Investigation of central versus peripheral effects of estradiol in ovariectomized mice

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Abstract

It is generally believed that estrogens exert their bone sparing effects directly on the cells within the bone compartment. The aim of the present study was to investigate if central mechanisms might be involved in the bone sparing effect of estrogens. The dose–response of central (i.c.v.) 17 β -estradiol (E2) administration was compared with that of peripheral (s.c.) administration in ovariectomized (ovx) mice. The dose–response curves for central and peripheral E2 administration did not differ for any of the studied estrogen-responsive tissues, indicating

that these effects were mainly peripheral. In addition, ovx mice were treated with E2 and/or the peripheral estrogen receptor antagonist ICI 182,780. ICI 182,780 attenuated most of the estrogenic response regarding uterus weight, retroperitoneal fat weight, cortical BMC and trabecular bone mineral content ($P < 0.05$). These findings support the notion that the primary target tissue that mediates the effect of E2 on bone is peripheral and not central.

Journal of Endocrinology (2005) **187**, 303–309

Introduction

Estrogens are of importance for the regulation of bone metabolism in both males and females. It is generally believed that estrogens exert their bone sparing effects directly on the cells within the bone compartment. However, some studies indicate that the effects of estrogens on bone might be indirect and mediated via other tissues (Vanderschueren *et al.* 2004). An indirect mechanism is supported by the fact that the effects of estrogens *in vitro* on cultured osteoblasts/osteoclasts, are often small and sometimes only seen when the cells are transfected with a high number of estrogen receptors (Vanderschueren *et al.* 2004), while the *in vivo* effects of estrogens on bone are more pronounced (Clark *et al.* 2004). In addition, the bone sparing effect of estrogens on bone is partly lost after hypophysectomy (Yeh *et al.* 1997) and it has been suggested that estrogens increase bone mass partly through the stimulation of pituitary growth hormone secretion (Vanderschueren *et al.* 2004). Furthermore, it has been suggested that the hypothalamus might be of importance

in the regulation of not only fat mass but also bone mass, and that osteoporosis may at least in part be a hypothalamic disease (Baldock *et al.* 2002, Takeda *et al.* 2002). Animal experiments have shown that the inactivation of central leptin receptors (Takeda *et al.* 2002), or of hypothalamic neuropeptide Y Y2 receptors (Baldock *et al.* 2002), resulted in increased bone mass. It was proposed that activation of either of these receptors causes a tonic inhibition of bone mass. Furthermore, we have recently demonstrated that inactivation of another hypothalamic receptor, the melanin concentrating hormone receptor-1, results in decreased bone mass (Bohlooly *et al.* 2004), further supporting the notion that the hypothalamus is an important target organ and sensor for factors regulating bone mass. Thus, one may speculate that the effects of estrogens on bone might include hypothalamic mechanisms. In a previous preliminary investigation, it was indicated that the pituitary gland might be involved in the estrogenic effect on bone. Estrogen treatment to rats that were both hypophysectomized and ovariectomized (ovx) did not result in any beneficial effects on bone mass,

suggesting that a pituitary product is either obligatory or a critical co-factor for estrogen-related effects on bone (Bryant *et al.* 1994).

The aim of the present study was to investigate the relative contribution of peripheral versus central estrogen receptors in the mediation of the effects of 17 β -estradiol (E2) on bone and on some other estrogen-responsive tissues. Two different approaches were used to investigate the relative importance of estrogen receptors in the peripheral versus central estrogen receptors. We first compared the dose–response of central E2 administration with that of peripheral E2 administration in ovx mice. In addition, ovx mice were treated with E2 and/or the peripheral estrogen receptor antagonist ICI 182,780. We demonstrate that the primary target tissue for that mediates the effect of E2 on bone is peripheral and not central.

Material and Methods

Animals

Female C57BL/6 mice were used in all experiments (Taconic M&B, Skensved, Denmark). The animals were maintained under a strict 12 h light:12 h darkness cycle at 22 °C with standard rat food pellets (Lactamin AB, Stockholm, Sweden) and tap water available *ad libitum*. The animal experiments were approved by the local Ethics Committee for animal studies at Göteborg University.

Experiment 1 In the first dose–response study, 8-week-old C57BL/6 mice were ovariectomized under isoflurane (Baxter Medical AB, Kista, Sweden) anaesthesia. The ovx model for osteoporosis is included in the FDA guidelines for preclinical and clinical evaluation for agents used for the treatment of postmenopausal osteoporosis (Thompson *et al.* 1995, Andersson *et al.* 2001). The mice were treated with increasing doses (0, 0.01, 0.1, 1.0, 10 and 100 μ g/kg per day; $n=5$ mice per group) of E2 (cyclodextrin encapsulated E2, Sigma) for 2 weeks. E2 was administered using s.c. implanted osmotic minipumps (Model 1002, Alzet, Cupertino, CA, USA). Control mice received infusion of the vehicle (20% (w/v) 2-hydroxypropyl- β -cyclodextrin, Sigma) (Yaksh *et al.* 1991).

Experiment 2 In the second study, the dose–response effect of i.c.v. infused E2 was compared with the dose–response effect of s.c. infused E2. 214 8-week-old C57BL/6 mice (Taconic) were ovariectomized under isoflurane anaesthesia. One week after ovariectomy the mice were anaesthetized with isoflurane and a brain infusion cannula (Alzet) was stereotactically implanted into the left ventricle using the following coordinates from *Bregma*: 0.46 mm posterior, 1.0 mm lateral and 2.2 mm ventral (Hof *et al.* 2000). The brain infusion cannula was secured with two supporting screws and acrylic dental cement (AgnTho's, Lidingö, Sweden) to the calvarial

surface. The brain infusion cannula was connected to a dorsally implanted s.c. osmotic minipump (Alzet) with a polyvinylchloride tube (Alzet). In addition, a second osmotic minipump was implanted s.c. on the back of each mouse (for peripheral administration). The mice were treated for 12 days with increasing doses (0, 0.01, 0.03, 0.1, 0.3, 1.0, 3, 30 and 100 μ g/kg per day) of E2 (cyclodextrin encapsulated E2, Sigma) or vehicle (20% (w/v) 2-hydroxypropyl- β -cyclodextrin, Sigma) (Yaksh *et al.* 1991) via either the central (i.c.v.) or the peripheral (s.c.) route of administration. In order to avoid confounding factors, all animals received continuous infusion via both administration routes simultaneously (i.e. E2 i.c.v. and vehicle s.c. or vice versa).

Experiment 3 In the third study, 13-week-old C57/BL6 mice (M&B, Uppsala, Sweden) were ovariectomized two weeks before the start of treatment. The treatment groups ($n=7$ –10 mice per group) were: (i) ovx+vehicle (Miglyol 812 (fractionated coconut oil), Recip AB, Årsta, Sweden); (ii) ovx+E2 (30 μ g/kg per day, Sigma); ovx+ICI 182,780 (8.3 mg/kg per day; Tocris Cookson Ltd, Bristol, UK) and ovx+E2+ICI 182,780. The drugs were administered s.c. on a daily basis during 3 weeks. Organs and tissues were removed and wet weights recorded. The left femur and tibia were dissected out and fixed in 10% formalin, washed thoroughly and stored in 70% ethanol until bone density measurements were performed.

Peripheral Quantitative Computerized Tomography (pQCT)

pQCT was performed with the Stratec pQCT XCT Research M (v5.4B; Stratec Medizintechnik GmbH, Pforzheim, Germany) operating at a resolution of 70 μ m as previously described (Windahl *et al.* 1999, Andersson *et al.* 2001). Trabecular bone mineral density (BMD) was determined *ex vivo*, with a metaphyseal pQCT scan of the proximal tibia. The scan was positioned in the proximal tibial metaphysis at a distance corresponding to 3% of the total length of the tibia, in the distal direction from the proximal growth plate. The trabecular bone region was defined as the inner 45% of the total cross-sectional area. Cortical bone mineral content (BMC) was determined *ex vivo* with a mid-diaphyseal pQCT scan of the femur.

Statistical analyses

All data are presented as mean $\pm$ S.E.M. Differences in E2 sensitivity between different tissues were tested using two-way ANOVA in which dose and tissue were regarded as separate treatments. Differences between i.c.v. and s.c. administration were tested using two-way ANOVA in which dose and administration route were regarded as separate treatments. Differences in E2 response with or without ICI 182,780 were calculated by Student's *t*-test.

Table 1 Effect of a high dose 17 β -estradiol (100 μ g/kg per dose) on well known estrogen-responsive tissues. Values are means $\pm$ S.E.M. ($n=5$ mice per group). Statistical significance was assessed for the difference between vehicle treatment and 17 β -estradiol (E2) treatment

	Ovx+vehicle	Ovx+E2
Uterine weight (mg)	17.4 $\pm$ 0.6	129.2 $\pm$ 9.1***
Trabecular BMD (mg/cm ³)	140 $\pm$ 17	367 $\pm$ 5.5***
Cortical BMC (mg/mm)	0.85 $\pm$ 0.03	1.02 $\pm$ 0.06*
Retroperitoneal fat weight (mg)	134 $\pm$ 22	52.8 $\pm$ 5.1**
Thymus weight (mg)	75.8 $\pm$ 6.6	18.6 $\pm$ 1.0***

* $P<0.05$; ** $P<0.01$; *** $P<0.001$ (Students t -test).

Results

Tissue sensitivity for E2 treatment

The effects of a high dose of peripherally administered E2 (100 μ g/kg per day) on several well known estrogen responsive tissues were first investigated in ovx mice. As expected, the E2 treatment increased the uterine weight (743% of vehicle), the trabecular BMD (262% of vehicle) and the cortical BMC (121% of vehicle) while it reduced the weights of the retroperitoneal fat depot (39% of vehicle) and the thymus (25% of vehicle, Table 1). The affect of estrogen on cortical BMC was due to an effect on cortical bone size while cortical volumetric BMD was unaffected (data not shown). In addition, dose-response experiments of the effect of peripherally administered E2 on these estrogen-responsive tissues were performed

(Figure 1). These experiments demonstrated that the uterus (ED₅₀ 0.9 μ g/kg per day) and the cortical bone (ED₅₀ 0.9 μ g/kg per day) were the most sensitive tissues with regard to E2 treatment. In contrast, the trabecular bone (ED₅₀ 12 μ g/kg per day) was more than 10 times less sensitive than the uterus and the cortical bone ($P<0.01$). The estrogen sensitivity was intermediate for the effects of E2 on thymus weight (ED₅₀ 1.9 μ g/kg per day) and retroperitoneal fat weight (ED₅₀ 1.7 μ g/kg per day; Fig. 1). Thus, the uterus and the cortical bone were the most sensitive tissues, thymus and retroperitoneal fat were of intermediate sensitivity and trabecular bone was the least sensitive tissue with regard to E2 treatment.

Comparison of the dose-response effects of centrally versus peripherally administered E2

To investigate whether the effects of E2 on the above mentioned estrogen-responsive tissues were primarily mediated via a central mechanism, ovx mice were treated with increasing doses of E2 administered either s.c. or i.c.v. We hypothesized that a centrally mediated effect of E2 should result in a left shift of the dose-response curve for i.c.v. administration compared with s.c. administration (i.e. ED₅₀ for i.c.v. should be lower than ED₅₀ for s.c.), while no such left shift should occur for a peripheral effect. No significant difference between the s.c. and i.c.v. route of administration was seen for the effect of E2 on uterine weight, thymus weight, trabecular BMD or cortical bone (two-way ANOVA). Thus, the dose-response curves for centrally versus peripherally administered E2 did not

Figure 1 Tissue sensitivity for estradiol treatment. The dose-response effect of peripherally administered E2 was determined in estrogen responsive target organs. All organs responded in a dose-dependent manner to E2. Estrogen increased uterine weight, cortical bone mineral content (BMC) and trabecular bone mineral density (BMD) while it decreased retroperitoneal fat weight and thymus weight. Values are expressed as a % of the maximal effect of E2.

Figure 2 Comparison of the dose-response effects of central versus peripheral administered E2. Dose-response effect of 17β-estradiol following peripheral (s.c.) and central (i.c.v.) administration of 17β-estradiol on (A) uterine weight, (B) thymus weight (C) trabecular bone mineral density (BMD) and (D) cortical bone mineral content (BMC). Ovx mice were treated with various doses of 17β-estradiol for 14 days. 17β-estradiol was administered either through the s.c. or the i.c.v. route using subcutaneously implanted osmotic minipumps. Values are expressed as a mean ± S.E.M % of maximal effect of 17β-estradiol.

differ for any of the studied estrogen-responsive tissues (Fig. 2).

Investigation of central versus peripheral E2 effects, using a peripheral estrogen receptor antagonist (ICI 182,780)

The central versus peripheral estrogen effects on selected estrogen responsive tissues were also investigated using an alternative approach. Ovx mice were treated with E2 (30 μg/kg per day, and/or the estrogen receptor antagonist

ICI 182,780 (8.3 mg/kg per day). It has previously been shown that ICI 182,780 does not pass the blood-brain barrier (BBB) Wakeling *et al.* 1991, Wakeling & Bowler 1992, Wade *et al.* 1993, DeFriend *et al.* 1994, Thomas *et al.* 1994). Thus, ICI 182,780 is a peripheral estrogen receptor antagonist. As expected, the E2 treatment increased the uterine weight (1428 ± 90% of vehicle, $P < 0.05$), the trabecular BMD (131 ± 6% of vehicle, $P < 0.05$) and the cortical BMC (109.6 ± 1.8% of vehicle, $P < 0.05$) while it reduced the weight of the

Figure 3 Investigation of central versus peripheral E2 effects, using a peripheral estrogen receptor antagonist (ICI 182,780). The effects of E2 in the absence or presence of estrogen receptor antagonists were compared. Values are expressed as mean $\pm$ S.E.M. % effect of E2 in estrogen treated ovx mice compared with vehicle treated ($n=7-10$). * $P<0.05$, ** $P<0.01$ E2 versus E2+ICI 182,780 students t -test.

retroperitoneal fat depot ($47 \pm 6\%$ of vehicle, $P<0.05$) and the thymus ($53 \pm 5\%$ of vehicle, $P<0.05$). We then calculated to which extent ICI 182,780 could attenuate the response to E2 in these tissues. A high degree of attenuation would indicate that the effect on the specific tissue is peripherally mediated (PM) since the estrogen antagonist does not cross the BBB. ICI 182,780 attenuated most of the estrogenic response regarding uterus weight (PM $\geq 92\%$, $P<0.05$), retroperitoneal fat weight (PM $\geq 85\%$, $P<0.05$), cortical BMC (PM $\geq 92\%$, $P<0.05$) and trabecular BMD (PM $\geq 61\%$, $P<0.05$) while the attenuation was intermediate for the thymus (PM $\geq 54\%$, not significant, Fig. 3).

Discussion

Besides being important for the reproductive system, estrogens are of importance for the regulation of the bone mass as demonstrated by the pronounced bone loss in various estrogen deficient states, such as postmenopausal osteoporosis and after ovariectomy (Riggs *et al.* 2002). Despite extensive studies, the exact mechanism behind ovx-induced bone loss is not fully understood. It is generally believed that estrogens exert their bone sparing effects directly on the cells within the bone compartment. The bone sparing effects of estrogen in rodents are believed not only to be mediated through inhibition of bone resorption, but also through stimulation of bone formation (Chow *et al.* 1992). However, some studies indicate that the effects of estrogens on bone are indirect and may include central mechanisms (Yeh *et al.* 1997, Baldock *et al.* 2002, Takeda *et al.* 2002, Bohlooly *et al.* 2004, Sainsbury *et al.* 2003, Vanderschueren *et al.* 2004).

Our present findings support the concept that most of the E2 effect on bone is primarily mediated via peripheral and not central estrogen receptors.

We first investigated the magnitude and the sensitivity of the E2 effect in different estrogen responsive tissues when E2 was administered peripherally (s.c.). As expected the largest estrogen response compared with control was seen for the uterine weight and for the trabecular BMD, while the maximal response for the cortical BMC and on fat mass was of minor magnitude but still highly statistically significant. However, regarding the sensitivity for E2, it was shown that the uterus and the cortical bone were the most sensitive tissues while the trabecular bone was more than 10 times less sensitive (lower ED₅₀ value for cortical bone and uterus than in trabecular bone). The sensitivity of fat and thymus was intermediate. Thus, a different tissue pattern was observed for the maximal magnitude of E2 response compared with the sensitivity to E2. The higher sensitivity for E2 in cortical bone than in trabecular bone might indicate that the mechanism of action for E2 in these two bone compartments differs. However, one can not exclude that the biological and clinical relevance of a specific dose of estradiol on trabecular BMD (with a relatively high ED₅₀) might be of more importance than that on cortical BMC (with a relatively low ED₅₀), due to the fact that the maximal response is much higher for trabecular BMD than that for cortical BMC. It has previously been suggested that the cortical BMC but not the trabecular BMD is dependent on circulating IGF-I (Sjögren *et al.* 2002) and, therefore, one might speculate that the stimulatory E2 effect on cortical bone (but not that on trabecular bone) is IGF-I-dependent.

Since the ovariectomized mouse model is frequently used for the tissue specific evaluation of selective estrogen receptor modulators, the need for a well characterized mouse model becomes apparent. The present study provides important information regarding the dose-response of estrogen on several estrogen-responsive tissues, including the uterus, the thymus, the retroperitoneal fat depot as well as the cortical and the trabecular bone compartments separately. Furthermore, due to the development of a large number of genetically engineered mouse models, the ovariectomized mouse model has been frequently used as an animal model for postmenopausal osteoporosis (Lindberg *et al.* 2002, Hikiji *et al.* 2004, Usui *et al.* 2004). It is therefore surprising that very few studies have characterized the dose-response effect of E2 in different tissues in this model (Mödder *et al.* 2004). Most of the genetically engineered mouse models have been bred to the C57BL/6 background strain. Thus, the mouse in general and the C57BL/6 strain in particular, needs to be further characterized with respect to the dose-response of estrogens in different tissues. In a recent study, the dose-response of E2 was studied in C57BL/6 mice (Mödder *et al.* 2004). This study demonstrated that the relative effects of E2 on bone and the uterus are highly

dependent on the particular experimental conditions used, with a relatively high uterine E2 sensitivity in 3-month-old mice but a relatively low uterine E2 sensitivity in 6-month-old mice (Mödder *et al.* 2004). In the present study, using 2-month-old C57BL/6 mice, we found that the uterus was highly E2 sensitive compared with the trabecular bone. Although we may not be able to compare these studies, it seems clear that the uterus in young mice is more sensitive to E2 than that of older mice whereas the estrogenic response in bone is not so much affected by age.

In the present study, we hypothesized that a centrally mediated effect of E2 on a tissue should result in a left shift of the dose–response curve for i.c.v. administration compared with s.c. administration (i.e. ED₅₀ for i.c.v. should be lower than ED₅₀ for s.c.), while no such left shift should occur for a peripherally mediated effect. The dose–response curves for central, compared with the peripheral administration of E2 did not differ for any of the studied estrogen-responsive tissues, supporting the notion that these effects were mainly peripherally mediated. Our second approach demonstrated that the peripheral estrogen receptor antagonist ICI 182,780 attenuated most of the estrogenic response regarding uterine weight, retroperitoneal fat weight, cortical BMC and trabecular BMD, further supporting the notion that the primary target tissues for the mediation of the effect of E2 are peripheral and not central.

In this study, we have used s.c. implanted osmotic minipumps to deliver E2 to the mice. The osmotic minipumps can deliver a constant dose of E2 with a plasma profile similar to that of a slow release pellet, which is commonly used in long-term *in vivo* studies (Fara & Urquhart 1984, Ebling *et al.* 2000). The major advantage of using osmotic minipumps is that they can be connected to a brain infusion cannula, enabling continuous long term i.c.v. administration. Thus, with the minipumps we were able to avoid much of the daily handling trauma and anesthesia that would occur if the mice would have been given daily i.c.v. injections. A limitation with the present study is that the effects of central and peripheral estradiol treatment on food intake and GH-secretion, which might be involved in some of the estrogenic effect on bone, not were analysed.

In conclusion, the sensitivity for E2 is tissue-dependent and our findings indicate that the primary target tissue for the mediation of the effect of E2 on bone is peripheral and not central.

Acknowledgements

The authors extend their appreciation to Anette Hansevi and Maud Petersson for their technical assistance. We would also like to thank the SWEGENE Center for Bio-Imaging (CBI) for technical support regarding image analysis.

Funding

This study was supported by the Swedish Research Council, the Swedish Foundation for Strategic Research, the Tore Nilson Foundation, SWEGENE, European Commission Grant QLK4-CT-2002-02528, the Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, Petrus and Augusta Hedlunds Foundation and the Novo Nordisk Foundation. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 10 June 2005

Accepted 5 August 2005