RESEARCH

Effects of the selective GPER1 agonist G1 on bone growth

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Abstract

Estrogens may affect bone growth locally or systemically via the known estrogen receptors ESR1, ESR2 and G protein-coupled estrogen receptor 1 (GPER1). Mouse and human growth plate chondrocytes have been demonstrated to express GPER1 and ablation of this receptor increased bone length in mice. Therefore, GPER1 is an attractive target for therapeutic modulation of bone growth, which has never been explored. To investigate the effects of activated GPER1 on the growth plate, we locally exposed mouse metatarsal bones to different concentrations of the selective GPER1 agonist G1 for 14 days *ex vivo*. The results showed that none of the concentrations of G1 had any direct effect on metatarsal bone growth when compared to control. To evaluate if GPER1 stimulation may systemically modulate bone growth, ovariectomized C57BL/6 mice were treated with G1 or β -estradiol (E2). Similarly, G1 did not influence tibia and femur growth in treated mice. As expected, E2 treatment suppressed bone growth *in vivo*. We conclude that ligand stimulation of GPER1 does not influence bone growth in mice.

Key Words

- growth plate
- growth
- chondrocytes
- estrogen
- GPER1
- G1
- bone

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Introduction

Estrogen signaling is mediated via two nuclear and one membranous estrogen receptors (ER). Nuclear receptors ER alpha and beta (ESR1 and ESR2) are activated by the binding of specific ligands, followed by the activation of gene transcription (1). In contrast to the classical nuclear receptor signaling, membranous ERs exert their rapid effects via posttranslational modification and recruitment of transcription factors to their DNA-binding sites (2). In contrast to the nuclear ERs, activation of GPER1, a transmembrane ER, leads to rapid changes in the concentrations of messenger molecules and regulation of kinase pathways.

Several studies have found that GPER1 is widely expressed in human tissues, such as, lungs, heart, and the immune system, including myeloid and lymphoid immune cells (3, 4). Moreover, important functional roles

of GPER1 in the reproductive, immune, cardiovascular, endocrine and nervous systems have previously been reported (5, 6). Furthermore, expression of GPER1 has also been reported in human stem cells (7). In addition to the physiological effects, GPER1 might be involved in various estradiol (E2)-dependent diseases, such as atherosclerosis, osteoporosis, autoimmunity and tumors of the reproductive system (4).

With regard to connective tissues, the expression of ESR1, ESR2 and GPER1 has previously been demonstrated in human bone and growth plate cartilage (8, 9) as well as in rat spinal and tibial growth plate chondrocytes (10). Moreover, cytoplasmic expression of GPER1 has been detected in human osteoblasts, osteocytes and osteoclasts (9). Expression of GPER1 has also been found in the cellular compartments outside the nuclei and treatment with E2 had a protective effect against mitophagy via the GPER1 signaling pathway in mouse ATDC5 chondrocytes

(11). Earlier *in vitro* studies have shown that E2 binds to GPER1 (12) and stimulates growth of ESR1-negative breast and endometrial tumor cells (13, 14). Furthermore, a high dose of E2 inhibited longitudinal bone growth in WT but not in *Gper1*^{-/-} mice, suggesting a physiological function of GPER1 in E2-signaling in the growth plate (15). Moreover, male *Gper1*^{-/-} mice were reported to have increased bone mineralization and proliferation in the growth plate (16). Based on these data, we hypothesized that modulation of GPER1 activity may be an attractive therapeutic strategy in children with abnormal growth including extreme short and tall stature. Since E2, the main natural GPER1 agonist, can influence growth plate cartilage and bone growth via both local and systemic routes (1), we designed two distinct experiments to test whether a selective GPER1 agonist G1 may affect bone growth. To study any local effects, mouse metatarsal bones were treated with G1 *ex vivo*. To explore any systemic bone growth effects of the GPER1 agonist, ovariectomized (OVX) female mice were challenged with G1.

Materials and methods

Reagents

G protein-coupled estrogen receptor 1 (GPER1) selective agonist G1 was obtained from Merck Chemicals. Bovine serum albumin (BSA), β -glycerophosphate disodium salt hydrate and 17 β -estradiol-3-benzoate (E2) were purchased from Sigma-Aldrich. Phosphate buffer saline (PBS), DMEM/F12 phenol red-free medium and gentamycin were bought from Invitrogen. L-Ascorbic acid A4403 and β -glycerophosphate disodium salt hydrate G 9422 were purchased from Sigma.

Primary rabbit anti-PCNA antibody (ab18197) was provided by Abcam. Normal donkey serum and secondary CY3-conjugated AffiniPureF(ab) Fragment Donkey Anti-Rabbit IgG antibody were purchased from Jackson ImmunoResearch Laboratories. Fluorescence mounting medium was obtained from Dako. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Sigma-Aldrich.

Animals

Four-day-old mouse pups C57BL/6 were purchased from Charles River Laboratories. Pups were killed 1h after arrival to the animal facility at Karolinska University Hospital in Solna, Sweden. For *ex vivo* organ cultures,

the three middle metatarsal bones were dissected out from each hind paw and cultured as described below ($n=9$ /group). The study was approved by the ethical committee for animal experiments at the Karolinska Institutet, Solna (permit no. N 254/10). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

For *in vivo* studies, 12-week-old female C57BL/6 mice ($n=9-10$ /group) were purchased from Charles River Laboratories. Animals were housed in the animal facility at the University of Gothenburg, Sweden and kept in 12-h day and night condition with standard chow and tap water *ad libitum*. The study was approved by the ethical committee for animal experiments at the University of Gothenburg (permit no. 205-2007).

Organ culture

After dissection, metatarsal bones were transferred to 24-well plates and cultured in 1 mL of phenol red-free and estrogen-free DMEM/F12 medium supplemented with 0.1% BSA, 1 mM β -glycerophosphate, 0.05 mg/mL ascorbic acid and 20 μ g/mL gentamicin added at 37°C under a humidified atmosphere containing 5% CO₂ according to the protocol established by us previously (17). The bones were co-cultured with either vehicle (DMSO) or G1 (1, 10, 100 and 300 nM) dissolved in DMSO. The medium was changed every other day. Digital pictures were captured on day 0, 2, 5, 7, 9, 12 and 14 using a Hamamatsu C4742-95 digital camera attached to a Nikon SMZ-U microscope. The length of each metatarsal bone was measured blindly using the ImageJ software (NIH). The increase in bone length was expressed as a delta of the length at the day of dissection (day 0=baseline).

Ovariectomy and *in vivo* treatment

Mice were randomized into four different groups. Three groups were OVX and one group was sham-operated. Both ovariectomy and sham operations were performed at 12 weeks of age under intraperitoneal anesthesia with ketamine (Ketalar; Pfizer) and medetomidine (Domitor; Orion Pharma, Espoo, Finland). Carprofen (Orion Pharma) was used postoperatively for pain relief. A midline incision was followed by flank incisions of the peritoneum, and the ovaries were removed with sterile scissors. The skin incisions were closed with metal clips. The sham-operated mice were treated in the same way, except that the ovaries were not removed. Sham-operated mice were subcutaneously (s.c.) injected with 100 μ L of

vehicle (10% ethanol and 90% Miglyol 812; Omya Peralta, Hamburg, Germany) ($n=10$). OVX mice were treated s.c. with 100 μ L of E2 (1 μ g/mouse; 0.04 mg/kg/day) ($n=9$), G1 (5 μ g/mouse; 0.2 mg/kg/day) ($n=9$) or vehicle ($n=10$). Biological activity of G1 at the chosen well-established dose has previously been demonstrated *in vivo* (18, 19). All groups received treatments 5 days per week for 4 weeks. The data from E2 and control groups have been previously published by our group (20).

Quantitative histology of tibia growth plates from *in vivo*-treated mice

After killing, bones were dissected out and the length of the tibiae and femora was measured with a pocket Vernier caliper (Helios-Preisser, Gammertingen, Germany). The tibiae were fixed in 4% phosphate-buffered formalin for 24 h, decalcified in 10% EDTA for 2 weeks and stored in 70% ethanol. For histological analysis, tibiae were embedded in paraffin and 5 μ m thick sections were prepared from the paraffin blocks and stained with Alcian blue/van Gieson. The quantitative analysis was performed by measuring two-third of the growth plate sections at 10 $\times$ magnification. Images were captured by a Nikon Eclipse E800 light microscope (Nikon) connected to the digital camera. The heights of the tibia growth plate, proliferative and hypertrophic zones were calculated as an average of 20 measurements per growth plate. Hypertrophic chondrocytes were defined as cells with a height larger than 7 μ m. All measurements were made in a blind manner using the Image Pro Plus, version 6.3 software.

Immunofluorescence for the detection of PCNA expression

For immunofluorescence, the tibia growth plate sections were deparaffinized for 40 min at 60°C, rehydrated in xylene, 100% ethanol, 95% ethanol and 75% ethanol, for 5 min in each solution and finally washed with water. Sodium citrate buffer (10 mM, pH 6.0) was used for antigen retrieval for 15 min at 95°C. Following the retrieval, the slides were incubated with 1.5% donkey serum in PBS for 1 h at room temperature (RT), with primary rabbit anti-PCNA antibody (1:250 dilution) overnight at 4°C and with secondary donkey anti-rabbit antibody (1:250 dilution) for 1 h at RT. Nuclear staining with DAPI was performed for 15 min at RT. After the staining, the slides were mounted with Dako Fluorescence Mounting Medium.

TUNEL assay (apoptosis detection)

The TUNEL assay was performed on tibia growth plate sections according to the manufacturer's instructions. Briefly, apoptotic chondrocytes were identified by TUNEL immunohistochemistry applying the TdT-FragEL DNA fragmentation kit (Oncogene Research, Boston, MA, USA) according to the manufacturer's protocol. Alexa-546 (red fluorescence) (Invitrogen)-positive cells represented apoptotic chondrocytes.

Statistical analysis

All the data are presented as means $\pm$ S.E.M., and the differences were calculated by one-way ANOVA using the SPSS software by multiple comparisons vs control group (Holm–Sidak method). *** $P<0.001$, ** $P<0.01$ were considered as statistically significant.

Results

Effect of G1 treatment on mouse metatarsal bone growth

Four-day-old mouse metatarsal bones were cultured with either vehicle or the GPER1 agonist G1 at concentration 1, 10, 100, or 300 nM for 14 days. Measurements of bone lengths over the 14-day period of culture showed that none of the tested G1 concentrations had any effect on metatarsal bone growth (Fig. 1A, B, C and D).

Effects of E2 and G1 on tibia and femur growth *in vivo*

The effects of vehicle, E2 and G1 on tibia and femur lengths were analyzed in OVX mice. As expected, E2 inhibited both tibia and femur growth in OVX mice compared to the vehicle treated OVX group ($P<0.01$, Fig. 2A and B), whereas ovariectomy promoted bone growth in vehicle compared to sham-operated mice ($P<0.01$, Fig. 2A and B). In contrast, G1 treatment did not affect tibia and femur lengths in OVX mice compared to vehicle-treated OVX mice (Fig. 2A and B).

Effects of *in vivo* exposure to E2 and G1 on tibia growth plate morphology

To analyze the effects of E2 and G1 on tibia growth plate morphology, OVX mice were treated with

Figure 1

Effects on bone growth of the GPER1 agonist G1 in ex vivo cultured mouse metatarsal bones. The metatarsal bones were treated with 1 nM (A), 10 nM (B), 100 nM (C) or 300 nM (D) of G1 or vehicle. No significant effects of G1 were seen at any of the concentrations ($n = 9$). All the data are presented as means $\pm$ s.e.m., and the differences were calculated by one-way ANOVA.

E2, G1, or vehicle. As expected, histological analyses showed that the growth plate was narrower in sham-operated mice compared to vehicle-treated OVX mice ($P < 0.01$, Figs 3A, B and 4A). Treatment of OVX mice with E2 decreased growth plate height to a similar size as seen in sham-operated controls (Figs 3A, C and 4A). In contrast to E2, G1 treatment did not affect growth plate height compared to vehicle-treated OVX mice (Figs 3B, D and 4A).

Morphometric analyses showed that heights of both proliferative and hypertrophic zones were decreased in E2-treated OVX mice compared to the vehicle group ($P < 0.001$, Fig. 4B and C). In contrast, G1 treatment did not affect proliferative and hypertrophic zone heights compared to the vehicle group (Fig. 4B and C). As expected, proliferative and hypertrophic zone heights

were decreased in sham-operated mice compared to vehicle-treated OVX animals ($P < 0.001$, Fig. 4B and C).

Effects of *in vivo* treatment with E2 and G1 on chondrocyte proliferation and apoptosis

The histological analysis of PCNA staining of tibia growth plate cartilage showed decreased proliferation in E2-treated OVX mice compared to the vehicle group. Furthermore, proliferation was also suppressed in sham-operated mice compared to the OVX vehicle group ($P < 0.001$, Fig. 5A). In contrast, G1 treatment did not affect chondrocyte proliferation compared to the vehicle group (Fig. 5A). Effects of E2 and G1 on chondrocyte apoptosis were studied by TUNEL assay. No significant differences in apoptosis level were observed between the treatment groups (Fig. 5B).

Figure 2

Effects of E2 and G1 on tibia and femur lengths. The study groups included ovariectomized mice treated with E2 (0.04 mg/kg/day), G1 (0.2 mg/kg/day) or vehicle alone and sham-operated mice. Vehicle vs E2, vehicle vs sham, $**P < 0.01$. Tibia length (A) and femur length (B) ($n = 9-10$). All the data are presented as means $\pm$ s.e.m., and the differences were calculated by one-way ANOVA.

Figure 3

Effects of E2 and G1 on tibia growth plate morphology. Alcian blue/van Gieson staining of mouse proximal tibia growth plate obtained from either sham-operated or ovariectomized mice treated with E2 (0.04 mg/kg/day), G1 (0.2 mg/kg/day), or vehicle. H, hypertrophic zone; P, proliferative zone; R, resting zone. Treatment groups: sham (A), vehicle (B), E2 (C), and G1 (D). Magnification: 40 $\times$.

Discussion

Our data clearly show that the selective GPER1 agonist G1 did not affect growth plate cartilage or bone growth

at different concentrations in *ex vivo*-cultured mouse metatarsal bones. Furthermore, *in vivo* study in OVX mice has reported that ligand activation of GPER1 by the systemic administration of G1 did not influence either

Figure 4

Effects of E2 and G1 on tibia growth plate height in different zones. The study groups included ovariectomized mice treated with E2, G1 or vehicle (DMSO) and sham-operated mice. Growth plate height (A) and height of proliferative (B) and hypertrophic (C) zones. Vehicle vs E2, vehicle vs sham: *** $P < 0.001$ ($n = 9-10$). All the data are presented as means $\pm$ s.e.m., and the differences were calculated by one-way ANOVA.

Figure 5

Effects of E2 and G1 on proliferation and apoptosis in tibia growth plate chondrocytes. The effects on proliferation and apoptosis were analyzed by PCNA staining (A) and TUNEL assay (B). Vehicle vs E2, vehicle vs sham: *** $P < 0.001$. No significant difference was seen between vehicle and G1 ($n = 9-10$). All the data are presented as means $\pm$ S.E.M., and the differences were calculated by one-way ANOVA.

tibia or femur growth as well as growth plate height, chondrocyte proliferation or apoptosis. In contrast, E2 suppressed both tibia and femur growth in OVX mice.

Despite the observed expression of GPER1 in mouse and human growth plate chondrocytes (8, 11) and bone elongation in the *Gper1*^{-/-} mice (16), the therapeutic potential for GPER1 agonists has not yet been investigated. To address this gap of knowledge, we chose a well-established experimental model of *ex vivo*-cultured postnatal mouse metatarsal bones to study local effects on the bone growth. By testing different G1 concentrations, we found that G1 had no direct effects on metatarsal bone growth compared to control. To investigate if GPER1 stimulation influences bone growth through systemic actions, OVX mice were treated with G1. Similarly to the *ex vivo* results, G1 treatment did not affect tibia and femur growth *in vivo*. Applied as a positive control, E2 treatment suppressed bone growth and growth plate height as expected.

Our initial hypothesis that the GPER1 agonist may affect bone growth was based on earlier reported correlation between the expression of GPER1 in the growth plate and pubertal stage in children (8) as well as suppressive effects of high doses of E2 on bone growth in WT, but not in *Gper1*^{-/-} mice (15). Other studies have shown that GPER1 may regulate E2-mediated activation of transcription (21, 22) and insulin release in female mice (23). Furthermore, earlier *in vitro* studies have demonstrated that E2 may suppress chondrogenic differentiation of mesenchymal stem cells via GPER1 (24). Nevertheless, our present data showed that the GPER1 agonist G1 did not influence chondrocyte proliferation when applied locally to the *ex vivo*-cultured metatarsal bones.

Earlier published data on the expression and effects of GPER1 in different organs suggested systemic functions of ligand-activated GPER1 (5, 25). To evaluate potential systemic ligand-mediated actions of GPER1 on bone growth, we applied an experimental model of OVX female mice since the removal of endogenous E2 helps to identify G1-mediated effects. Our present *in vivo*

study allowed us to conclude that systemic G1-mediated stimulation of GPER1 does not influence the growth of long bones. Furthermore, *in vivo* G1 treatment did not affect the heights of the growth plate or the proliferative or hypertrophic zones. A minor elongation of the hypertrophic zone was observed in the G1-treated group when compared to vehicle, although this difference was not significant. Therefore, we do not expect that G1 would affect the terminal chondrocyte differentiation in the growth plate. Moreover, systemic G1 treatment did not influence chondrocyte proliferation or apoptosis. Altogether, our data suggest that GPER1 is unlikely directly involved in the regulation of bone growth, despite the demonstrated high expression of GPER1 in the human and rodent growth plate (8, 10). Nevertheless, alterations of GPER1 expression during the course of the experiment cannot be excluded.

As G1 has been shown to inhibit the activity of nuclear ERs (26), this potential cross-reactivity should be considered when evaluating G1 effects in the growth plate where all three ERs are expressed. An earlier study indeed suggested that GPER1 might interact with the nuclear ESR1 (27). Moreover, as earlier published, high doses of E2 inhibited bone growth in WT but not in *Gper1*^{-/-} mice (15). In contrast to that study, we here used a selective GPER1 agonist G1 to specifically activate GPER1. This approach allowed us to investigate ligand-mediated effects of GPER1 signaling in the growth plate. Several earlier findings have revealed the complexity of GPER1-mediated effects (28, 29, 30). In addition to the ligand-mediated effects, GPER1 may act in a ligand-independent manner in the growth plate. An earlier study demonstrated ligand-independent stimulation of proliferation in ovarian tumor cells by GPER1 (28). Our present data cannot exclude ligand-independent effects of GPER1 in the growth plate. A similar dose of G1 has been used in several earlier animal studies, where significant systemic effects were documented (18, 19, 29, 30).

Thus, the present study did not confirm our hypothesis that ligand-activated GPER1 influences bone growth.

Although we performed a dose–response *ex vivo* study in cultured bones and tested the previously *in vivo* validated dose and treatment duration of G1 in mice, we cannot completely exclude that using younger mice or treating with other concentrations of G1 for longer time would lead to another conclusion.

Altogether, we conclude administration of the selective GPER1 agonist G1, either *ex vivo* or *in vivo*, did not affect bone growth or growth plate morphology. Our present data are in line with earlier published results suggesting that E2 primarily modulates bone growth via ESR1.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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