept in a light- and temperature-controlled room (12 h light : 12 h darkness, 20–22 °C) with food and water available *ad libitum*. Experiments were approved by the local ethics committee for animal experiments.

Experiment 1

Twenty-four 26-day-old female Wistar rats were ovariectomized by the dorsal approach (*n*=11) or sham operated (*n*=13). During the experiment, body weight was measured weekly and after 3 weeks the animals were killed using a fatal dose of pentobarbital sodium (Nembutal, Sanofi Sante Animale, Maassluis, Netherlands). Rats enter sexual maturation around day 30, and maturation takes about 4 weeks. This means that at the start of the experiment rats were still sexually immature, whereas at the end of the experiment they were halfway to sexual maturation. Approximately 50% of the animals from each experimental group (OVX: *n*=5; SHAM: *n*=6) were fixed *in vivo* (2% paraformaldehyde in 0.1 M phosphate buffer supplemented with 75 mM lysine monohydrochloride and 10 mM Na periodate) as described previously (24). Tibiae were isolated and fixed in the same fixative for 24 h. Next, they were decalcified in 15% EDTA, including 0.5% paraformaldehyde for 4 weeks and processed for paraffin embedding. Tibiae were cut in halves in a sagittal orientation, processed for paraffin embedding and 5 µm sections were cut mid-sagittally. From the other half (*n*=6), tibiae were collected and the growth plates were carefully excised, avoiding contamination with other cell types. The obtained growth plate material was frozen rapidly and stored at -80 °C until use.

Experiment 2

Twenty-four female and 24 male 26-day-old Wistar rats were implanted with a slow-release pellet (Innovative Research of America, Saratosa, FL, USA) subcutaneously between the scapulae, releasing 0.5 mg of E2 (*n*=6), 5 mg of DE (*n*=6) or 5 mg of 2-MeO-E2 (*n*=6) over a 21-day period. A fourth group received a placebo pellet (*n*=6).

The doses of DE and 2-MeO-E2 were 10 times higher than that for E2, since they have a lower affinity for the receptor (18;25). During the experiment, body weight was measured weekly, and after 3 weeks the animals were killed using a fatal dose of pentobarbital sodium (Nembutal, Sanofi Sante Animale). All animals were fixed *in vivo* and tibiae were processed as mentioned in the first experiment.

Measurements

In both experiments, body weight gain (g), tibial bone length (cm), growth plate width (total, proliferating zone and hypertrophic zone: all in mm), ratio between proliferating and hypertrophic zone width (P/H ratio), the number of chondrocytes and cell diameter (µm) in each zone (except DE and 2-MeO-E2 treated animals), as well as wet weights of thymus (g) were determined. In the second experiment, uterus, testis and liver wet weight (g) were also measured. Values were expressed as means ± S.D.

PCNA and ER immunohistochemistry

The immunohistochemical protocol performed for both ERs has been published earlier (24). For

the detection of PCNA, growth plate sections were incubated in 10 mM citrate buffer (pH 6.0) at 95 °C for 10 min followed by 30 min of cooling down. After blocking, sections were incubated overnight at 4 °C in 0.5% locking buffer with the mouse monoclonal PCNA antibody (Clone PC-01; Calbiochem, San Diego, CA, USA; 1:5000), the rabbit polyclonal ERα antibody (Clone MC-20: Santa Cruz Technologies, Santa Cruz, CA, USA; 1:50) or the goat polyclonal ERβ antibody (Clone Y-19; Santa Cruz; 1:50). After a number of secondary antibody steps, staining was visualized with AEC (0.2 mg/ml in acetate buffer pH 5.2 with 0.04% H2O2; Sigma) for 3 min. After counterstaining with hematoxylin, the sections were embedded in aquamount (BDH, Poole, Dorset, UK). Pictures of growth plate sections from rats in two consecutive experiments were taken with a Nikon DXM 1200 digital camera using the same settings and the average number of positive cells in a fixed window was determined using Image-Pro-Plus software (Media Cybernetics Inc., Silver Springs, MD, USA). Next, mean values± S.D. were calculated and tested for significance (P<0.05) versus control groups, using the Student t-test.

RNA isolation and cDNA synthesis

Total RNA was extracted from growth plate material according to Chomczynski & Sacchi (1987). Subsequently, RNA samples were treated with RNase-free DNase for 15 min at 37 °C to remove residual DNA contamination (Promega, Madison, WI, USA). The concentration of RNA was determined spectrophotometrically.

One microgram of RNA was denatured (10 min at 70 °C followed by 5 min on ice) and reverse transcribed in a 20 µl reaction containing first strand buffer (75 mM KCl, 3 mM MgCl2 and 50 mM Tris–HCl, pH 8.3), 5 mM DTT, 0.375 mM dNTPs, 200 ng of random hexanucleotides (all from Life Technologies, Breda, the Netherlands), 1 unit of RNasin (Promega) and 2.5 units of M-MLV reverse transcriptase (Life Technologies) at 37 °C for 60 min and denatured again at 70 °C for 10 min. A second addition of 1 unit of RNasin and 2.5 units of M-MLV reverse transcriptase was performed in each tube and the reaction was allowed to proceed at 37 °C for 30 min followed by inactivation of the enzymes by incubation at 70 °C for 10 min. Samples were diluted and stored until use at -20 °C.

Real-time PCR

Three microliters of cDNA were amplified in a total volume of 25 µl containing 1* buffer A, 3.5 mM MgCl2 (Applied Biosystems, Foster City, CA, USA), 0.2 mM dNTPs (Roche, Basel, Switzerland), 50 pmol/ml of the forward and reverse primer (Eurogentec, Seraing, Belgium), 5 pmol/ml probe and 5U/ml AmpliTaq Gold (Applied Biosystems).

The probe sequences for the household gene, rat porphobilinogen deaminase (PBGD; 5'-CCA-GCTGAC- TCT-TCC-GGG-TGC-CCA-C-3') and rat IGF-I (5'-ACA-GGC-TAT-GGC-TCC-AGC-ATTCGG-A-3') were labeled with the reporter dye FAM on the 5' and with the quencher dye TAMRA on the 3' site. The primer sequences for rat PBGD were: forward (5'-ATG-TCC-GGT-AAC-GGC-GGC-3') and reverse (5'-CAA-GGT-TTT-CAG-CAT-CGC-TAC-CA-3'); and for rat IGF-I: forward (5'-TCA-GTT-CGT-GTGTGG- ACC-AAG-3') and reverse (5'-TCA-CAGCTC- CGG-AAG-CAA-C-3'). The amplified products for PBGD and IGF-I were 135 and 117 bp long respectively.

For standardization, both PBGD and IGF-I primer sets were tested with different amounts of cDNA to determine the suitable amount of cDNA to be used in the quantitative analysis. The Taqman reaction included 40 thermal cycles consisting of 15 s at 95 °C and 1 min at 60 °C and was run on an ABI Prism 7700 Sequence Detector (Applied Biosystems). Finally, the Ct value (number of cycles to reach threshold value) was determined for all samples and mean values_S.D. were calculated for each experimental group.

Results

OVX experiment

We performed OVX in 26-day-old female rats and assessed several parameters concerning longitudinal growth 3 weeks later, which are summarized in Table 1.

At the start of the experiment, the rats were still sexually immature and 3 weeks later, they were halfway to sexual maturation. OVX caused significant increases in body weight gain (Fig. 1). When examining longitudinal growth, both tibial length (Fig. 2A) and growth plate width (Fig. 2B) increased significantly compared with sham-operated rats. The wider growth plates in OVX animals were associated with an increased proliferating zone width, while the width of the hypertrophic zone was unaltered. The ratio between the width of the proliferating and the hypertrophic zone (P/H ratio) increased in the OVX animals (Table 1). The increase in the proliferating zone was associated with a significant increase in cell number with a decreased cell diameter compared with sham. Cell number and diameter were unchanged in the hypertrophic zone (Table 1). Representative HE-stained sections (Fig. 2C) from an OVX (b) and sham-operated (a) rat illustrate the results in Fig. 2B. To further address the increased proliferating zone width after OVX, PCNA immunohistochemistry and real-time PCR for IGF-I on cDNA from growth plate preparations were performed. Based on an analysis of two mid-sagittal growth plate sections from the sham (n=5) and OVX group (n=7), the number of PCNA-positive cells significantly increased after OVX compared with SHAM (Fig. 3A; 266_37 vs 181_49 cells; P=0.019). A representative picture of a PCNA-stained growth plate section of a sham-operated and an OVX rat is shown in Fig. 3B (a and b respectively). PCNA immunostaining was primarily found in the proliferating zone, although some positive cells were detected in the hypertrophic zone. Using real-time PCR on cDNAs from dissected growth plates (n=6), amounts of cDNA were adjusted for expression of the housekeeping gene PBGD. The difference between the expression levels of IGF-I and PBGD (Ct value) in each rat for the OVX versus SHAM rats did not reach significance (0.62_0.61 vs 0.36_0.79; P=0.53), which was underscored by a near identical IGF-I expression level (OVX/SHAM ratio=0.83). Thymus wet weight of OVX rats was significantly increased compared with sham.

Estrogen supplementation experiment

As a mirror experiment, either E2 (0.5 mg/21 days), DE or 2-MeO-E2 (both 5 mg/21 days) was supplemented to 26-day-old female and male rats for 3 weeks (n=6). The effects of estrogen supplementation are summarized in Table 1. Compared with placebo rats, administration of E2 via slow-release pellets caused a significant decrease in body weight gain in females and males (Fig. 1). Longitudinal growth was affected by E2, exemplified by a significant decrease in tibial length (Fig. 2A) and growth plate width (Fig. 2B) in both genders. In females, the reduced width of the growth plate was caused by a significant decrease of both the proliferating zone and the hypertrophic zone (Fig. 2B), with an unaltered P/H ratio compared with placebo. In males, however, the reduction of the hypertrophic zone width was more severe than of the proliferating zone, leading to significantly higher P/H ratio compared with controls (Table 1). In both sexes, the decrease in width of the proliferating and hypertrophic zone was associated with a significant decrease in cell number with unchanged cell diameter compared with placebo (Table 1). Representative HE-stained growth plate sections are shown in Fig. 2C (panels c–f). E2 tended to decrease the number of PCNA immunopositive cells in female (Fig. 3A; 40.8 vs 72.5 cells and Fig. 3B: c and d) but not in male (Fig. 3A: 105.2 vs 88.0 cells and Fig. 3B: e and f) rats (n=6). In most of the growth plates studied, staining for PCNA seemed to be dominant in pre-hypertrophic and even hypertrophic chondrocytes, especially in the males (Fig. 3B: c–f). Due to large inter-animal

variation in expression levels, the results did not reach significance. Estrogen supplementation decreased thymus wet weight in females and males and increased uterus or decreased testis wet weight respectively. No effect was observed on liver wet weight (Table 1).

Supplementation of DE led to a number of significant effects (Table 1), albeit to a much lesser extent than E2. Body weight gain significantly decreased compared with placebo in both sexes (Fig. 1). Furthermore, in females there was a trend towards significance concerning decrease of tibial length (Fig. 2A). Despite an unaltered total growth plate width, the width of the proliferating zone was significantly reduced leading to a lower P/H ratio compared with placebo. In DE-supplemented males, tibial length was significantly reduced (Fig. 2A), whereas at the level of the growth plate no changes were evident. When 2-MeO-E2 was supplemented, no effects were seen on body weight gain (Fig. 1) and tibia length (Fig. 2A) in females and males. However, at the level of the growth plate, females had a significantly reduced proliferating zone width (Fig. 2B), leading to a reduced P/H ratio (Table 1), whereas in males the significantly reduced total growth plate width seemed to be associated primarily with a decreased width of the hypertrophic zone, which showed a trend towards significance (Fig. 2B). Due to the small changes in growth plate width, we did not determine the number of cells in the different zones in the DE and 2-MeO-E2 supplemented rats. DE and 2-MeO-E2 had no effect on thymus, uterus, testis or liver wet weight compared with placebo controls.

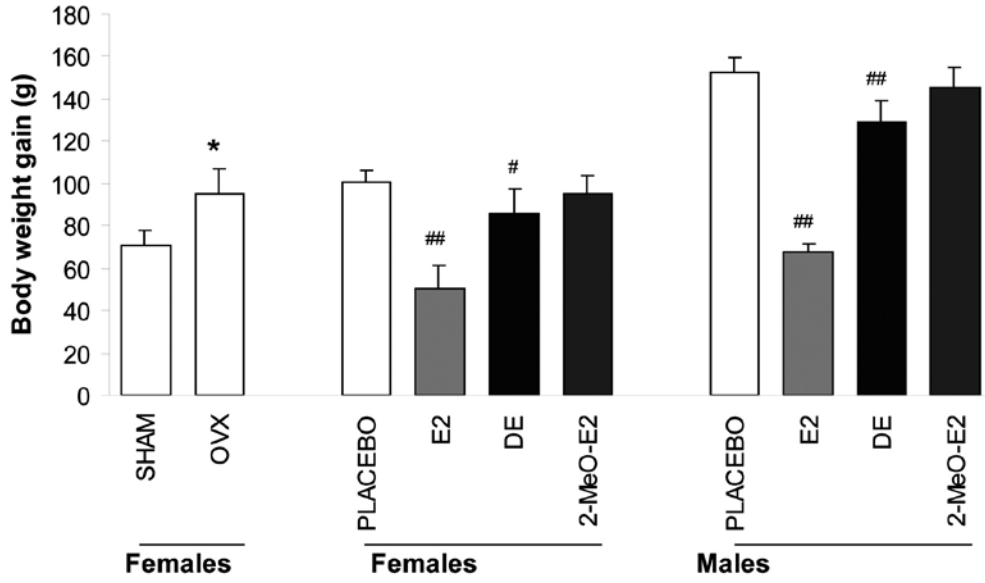

Figure 1

Effects of OVX and estrogen supplementation on body weight gain. Body weight gain was determined 3 weeks after OVX or E2, DE or 2-MeO-E2 supplementation of 26-day-old female and male rats. Values are given as means SD. *P<0.05 compared with sham; *P<0.05 and **P<0.001 compared with placebo.

Table 1 Effect of OVX or supplementation of E_2, DE or 2-MeO_2 for 3 weeks on parameters of longitudinal growth and other characteristics in 26-day-old rats

	OVX experiment		**E_2, DE and 2-MeO_2 supplementation experiment**							
	Females		Females				Males			
	Sham	OVX	Placebo	E_2	DE	2-MeO-E_2	Placebo	E_2	DE	2-MeO-E_2
Parameter										
Body weight gain (g)	71·0	95·4**	100·4	50·1***	85·8*	94·9	152·8	67·5***	128·6***	145·4
Tibia length (cm)	3·24	3·35***	3·22	3·06***	3·15†	3·23	3·42	3·11***	3·29**	3·41
Total growth plate width (mm)	0·34	0·41**	0·39	0·24***	0·36	0·37	0·50	0·31***	0·48	0·45*
Proliferating zone (P) width (mm)	0·18	0·23**	0·20	0·13***	0·17*	0·16*	0·23	0·15***	0·21	0·20
Hypertrophic zone (H) width (mm)	0·17	0·18	0·19	0·11***	0·20	0·21	0·28	0·16***	0·27	0·25†
P/H ratio	1·04	1·37*	1·08	1·12	0·88*	0·79**	0·82	0·96*	0·80	0·81
Proliferating chondrocyte number	10·47	16·19*	19·72	12·39***	N.D.	N.D.	24·22	14·78***	N.D.	N.D.
Hypertrophic chondrocyte number	6·07	6·56	7·33	4·06***	N.D.	N.D.	9·22	5·61***	N.D.	N.D.
Proliferating chondrocyte diameter (μm)	16·81	13·72*	10·17	10·18	N.D.	N.D.	9·40	10·27	N.D.	N.D.
Hypertrophic chondrocyte diameter (μm)	27·59	28·10	25·54	27·86	N.D.	N.D.	30·42	28·27	N.D.	N.D.
Thymus wet weight (g)	0·51	0·86**	0·61	0·23***	0·56	0·59	0·81	0·33***	0·72	0·83
Uterus/Testis wet weight (g)	N.D.	N.D.	0·16	0·30*	0·18	0·21	0·97	0·45***	0·98	1·01
Liver wet weight (g)	N.D.	N.D.	4·68	4·97	4·88	4·20	4·52	4·47	4·62	4·30

Values are expressed as means. *P<0·05, **P<0·01, ***P 0·001, †trend towards significance (P<0·1) compared with respective control (Student's t-test).
N.D. not determined.

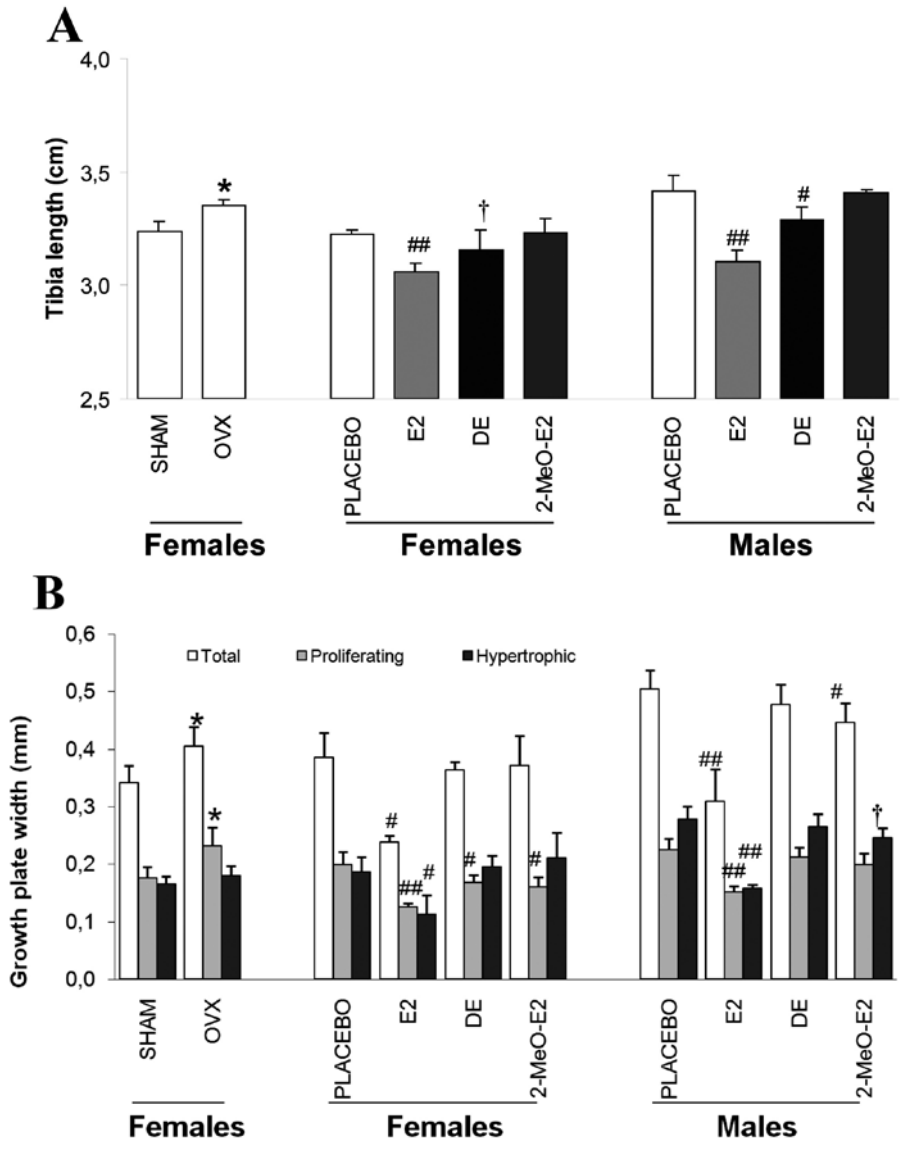
A
Tibia length (cm)
4,0
3,5
3,0
2,5
SHAM
OVX
PLACEBO
E2
DE
2-MeO-E2
Females
Females
Males
B
Growth plate width (mm)
Total
Proliferating
Hypertrophic
0,6
0,5
0,4
0,3
0,2
0,1
0,0
C
a
b
c
d
e
f

Figure 2 (page 38)

Effects of OVX and estrogen supplementation on tibia length and growth plate width. (A) Tibia length was measured 3 weeks after OVX or E2, DE or 2-MeO-E2 supplementation of 26-day-old female and male rats. (B) Total, proliferating zone and hypertrophic zone widths were determined 3 weeks after OVX or E2, DE or 2-MeO-E2 supplementation of 26-day-old female and male rats. (C) Representative growth plate images of a sham-operated (a) and OVX rat (b) as well as female and male placebo controls (c and e respectively) and E2-supplemented (d and f respectively) rats. The added lines in (a) indicate the borderlines between the growth plate and the epi- and metaphysis and the transition between proliferating and hypertrophic chondrocytes and were used to determine the width of the proliferating and hypertrophic zones. Bars represent 50 μm. Values are given as means_SD. *P<0.05 compared with sham; *P<0.05, **P<0.001 and † trend towards significance (P<0.1) compared with placebo.

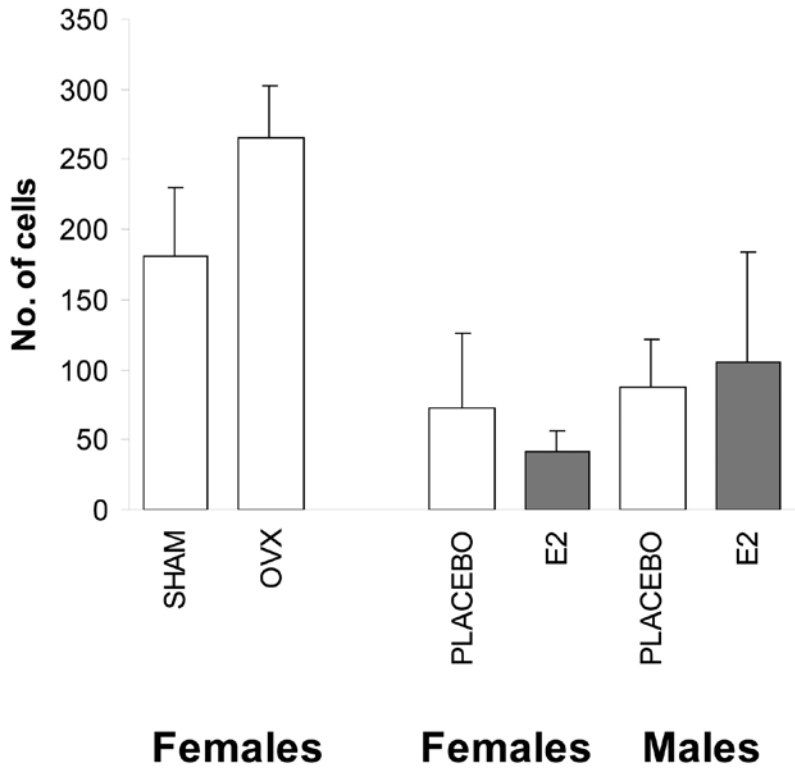

Figure 3

Effects of OVX on PCNA staining in the growth plate. (A) After immunohistochemistry for PCNA, the number of positively stained chondrocytes was determined 3 weeks after OVX of 26-day-old female rats and in E2-supplemented female and male rats by image analysis. Values are given as means_S.D. *P<0.05 compared with SHAM. (B) Representative growth plate images of a sham-operated (a), OVX rat (b), female placebo (c), female with E2 supplementation (d), male placebo (e) and male with E2 supplementation (f). Note the increased number of immunopositive nuclei in the growth plate of the OVX rat, predominantly in the proliferating zone (b) compared with sham (a). Bars represent 50 μm.

ERα and β expression in growth plates

Previously, we have demonstrated ER α and β protein expression in the growth plate of the rat (van der Eerden *et al.* 2002). To study whether the expression of the ERs is regulated by OVX or E2, supplementation immunohistochemistry was performed. Positive staining for ER α and β was predominantly detected in late proliferating and early hypertrophic chondrocytes. No differences in staining intensity were seen between the sham (data not shown), placebo (Fig. 4A and D) or OVX rats (Fig. 4B). In contrast, E2 supplementation caused a downregulation of ER α and β staining in growth plates of females and males (Fig. 4C and E respectively). In contrast, E2 supplementation increased ERα and β staining in osteoblastic cells of the primary spongiosum (Fig. 4C and E). Negative controls, including pre-incubation with a corresponding peptide and omission of the first antibody (Fig. 4F), resulted in absence of staining.

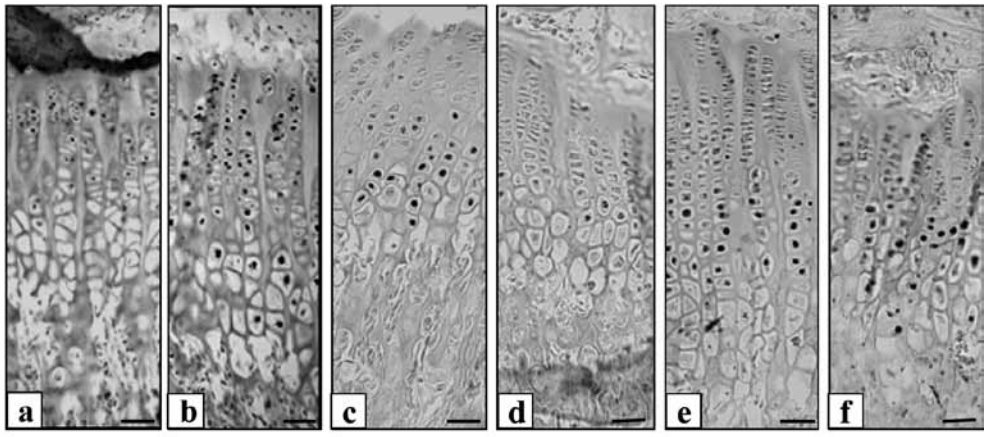

Figure 4

Effects of OVX and E2 supplementation on ERα and β staining in the growth plate. Growth plate sections from the different animal groups were stained for ERα or β. Positive staining was predominantly found in late proliferating (P) and early hypertrophic (H) chondrocytes. No differences in staining intensity were seen between the female placebo (A), OVX (B) or male placebo (D). E2 reduced ERα and β staining in females (C) and males (E) in the growth plate, whereas it increased the expression in osteoblasts of the primary spongiosum (see arrows in C and E). Omission of the first antibody (F) resulted in absence of staining. S, stem cells. Bars represent 50 μm.

Discussion

We recently found that in female and male rats before sexual maturation (1 and 4 weeks of age), both ER α and ER β were expressed in the tibial growth plate at the mRNA and protein level, implying that this tissue may be responsive to estrogens before sexual maturation (12). To assess this, we either ovariectomized or supplemented female and male rats with E2, DE or 2-MeO-E2 at 26 days of age and analyzed several parameters of longitudinal growth and chondrocyte proliferation 3 weeks later. Rats usually enter sexual maturation around day 30, and maturation takes approximately 4 weeks, implying that rats were still sexually immature at the start of our experiments, while they were halfway to sexual maturation when the experiments were terminated.

OVX of 26-day-old rats caused strong increments in body weight gain, tibia length and growth plate width over a 3-week period compared with sham. The increased growth plate width was explained by a widening of the proliferating zone, which was associated with an increased cell number as well as an increased number of PCNA immunopositive cells. In contrast to OVX, E2 supplementation in female rats of the same age resulted in opposite effects: a dramatically reduced body weight gain, tibial length and growth plate width, the latter being caused by both a reduction in the proliferating zone and hypertrophic zone width. Concomitant with the reduced proliferating zone, E2 tended to decrease the number of PCNA positive chondrocytes as well, although this did not reach significance. In male rats, the parameters of longitudinal growth were similarly decreased compared with females. However, no reduction of PCNA-positive cells was observed.

Turner and co-workers demonstrated increased longitudinal growth rates in ovariectomized 7-week-old sexually maturing rats, whereas subsequent estrogen supplementation reversed this (13). The lowest effective dose of E2 was lower than in our study (1.5 vs 23 μg/day), but these rats were previously ovariectomized (13). Moreover, two reports in which intact 3-month-old female and 2-month-old sexually mature male rats received approximately 200 μg/day E2, demonstrated a substantial decrease in longitudinal growth (14;15). Our data extend previous data, claiming that as well as in sexually maturing or mature rats, E2 also inhibits longitudinal growth before and at the onset of sexual maturation. OVX of 26-day-old rats results in enhanced parameters of longitudinal growth, despite very low endogenous estrogen levels, suggesting that even in this period, E2 may have an inhibitory effect on growth.

The change in tibial length after OVX or E2 supplementation coincided with changes in growth plate width. Estrogen removal in females caused an increase in proliferating zone width and therefore total growth plate width, whereas estrogen supplementation reduced the width of both the proliferating and hypertrophic zone in both genders. Moreover, the increased width of the proliferating zone after OVX paralleled the increased PCNA staining, whereas E2 supplementation did not significantly alter the number of immunopositive cells in the growth plate. In males, the immunostaining in the hypertrophic zone was quite high, especially in the E2- treated rats, which contributed to an absence of an effect of E2 on PCNA staining compared with placebo. In the E2-supplementation experiment, and to a lesser extent in the OVX experiment, PCNA-positive nuclei were abundantly observed in the pre- and hypertrophic zone. In a study by Aizawa and co-workers, the same phenomenon has been described in rabbit growth plates (26). It appears that subtle changes in the fixation procedure as well as the fact that chondrocytes have a long G1 phase in relation to the S, G2 and M phase contribute to the relatively high number of PCNA-positive hypertrophic chondrocytes (26). In addition, we speculate that E2 may have a sexually dimorphic

effect on the cell cycle G1 phase duration in chondrocytes (27;28). The inhibitory effect of E2 on chondrocyte proliferation is well established. It has been shown that thymidine uptake is reduced following E2 administration in sexually maturing rats and PCNA staining is increased after OVX in sexually immature rabbits (13;16). In the latter study, besides increased PCNA staining, serum IGF-I levels were increased, suggesting that the proliferating effect of OVX was caused in part by serum IGF-I (16). In our study, we did not determine serum levels of IGF-I, since a dramatic decrease of total serum IGF-I did not affect body weight and longitudinal growth in liver-specific IGF-I knockout mice (29). Instead, local effects of IGF-I seemed more important for longitudinal bone growth. Therefore, we quantified the amount of IGF-I mRNA in preparations from tibial growth plates using real-time PCR. We found no difference in relative IGF-I mRNA levels between OVX and sham. This suggests that the effects of E2 status on chondrocyte proliferation cannot be explained by influencing local IGF-I expression. However, we cannot exclude changes in expression levels of other members of the IGF family (IGF-II, IGF-I and II receptors and IGF binding proteins) in the growth plate caused by E2. Removal of E2 has no effect on the width of the hypertrophic zone, whereas E2 administration reduces both proliferating and hypertrophic zone widths. The reduced width of the hypertrophic zone may be a direct effect of E2 itself but it may also be secondary to an effect on chondrocyte proliferation. It was reported that estrogen reduces matrix synthesis, which has been shown to contribute to an age-related decline in longitudinal growth (30). This may be in line with our study, since OVX decreased the cell diameter in the proliferating zone, despite an increase in its width, indicating that matrix synthesis is downregulated when estrogen is deficient. In contrast, it has been shown that E2 stimulates 35S uptake by chondrocytes, implying increased chondrocyte differentiation (31;32). An explanation for a reduced width of the hypertrophic zone could be that estrogen causes the rate of apoptosis or vascular invasion to exceed the rate of cartilage matrix synthesis (21). We recently demonstrated that ER expression levels were relatively unaffected by endogenous estrogen levels (12). In this study, we found that OVX did not affect ER α or ER β expression. However, treatment with a medium dose of E2 caused a reduction in the expression levels of both receptors in male and female rats. From these findings, one may conclude that a substantial rise in endogenous estrogen levels is apparently required to elicit alterations in receptor expression in the rat growth plate. Interestingly, the raised E2 levels increased receptor expression in osteoblasts of the primary spongiosum. This suggests that E2 can either down- or upregulate the expression of its own receptor, depending on the cell type.

From our data we cannot exclude effects of E2 on the somatotropic axis. It has been shown that estrogen augments GH secretion, especially by increasing the secretory
pulse mass (4;6). In addition, anti-estrogens lower the basal GH secretion (5). However, direct effects are also likely to occur since the P/H ratio increases strongly following OVX, whereas in dwarf rats (dw/dw), which have reduced GH levels to 5% of normal, the P/H ratio is similar to that in normal rats and this ratio is not affected by subsequent GH treatment (33). These results suggest that E2 inhibits longitudinal bone growth most likely by direct effects on chondrocyte proliferation and differentiation in female and male rats before sexual maturation. Since E2 removal causes such dramatic effects on longitudinal growth, it seems evident that despite its low endogenous levels, E2 may play an important role in controlling longitudinal growth in these animals at this stage.

In analogy with E2 supplementation, we have used the synthetic compound DE, which, based on its lower binding affinity compared with E2, was administered at a

dose 10 times higher than E2 (5 mg/21 days) (18). Despite the fact that supplementation of DE after OVX would result in more pronounced effects, we chose intact rats, since this mimics better the situation when it would be used clinically. DE was recently recognized as an activator of nongenomic estrogen-like signaling (ANGEL) and exhibits anti-apoptotic effects on osteoblasts and osteocytes *in vitro* (19). Moreover, DE increases BMD and bone strength in both estrogen-replete and estrogen-deficient mice (20).

Although to a lesser extent than E2, DE decreased body weight gain in both sexes, tibial length (trend towards significance in females) and growth plate width (not significant in males). In addition, females had a decreased proliferating zone width combined with a reduced P/H ratio. However, despite a 10 times higher dose for DE compared with E2, no effects were found on wet weights of thymus, uterus and testis, whereas E2 severely affected these organs. This suggests that in the classical estrogen target tissues genomic effects are most important, whereas in bone and cartilage genomic and nongenomic pathways mediate the effect of estrogen. These results imply a role for genomic and nongenomic signaling in the regulation of body weight gain, tibia length and growth plate width, although genomic signaling prevails.

In addition to DE, 2-MeO-E2 was administered at a similar dose used for DE, since this naturally occurring metabolite of E2 has a very low binding affinity compared with E2 (25). Our 2-MeO-E2 supplementation experiment was comparable to that by Turner & Evans (21) using intact rats, but there were some differences. The rats in our study were sexually immature at the start of the experiment, whereas the female rats used by Turner & Evans were sexually mature (3 months). Moreover, we used slow-release pellets with a relatively low dose (230 µg/day), establishing relatively constant levels during the experiment, while Turner administered 2-MeO-E2 orally (20 mg/day), not knowing precisely what amount the gastro-intestinal tract absorbs. Furthermore Turner & Evans demonstrated dramatically reduced longitudinal bone growth and growth plate width in the 3-month-old sexually mature intact female rats. Interestingly, no effects were found on radial bone growth and cancellous bone turnover or on uterus wet weight (21). Our results were less pronounced but a significant growth plate width reduction was observed in male rats, which was primarily explained by a reduction of the hypertrophic zone width (trend towards significance). Growth plate width in females did not alter but the proliferating zone width was significantly reduced and, subsequently, the P/H ratio. We found no effects of 2-MeO-E2 on body weight gain, tibial length and wet weights of thymus, uterus/testis or liver. From these results, we may conclude that, although not as pronounced as in an earlier study, effects of 2-MeO-E2 seem to be limited to the regulation of growth plate parameters only, suggesting that it might be used as a SERM in the future.

In conclusion, E2 may exert effects on longitudinal growth before and at the onset of sexual maturation, despite very low serum levels at these stages. There may be a role for nongenomic signaling in body weight gain, tibial length and growth plate width, explaining some of the effects on longitudinal growth, but genomic signaling prevails. DE administration in ovariectomized rats should provide more evidence on the relative contribution of genomic and nongenomic signaling in longitudinal growth.

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