The role of the G protein-coupled receptor GPR30 in the effects of estrogen in ovariectomized mice


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Estrogens exert a variety of important physiological effects, which have been suggested to be mainly mediated via the two known nuclear estrogen receptors (ERs), ERα and ERβ. In vitro studies (26, 31) suggest that the membrane G protein-coupled receptor (GPCR) GPR30 is also a functional ER. GPR30 was shown to bind estradiol (E2) with high affinity in vitro (26, 31) and to mediate estrogen-promoted proliferative signaling in an estrogen-sensitive but ER-negative breast cancer cell line (7, 15) and human endometrial cells (40). Furthermore, it was shown in vitro to mediate estrogen-dependent kinase activation as well as transcriptional responses (24, 25). Using our recently developed GPR30−/− mouse model, we demonstrated that GPR30 deletion abolished E2-stimulated insulin release both in vivo in ovariectomized (OVX) adult mice and in vitro in isolated pancreatic islets (16). Furthermore, other researchers (35) have provided evidence that GPR30 participates in estrogen regulation of thymic atrophy. However, the in vivo role of GPR30 as a functional ER for the regulation of skeletal parameters, including bone mass and longitudinal bone growth is unknown.

Estrogens are of importance for the regulation of both trabecular and cortical bone mass in both genders (27, 34). This is demonstrated by the decrease in both trabecular and cortical bone mass that occurs in estrogen-deficient states, such as in postmenopausal women and after ovariectomy (OVX), which is prevented by estrogen treatment. ERα is the major ER mediating the bone-sparing effect of estrogens in both male and female mice (12, 14, 19, 29), whereas ERβ activation reduces ERα-mediated gene transcription in bone from female mice and promotes trabecular age-dependent bone loss in old female mice (13, 38). We (9) recently showed that GPR30 is expressed in human osteoblasts, osteoclasts, and osteocytes, although the physiological role of GPR30 in bone remains elusive.

In addition, estrogens affect longitudinal bone growth and promote growth plate fusion in humans (1, 18, 30). In rodents, the growth plates do not fuse directly after sexual maturation, but prolonged treatment with high levels of E2 has the capacity to inhibit longitudinal bone growth and to reduce the growth plate width (2, 32, 33). We (3) recently demonstrated that GPR30 immunoreactivity is found in the human growth plate with localization specifically in the resting and hypertrophic zones, which suggests that GPR30 might be involved in the regulation of longitudinal bone growth. Moreover, in contrast to ERα and ERβ that are both expressed in the growth plate at relatively similar levels throughout puberty (20, 21), the levels of GPR30 decline as puberty progresses (3). In addition, (16) recently demonstrated that gonadal intact female, but not male, GPR30−/− mice displayed a slightly reduced longitudinal bone growth, indicated by reduced femur length in the
GPR30−/− mice compared with wild-type (WT) mice. However, as the effect of E2 in OVX mice was not evaluated in our previous study, we could not determine if the affected femur length was a result of altered E2 responsiveness or as a result of an estrogen-independent mechanism modulating longitudinal bone growth.

Estrogens are also known to exert multiple effects on the development and regulation of the immune system (23). It is well established that estrogens are important for the development of the thymus and for estrogen-induced thymic atrophy during pregnancy (4). Exposure of adult mice to endogenous or exogenous estrogens induces a massive reduction of thymic weight (28). Furthermore, it is well known that OVX results in increased fat stores and body weight in female rodents, which can be prevented with estrogen and reproduced with anti-estrogen treatment in intact females (5). A fat-reducing effect of endogenous estrogens is supported by the fact that aromatase-inactivated as well as ERα-inactivated mice are obese (8, 11, 22).

The aim of the present study was to determine the possible in vivo role of GPR30 as a functional ER primarily for the regulation of skeletal parameters, including bone mass and longitudinal bone growth, but also for some other well-known estrogen-regulated parameters, including uterine weight, thymus weight, and fat mass.

METHODS

Animals

GPR30-deficient mice (GPR30−/−) were developed using a targeting strategy resulting in the deletion of the whole GPR30 open reading frame (16). The mice were backcrossed six generations into C57BL/6 genetic background. The mice were housed in a standard animal facility under controlled temperature (22°C) and photoperiod (12-h light-dark) and fed standard phytoestrogen-free pellet diet ad libitum. Littermates were used as control group.

Three-month-old female GPR30−/− and WT mice were either sham-operated or OVX. The OVX mice were then treated either with vehicle or increasing doses of E2 (0, 30, 70, 160, or 830 ng·mouse−1·day−1) for 4 wk. The three higher doses of E2 were administered using slow-release pellets (Innovative Research of America) and were chosen according to a study demonstrating affected estrogenic responses in a mouse model with inactivation of steroid receptor coactivator-1 (17). The lowest dose of E2 used (30 ng·mouse−1·day−1) was administrated by subcutaneous silastic implants (Silicrel Tubing; Degania Silicone, Jordan Valley, Israel; Ref. 36), and it is a dose that preserves the trabecular bone mineral density (BMD) to approximately sham levels in OVX mice. Animal care was in accordance with institutional guidelines. All animal experiments were approved by the Ethics Committees for Animal Research at Göteborg University and Karolinska Institutet.

Dual Energy X-ray Absorptiometry

Analyses of total body bone mineral density (BMD), spine BMD, total body fat mass, and total body lean mass were performed by dual energy X-ray absorptiometry (DXA) using the Lunar PIXImus mouse densitometer (Wipro GE Healthcare, Madison, WI) with the mice under inhalation anaesthesia with isoflurane (Forene; Abbot Scandinavia, Solna, Sweden).

Peripheral Quantitative Computer Tomography

Computer tomography scans were performed with the PQCT XCT RESEARCH M (version 4.5B; Norland), operating at a resolution of 70 μm as described previously (39). Trabecular BMD was determined ex vivo, with a metaphyseal peripheral quantitative computer tomography (PQCT) scan of the proximal tibia. The scan was positioned in the metaphysis at a distance from the distal growth plate corresponding to 3% of the total length of the tibiae, and the trabecular bone region was defined as the inner 45% of the total cross-sectional area. Bone lengths were measured with a slide calliper. Cortical bone parameters (cortical bone mineral content, cortical BMD, cortical thickness, periosteal circumference, and endosteal circumference) were analyzed in the mid-diaphyseal region of femur (37).

Quantitative Histology of Growth Plates

Distal femora were fixed in 4% paraformaldehyde, decalcified in 10% EDTA, and embedded in paraffin. Sections (5 μm thick) were stained with Alcian blue/van Gieson. Images were captured using a Nikon Eclipse E800 light microscope connected to a Hamamatsu digital camera C4742-95 and a computer. All histological measurements were performed in the central two-thirds of the growth plate sections using Olympus MicroImage software (version 4.0; Olympus Optical, Hamburg, Germany). The widths of the whole growth plate, the proliferative zone, and the hypertrophic zone were calculated as an average of 20 measurements per growth plate. Hypertrophic chondrocytes were defined by a height of >6 μm. The height of the terminal hypertrophic chondrocyte, the cell in the last intact lacuna, was measured in 24 different columns per growth plate and averaged (2).

Immunostaining for Type X Collagen and Proliferating Cell Nuclear Antigen

Collagen type X immunohistochemistry. Sections were deparaffinized and rehydrated, followed by antigen retrieval in citrate buffer (pH 6.0) at 85°C for 15 min. Peroxidase quench was performed in 3% H2O2 for 10 min at room temperature (RT). A second antigen retrieval was performed with Hyaluronidase (Sigma-Aldrich, Stockholm, Sweden; 5 mg/ml in PBS) for 30 min at 37°C. Blocking was performed in 3% horse serum (Cedarlane Laboratories, purchased from Nordic Biosite, Stockholm, Sweden) in PBS for 1 h at RT before incubation with primary antibody [collagen type X (X53), NC-1; Quartett, Berlin, Germany] overnight at 4°C. The sections were incubated with secondary antibody (biotinylated polyclonal rabbit anti-mouse, E0464; DakoCytomation, Glostrup, Denmark) for 1 h at RT, and the signal was enhanced with avidin-biotin complex (Vectastain ABC kit, PK-4001; Vector Laboratories, Burlingame, CA) and visualized by DAB staining (DAB kit, SK-4100; Vector Laboratories).

Proliferating cell nuclear antigen immunohistochemistry. Sections were deparaffinized and rehydrated, followed by antigen retrieval in citrate buffer (pH 6.0) at 85°C for 15 min. Peroxidase quench was performed in 3% H2O2 for 10 min at RT. Blocking was performed in 2% horse serum in 1% BSA in PBS for 1 h at RT before incubation with primary antibody [mouse monoclonal anti-PCNA, ab29; Abcam, Cambridge, UK] for 1 h at RT. The sections were incubated with secondary antibody (biotinylated polyclonal rabbit anti-mouse, E0464; DakoCytomation) for 1 h at RT, and the signal was enhanced with avidin-biotin complex (Vectastain ABC kit, PK-4001) and visualized by DAB staining.

Bone Marrow Cellularity and Cell Distribution

Bone marrow cells were harvested by flushing 5 ml PBS through the bone cavity of one femur using a syringe. After centrifugation at 515 g for 5 min, pelleted bone marrow cells were resuspended in Tris-buffered 0.83% NaHCl solution (pH 7.29) for 5 min to lyse erythrocytes and then were washed in PBS. Cells were resuspended in buffer [PBS containing 1% fetal bovine serum (FCS) and 0.1% sodium azide] before use. The total number of leucocytes from the bone marrow was calculated using an automated cell counter (Sys-
The cells were then subjected to fluorescence activated cell sorter (FACS) analysis (FACS) on a FACSCalibur (BD Pharmingen, Franklin Lakes, NJ) and analyzed using FlowJo software. Results are expressed as the number of positively stained cells per femur.

RESULTS

Role of GPR30 in the Effects of E2 on Bone Mass

DXA analyses demonstrated that E2 treatment (830 ng·mouse⁻¹·day⁻¹) increased total body (WT, +14%; GPR30⁻/⁻, +16%) and spine (WT, +28%; GPR30⁻/⁻, +32%) BMD to a similar extent in 3-mo-old OVX WT and GPR30⁻/⁻ mice compared with vehicle-treated mice (Table 1). Separate analyses of trabecular and cortical bone using peripheral quantitative computer tomography demonstrated that the E2-induced increase in BMD was due to both increased trabecular BMD and cortical bone mineral content, the latter as a result of both increased cortical thickness and cortical BMD (Fig. 1; Table 2). For all these E2-responsive bone parameters, the magnitudes of the estrogenic responses were very similar in the WT and the GPR30⁻/⁻ mice. To determine in detail if the estrogenic responses in bone were affected in GPR30⁻/⁻ and wild-type mice, four different doses of E2 (30, 70, 160, and 830 ng·mouse⁻¹·day⁻¹) were evaluated. However, the estrogenic responses in bone did not significantly differ between WT and GPR30⁻/⁻ mice for any of the tested E2 doses (Fig. 1 and data not shown).

### Table 1. Effects of estradiol on body composition and BMD analyzed by DXA

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<th>Wild Type</th>
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<th>GPR30⁻/⁻</th>
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<tr>
<td></td>
<td>Vehicle</td>
<td>E₂</td>
<td>Vehicle</td>
<td>E₂</td>
</tr>
<tr>
<td>Fat mass, g</td>
<td>6.8±0.7</td>
<td>4.5±0.3*</td>
<td>7.2±0.6</td>
<td>4.0±0.2*</td>
</tr>
<tr>
<td>Fat, %</td>
<td>24.5±1.7</td>
<td>17.9±1.2*</td>
<td>26.7±1.4</td>
<td>16.4±0.7*</td>
</tr>
<tr>
<td>Lean mass, g</td>
<td>18.8±0.5</td>
<td>18.6±0.4</td>
<td>17.7±0.3</td>
<td>18.2±0.3</td>
</tr>
<tr>
<td>Total body BMD, mg/cm²</td>
<td>51.8±0.8</td>
<td>59.1±1.1*</td>
<td>50.6±0.6</td>
<td>58.5±1.1*</td>
</tr>
<tr>
<td>Spine BMD, mg/cm²</td>
<td>51.5±1.5</td>
<td>66.2±2.2*</td>
<td>49.7±0.8</td>
<td>65.7±16*</td>
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Ovariectomized (OVX) GPR30⁻/⁻ and wild-type mice were treated with vehicle or estradiol (E₂; 830 ng·mouse⁻¹·day⁻¹; n = 8–9). Bone mineral density (BMD), fat mass, and lean mass were analyzed by dual-energy-X ray absorptiometry (DXA) after 4 wk of E₂ treatment. Magnitudes of the estrogenic responses did not significantly differ between GPR30⁻/⁻ and wild-type mice for any of these parameters. Values are means ± SE. *P < 0.05 vs. vehicle-treated OVX mice.

### Fig. 1. Estrogenic responses for some well-known estrogen-regulated parameters. Ovariectomized (OVX) GPR30⁻/⁻ and wild-type (WT) mice were treated with vehicle or different doses of estradiol (E₂; n = 5–22). Both GPR30⁻/⁻ and WT mice displayed clear estrogenic responses regarding (A) trabecular bone mineral density (BMD) in the distal metaphyseal region of the tibia (increased by E₂), uterine weight (increased by E₂; B), thymus weight (C; reduced by E₂), and retroperitoneal fat weight (D; reduced by E₂). Magnitudes of the estrogenic responses did not significantly differ between GPR30⁻/⁻ and WT mice for any of these parameters. Values are percentage of sham-operated mice and are means ± SE. *P < 0.05 vs. vehicle-treated OVX mice.
Parameters were analyzed mid-diaphyseal region of femur after 4 wk of E2 treatment. Magnitudes of the estrogenic responses did not significantly differ between GPR30−/− and wild-type mice for any of these parameters. Values are given as means ± SE. *P < 0.05 vs. vehicle-treated OVX mice.

Role of GPR30 in the Effects of E2 on Longitudinal Bone Growth

As expected, E2 treatment resulted in decreased femur length in adult OVX WT mice compared with vehicle-treated WT mice (P < 0.05; Fig. 2A). In contrast, E2 treatment did not affect femur length in GPR30−/− mice compared with vehicle-treated GPR30−/− mice (Fig. 2A). In an attempt to understand the mechanism by which E2 affects the growth plate, quantitative morphological studies were performed of the distal growth plate in femur. These studies revealed that E2 treatment reduced the heights of the total growth plate, the proliferative zone, the hypertrophic zone, and the terminal hypertrophic chondrocyte in WT mice but not in GPR30−/− mice compared with vehicle-treated mice (Fig. 2, B–E). Type X collagen immunostaining was evaluated as a differentiation marker of hypertrophic chondrocytes, confirming that the height of the hypertrophic zone was significantly reduced by E2 treatment in the WT mice but not in the GPR30−/− mice (Fig. 3A). However, the ratio of the height of type X collagen immunostaining divided by the height of the total growth plate was not affected by E2 treatment in WT or GPR30−/− mice (Fig. 3B), suggesting that the E2 effect, seen in WT but not GPR30−/− mice, was of a similar magnitude in the proliferative and hypertrophic zones in the growth plate. Proliferating cells were evaluated by immunostaining for PCNA. The number of PCNA-positive cells per millimeter growth plate width was significantly reduced by E2 in the WT mice (−50.4 ± 7.3%; P < 0.05) but not in the OVX GPR30−/− mice (−25.2 ± 15.4%; nonsignificant). However, the proportion of PCNA-positive growth plate chondrocytes was neither in the WT nor in the GPR30−/− mice significantly affected by E2 treatment, although there was a trend of an E2 effect in the WT mice (−27%; P = 0.16; Fig. 3C).

It should be noted that the vehicle-treated GPR30−/− mice displayed reduced growth plate height compared with vehicle-treated WT mice (Fig. 2B–D), and this was associated with a nonsignificant trend of reduced femur length in the vehicle-treated GPR30−/− mice (Fig. 2A).

Table 2. Effects of estradiol on cortical bone parameters analyzed by peripheral quantitative computer tomography

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<tr>
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<th>Wild Type</th>
<th>GPR30−/−</th>
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<tr>
<td></td>
<td>Vehicle</td>
<td>E2</td>
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<tr>
<td>Cortical BMC, mg/mm</td>
<td>1.07±0.04</td>
<td>1.27±0.04*</td>
</tr>
<tr>
<td>Cortical area, mm²</td>
<td>0.87±0.03</td>
<td>0.99±0.02*</td>
</tr>
<tr>
<td>Cortical BMD, mg/cm³</td>
<td>1.232±8</td>
<td>1.279±10*</td>
</tr>
<tr>
<td>Cortical thickness, mm</td>
<td>0.20±0.1</td>
<td>0.24±0.1*</td>
</tr>
<tr>
<td>Periosteal circumference, mm</td>
<td>4.97±0.06</td>
<td>4.90±0.04</td>
</tr>
<tr>
<td>Endosteal circumference, mm</td>
<td>3.71±0.04</td>
<td>3.40±0.05*</td>
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OVX GPR30−/− and wild-type mice were treated with vehicle or E2 (830 ng·mouse⁻¹·day⁻¹; n = 8–9). BMC, bone mineral content. Cortical bone parameters were analyzed mid-diaphyseal region of femur after 4 wk of E2 treatment. Magnitudes of the estrogenic responses did not significantly differ between GPR30−/− and wild-type mice for any of these parameters. Values are given as means ± SE. *P < 0.05 vs. vehicle-treated OVX mice.

Fig. 2. Role of GPR30 in the effects of estradiol on femur length and growth plate morphology. OVX GPR30−/− and WT mice were treated with vehicle or E2 (n = 5–16). Values are means ± SE. *P < 0.05 vs. OVX mice. †P < 0.05 vehicle GPR30−/− vs. vehicle WT. Femur length (A), quantitative morphological studies analyzing the total growth plate height (B), the height of the proliferative zone (C), the height of the hypertrophic zone (D), and the height of the terminal hypertrophic chondrocyte (E) were performed in the distal femur growth plate.
Role of GPR30 in the Effects of E2 on Fat Mass

DXA analyses revealed that E2 treatment reduced the total body fat mass in both OVX WT mice (−34%) and GPR30−/− mice (−44%) compared with vehicle-treated mice (Table 1). Dissections of the retroperitoneal and gonadal fat depots demonstrated that the magnitude of the effect of E2 on fat mass reduction was very similar in WT and GPR30−/− mice (Fig. 1D and data not shown). For none of the tested E2 doses did the estrogenic responses on fat mass differ significantly between WT and GPR30−/− mice (Fig. 1D and data not shown). For none of the tested E2 doses did the estrogenic responses on fat mass differ significantly between WT and GPR30−/− mice (Fig. 1D and data not shown).

Role of GPR30 in the Effects of E2 on Uterus Weight, Thymus Weight, and Bone Marrow Cellularity

All tested E2 doses increased the uterus weight and reduced the thymus weight significantly in both WT and GPR30−/− mice compared with vehicle-treated mice (Fig. 1, B and C). The magnitudes of the estrogenic responses in these two tissues did not significantly differ between the WT and GPR30−/− mice for any of the tested E2 doses (Fig. 1, B and C). In addition, E2 treatment reduced the bone marrow cellularity to a similar extent in OVX WT mice (−55%) and GPR30−/− mice (−59%) compared with vehicle-treated mice (Table 3).

Table 3. Effects of estradiol on bone marrow cellularity and cell distribution

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<th>Wild Type</th>
<th>GPR30−/−</th>
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<tr>
<td></td>
<td>BM cellularity</td>
<td></td>
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<tr>
<td></td>
<td>Vehicle</td>
<td>E2</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>22.2±1.1</td>
<td>23.7±1.5</td>
</tr>
<tr>
<td>CD19+ B cells</td>
<td>8.2±0.5</td>
<td>8.5±0.6</td>
</tr>
<tr>
<td>Pre B cell (B220−/−)</td>
<td>4.27±0.29</td>
<td>4.27±0.42</td>
</tr>
<tr>
<td>Pre B cell (B220+µ)</td>
<td>1.73±0.16</td>
<td>1.74±0.17</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>0.80±0.07</td>
<td>0.90±0.07</td>
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OVX GPR30−/− and wild-type mice were treated with vehicle or E2 (830 ng·mouse−1·day−1; n = 8–9). Bone marrow (BM) cells were stained with antibodies to CD3 for estimation of the number of T lymphocytes and CD19, B220, and µ for the number and maturation stage of B lymphocytes. Values are number of cells (10^6) per femur. Magnitudes of the estrogenic responses did not significantly differ between GPR30−/− and wild-type mice for any of these parameters. Values are means ± SE. *P < 0.05 vs. vehicle-treated OVX mice.

The number of CD19+ B lymphocytes was dramatically decreased after E2 treatment in both WT (−86%) and GPR30−/− (−80%) mice compared with vehicle treatment, and the different stages of maturation in B cell lymphopoiesis were also similarly affected by E2 treatment in WT and GPR30−/− mice (Table 3). E2 treatment decreased the number of CD3+ T cells in WT mice (−38%), and a similar decrease was found in GPR30−/− mice (−50%) compared with vehicle treatment. The magnitudes of the estrogenic responses did not significantly differ between GPR30−/− and WT mice for any of these parameters.

DISCUSSION

In vitro studies (24–26, 31) have demonstrated that the membrane GPCR GPR30 specifically binds E2 and that E2 via GPR30 activates a number of intracellular signaling events. To determine the in vivo role of GPR30 for estrogenic responses in bone and some other well-known estrogen-responsive tissues, GPR30−/− mice were developed (16). We herein demonstrate that the bone-sparing effect of E2 is independent on GPR30, while this receptor is required for a normal estrogenic response in the growth plate.

Previous studies (5, 8, 12, 14, 22, 29) have shown that the estrogenic responses on trabecular and cortical bone mass (increased by E2), bone marrow cellularity (decreased by E2), uterus weight (increased by E2), and fat weight (decreased by E2) are mainly ERα mediated. To determine the possible role of GPR30 in the effects of E2 on these mainly ERα-dependent parameters, OVX GPR30−/− and WT mice were treated for 4 wk with several different doses of E2. For all E2 doses evaluated, the estrogenic responses on all these well-known estrogen responsive tissues were normal in the GPR30−/− mice. These data clearly demonstrate that GPR30 is not a functional ER for the preservation of bone mass. In addition, GPR30 is not required for the effect of E2 on fat mass or uterus weight.

It was recently reported by Wang et al. (35) that the magnitude of the effect of E2 on thymic atrophy (reduction in thymus weight) was marginally reduced in GPR30−/− mice compared with WT mice, suggesting that GPR30 contributes to E2-induced thymic atrophy. In contrast, in the present study, with the use of another GPR30-inactivated mouse strain, the estrogenic response on thymus weight did not differ significantly at any of the four tested E2 doses between the GPR30−/− and WT mice. The precise reason for these conflicting results regarding the role of GPR30 for E2-induced thymic atrophy is presently unclear. However, it is important to note that in that study by Wang et al. one extremely high supra-physiological E2 dose (2.5 mg/60 days = 41,000 ng·mouse−1·day−1) was used, whereas the
present study tested four different E2 doses (30, 70, 160, or 830 ng·mouse\(^{-1}\)·day\(^{-1}\)) ranging from low physiological to slightly supraphysiological E2 concentrations (17, 35, 36). Therefore, we believe that additional studies are required to determine if GPR30, besides the well-documented role of ER\(\alpha\), contributes significantly to E2-induced thymic atrophy (6, 10, 35).

The key role of estrogens in the regulation of human longitudinal bone growth is supported by the findings that both males and females with estrogen deficiency, caused by a mutation in the aromatase gene, have nonfused growth plates and continue to grow after sexual maturation (18). One male patient with a point mutation in the ER\(\alpha\) had a similar growth phenotype, indicating that ER\(\alpha\) is required for the effects of estrogens on the human growth plate (30). In addition, we (2) have presented data from elderly female ER-inactivated mice indicating that not only ER\(\alpha\) but also ER\(\beta\) is involved in the regulation of the growth plate width. We recently demonstrated that gonadal intact GPR30\(^{-/-}\) mice displayed reduced femur length but as the effect of E2 in OVX mice was not evaluated in our previous study, we could not determine if the affected femur length was a result of altered E2 responsiveness or as a result of an estrogen-independent mechanism modulating longitudinal bone growth (16). In the present study, we show that GPR30 is required for a normal inhibitory effect of E2 on longitudinal bone growth and growth plate height in OVX mice. In the OVX WT mice, the heights of both the proliferative and hypertrophic zones of the growth plate were reduced by E2 treatment, while no such effect was seen in the GPR30\(^{-/-}\) mice. In a similar manner, the height of type X collagen immunostaining, reflecting a hypertrophic chondrocyte phenotype, was reduced by E2 in the WT mice but not in the GPR30\(^{-/-}\) mice. The number of PCNA-positive cells, reflecting proliferating cells, was significantly reduced by E2 in the WT mice but not in the GPR30\(^{-/-}\) mice. However, this was mainly a consequence of the fact that the growth plate height was reduced by E2 treatment in WT mice and, therefore, only a trend of a reduced proportion of PCNA-positive chondrocytes was seen by E2 treatment in WT mice. All these E2-induced effects, observed in the growth plates of WT but not GPR30\(^{-/-}\) mice, might have contributed in the E2 treatment to the reduced femur length observed in WT but not in GPR30\(^{-/-}\) mice treated with E2. Thus, estrogen-mediated regulation of longitudinal bone growth and growth plate morphology requires not only the function of nuclear ER but also the function of GPR30. However, it should be noted that the vehicle-treated GPR30\(^{-/-}\) mice displayed reduced growth plate height compared with vehicle-treated WT mice and that this was associated with a nonsignificant trend of reduced femur length in the vehicle-treated GPR30\(^{-/-}\) mice. Thus, one cannot exclude that the lack of significant E2 effect in the growth plates of GPR30\(^{-/-}\) mice is a consequence of the reduced growth plate activity already existing in vehicle-treated GPR30\(^{-/-}\) mice. A role of GPR30 in the growth plate is supported by our recent finding (3) that GPR30 immunoreactivity is present in the human growth plate with the highest expression in the hypertrophic zone. In addition, we (3) demonstrated that GPR30 immunoreactivity declined during pubertal progression, suggesting that GPR30 might be involved in the modulation of longitudinal bone growth during sexual maturation.

Although we have previously demonstrated that E2-stimulated insulin release is clearly lost in GPR30\(^{-/-}\) mice (16) and we here present evidence that GPR30 is required for a normal estrogenic response in the growth plate, several major E2-responsive tissues, including bone, fat mass, uterine weight, and thymus, display a normal E2 response in GPR30\(^{-/-}\) mice. The unaffected E2 response in the majority of investigated E2-responsive tissues in the GPR30\(^{-/-}\) mice indicates that GPR30 is not a major functional ER in these tissues, but one cannot exclude that there is a high level of redundancy in the estrogen receptor system in these tissues. Our results also suggest that further research should be done to determine if another major GPR30 ligand might exist in vivo.

In conclusion, these in vivo findings demonstrate that GPR30 is not required for the bone-sparing effect of E2 or for the effect of E2 on some other major well-known estrogen-regulated parameters such as fat mass, uterine weight, or thymus weight. In contrast, GPR30 is required for a normal estrogenic response in the growth plate. We propose that the GPCR GPR30 may work in concert with the nuclear ERs in mediating estrogenic effects in the growth plate. Thus, although GPR30 seems not to be a functional ER in several major ER-responsive tissues, it is mediating E2-stimulated insulin release and is required for a normal estrogenic response in the growth plate.

GRANTS

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REFERENCES

ROLE OF GPR30 IN THE EFFECTS OF ESTROGEN


