***In vivo* activation of gene transcription via oestrogen response elements by a raloxifene analogue**

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Abstract

Raloxifene is a selective oestrogen receptor modulator with tissue-specific effects. The mechanisms behind the effects of raloxifene are partly unclear, and the aim of the present study was to investigate whether raloxifene can activate the classical oestrogen-signalling pathway *in vivo* in three known oestrogen-responsive organs, uterus (reproductive organ), bone (non-reproductive organ) and thymus (immune organ). For this purpose, we have used reporter mice with a luciferase gene under control of oestrogen-responsive elements (EREs), enabling detection of *in vivo* activation of gene transcription via the classical oestrogen pathway. Three-month-old ovariectomized ERE-luciferase mice were treated with the raloxifene analogue (LY117018), oestradiol (OE₂) or vehicle for 3 weeks. Luciferase activation was measured in bone, uterus and thymus, and compared to bone parameters, and uterus and thymus

weights. The raloxifene analogue affected bone mineral density (BMD) to the same extent as OE₂, and both treatments resulted in increased luciferase activity in bone. As expected, OE₂ treatment resulted in increased uterus weight and increased uterine luciferase activity, while the effect of LY117018 on uterus weight and luciferase activity was modest and significantly lower than the effect of OE₂. LY117018 and OE₂ treatment resulted in similar luciferase activation in thymus. However, only OE₂ treatment resulted in thymic atrophy, while no effect on thymus weight was seen after LY117018 treatment. In summary, the raloxifene analogue LY117018 can activate the classical oestrogen pathway in bone, uterus and thymus *in vivo*, and this activation is associated with BMD and uterus weight, but not thymus weight.

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Introduction

Oestrogens are of importance for many functions in the body including regulation of the female reproductive system, skeletal growth and bone metabolism, and the immune system.

Oestrogens affect gene transcription in target cells via two main mechanisms, the classical and the non-classical pathway. In the classical pathway, oestrogens bind oestrogen receptors, α (ER α ; ESR1 as listed in the MGI Database) or β (ER β ; ESR2 as listed in the MGI Database), belonging to the nuclear hormone receptor superfamily (Green *et al.* 1986, Kuiper *et al.* 1996). The oestrogen-ER complexes form dimers and translocate into the cell nucleus where it interacts with oestrogen response elements (EREs), located in promoter regions of target genes to initiate gene transcription (Pettersson *et al.* 1997). The oestrogen-ER complex can also interact with other transcription factors, such as *Ap1*, *Sp1* and *Nfkb* (listed as *Nfkb* in the MGI Database), and initiate transcription without binding directly to DNA via the

non-classical pathways (Galien & Garcia 1997, Kushner *et al.* 2000). Oestrogens have been demonstrated to affect gene transcription in oestrogen-responsive organs, such as bone and uterus, via both classical and non-classical signalling pathways (Ciana *et al.* 2001, Jakacka *et al.* 2002, Syed *et al.* 2005, Windahl *et al.* 2007).

Raloxifene is a selective ER modulator (SERM) approved to prevent and treat osteoporosis in postmenopausal women, and in the US, it is also approved as prophylaxis against invasive breast cancer in postmenopausal women (Stefanick 2005). It is selective by having agonistic oestrogenic effects on blood lipids and bone formation (Sato *et al.* 1995), while it has antagonistic effects on reproductive tissues (Black *et al.* 1994, Cummings *et al.* 1999). Raloxifene binds both ER α and ER β (Glasebrook *et al.* 1993, Gize *et al.* 1997), and the importance of ERs for the physiological effects of raloxifene has been demonstrated in various cell types using the selective ER antagonist ICI182,780 (Ashby *et al.* 1997, Viereck *et al.* 2003). The interaction between raloxifene and ER causes structural differences in the

complex compared with the oestrogen-ER complex (Bryant *et al.* 1999), and these structural differences result in assembly of other coregulators (Cheskis *et al.* 1997), which causes the tissue-specific agonistic/antagonistic effects of raloxifene.

By which signalling pathway raloxifene mediates its oestrogen-like effects in various tissues is still not clearly understood. Most previous studies on how raloxifene exerts its effects have been performed *in vitro*. Several studies have reported lack of raloxifene signalling via ERE in uterine cancer cell lines. Instead, the non-classical pathway, involving *Ap1*, is suggested to be more important in these cells (Paech *et al.* 1997, Cheung *et al.* 2003). In cultured osteoblasts, it has also been demonstrated that raloxifene may not initiate transcription via the classical pathway (Yang *et al.* 1996b).

From a public health point of view, oestrogen has positive effects such as being bone preserving, but also negative effects, such as increasing the risk for breast cancer. To be able to understand the mechanisms behind these diverse effects, it is important to know by which intracellular pathways oestrogen and SERMs mediate their biological effects. Since oestrogen and SERM signalling is found to be cell type and cell context specific, it is important to study the signalling of these compounds *in vivo*.

To investigate whether raloxifene affects gene transcription in the oestrogen-responsive tissues, bone, uterus and thymus via the classical oestrogen pathway, we have used a transgenic 3×ERE-TAT-Luc (ERE-luciferase) mouse model that transcribes luciferase after binding of a ligand-bound ER to ERE (Lemmen *et al.* 2004).

Material and Methods

Animals and experimental procedures

The studies were performed on transgenic 3×ERE-TAT-Luc (ERE-luciferase) mice, on a mixed CBA×C56Bl/6J background (Lemmen *et al.* 2004). These mice have a luciferase reporter gene under the control of three consensus EREs coupled to a minimal TATA-box. ER binding to the EREs results in transcription of the enzyme luciferase, and the amount of transcription can subsequently be estimated using an enzymatic reaction (see protein preparation and luciferase analysis) enabling *in vivo* detection of classical oestrogen signalling. The ethical committee for animal experiments at Gothenburg University approved this study. Mice were kept, five to ten animals per cage, under standard environmental conditions, and fed standard laboratory chow and tap water *ad libitum*. Ovariectomy (ovx) operations were performed at 3 months of age through a midline incision of the skin and flank incision of the peritoneum. The skin incision was closed with metallic clips. Sham-operated animals had their ovaries exposed but not removed. Surgery was performed after the mice were anaesthetized with ketamine (Pfizer AB, Täby, Sweden) and medetomidine (OrionPharma, Espoo, Finland). Carprofen (OrionPharma)

was used post-operatively as a painkiller. At termination, the mice were anaesthetized for blood withdrawal and then killed by cervical dislocation.

Treatment

Short-term experiment Ovx mice were injected with a single s.c. dose of the raloxifene analogue LY117018 (60 µg/mouse, generous gift from Eli Lilly), raloxifene (raloxifene hydrochloride, 60 µg/mouse, Sigma, R1202-500MG), 17β-oestradiol (OE₂)-3-benzoate (1.0 µg/mouse, Sigma) or vehicle (Miglyol812 OmyaPeralta GmbH, Hamburg, Germany), 2 weeks after ovx and were thereafter terminated 10 h after injection. LY117018 differs from raloxifene at one position on the molecule with a piperidine ring on the basic side chain instead of a pyrrolidine ring and was therefore compared with raloxifene to determine whether the two compounds affect classical signalling differently. Sham-operated mice were injected with raloxifene (60 µg/mouse, Sigma) or vehicle (Miglyol812 OmyaPeralta GmbH) and terminated 10 h after the injection.

Long-term experiment To compare the activation of the classical oestrogen-signalling pathway with physiological effects mediated via oestrogen and the raloxifene analogue LY117018, mice were given s.c. injections for 3 weeks of LY117018 (60 µg/mouse per day, 5 days per week) or OE₂-3-benzoate (1.0 µg/mouse per day, 5 days per week) or vehicle. Treatment started 2 weeks after surgery.

Tissue collection

Organs (thymus and uterus) were collected and individually frozen in liquid nitrogen. Bone marrow was flushed from the bone cavity of one femur with 5 ml PBS, after detachment of the distal and proximal parts, and centrifuged (515 g, 5 min). The supernatant was removed, and the pelleted bone marrow cells were frozen. The cortical bone shafts remaining after removing the bone marrow were also frozen.

Protein preparation and luciferase analysis

Cortical bone was cut into small pieces using a scalpel prior to homogenization. The frozen tissues were homogenized, using a polytron (Pt 3100, model 27137059) in lysis buffer (25 mM Tris pH 7.8, 1.5 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM dithiothreitol and complete protease inhibitors, #1535101, Roche), and separated by centrifugation at 10 650 g for 30 min. The supernatant was stored at -20 °C until further analysis. Luciferase activation, measured as amount of emitted light in the luciferase assay, is proportional to the activation of gene transcription by ERs binding to the ERE and is therefore considered a measure of signalling via the classical pathway. Luciferase activity in preparations from bone, uterus and thymus was measured and related to the amount of protein. The luciferase activity was analysed using a standard Luciferase Assay (#E4030, Promega)

according to the manufacturer's instructions and measured on a GloMax 20/20 Luminometer (#E5311, Promega). The protein content was measured using BioRad DC protein assay (#500-0116, Promega), according to the manufacturer's instructions.

Assessment of bone mineral density

One femur from the long-term experiment was subjected to a peripheral quantitative computed tomography (pQCT) scan with a Stratec pQCT XCT Research M, software version 5.4B (Norland, Fort Atkinson, WI, USA) at a resolution of 70 μm , as previously described (Windahl *et al.* 1999). Trabecular bone mineral density (BMD) was determined with a metaphyseal scan at a point 3% of the length of the femur from the growth plate. The inner 45% of the area was defined as the trabecular bone compartment. Cortical BMD was determined with a mid-diaphyseal scan.

Real-time PCR analysis

RNA was isolated from the homogenate of femur, thymus and uterus from ovx animals treated with vehicle, OE_2 or raloxifene (short-term treatment, see treatment) using the RNeasy kit (Qiagen). Real-time PCR analyses were performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Stockholm, Sweden) and using Assay-on-Demand primer and probe sets (PE Applied Biosystems), labelled with the reporter fluorescent dye FAM. Predesigned primers and a probe labelled with the reporter fluorescent dye VIC, specific for 18S rRNA, were included in the reaction as an internal standard. The assay identification numbers were Mm01228180_m1 for insulin-like growth factor-1 (IGF1) and Mm00487425_m1 for c-fos. The amount of mRNA for each gene was calculated using a standard curve method following the manufacturer's instructions (PE Applied Biosystems) and adjusted of 18s rRNA.

Statistical analysis

Values are given as mean $\pm$ S.D. For statistical evaluation, the non-parametric Kruskal–Wallis followed by χ^2 post test was

used when comparing more than two groups, and when two groups were compared, the Mann–Whitney test was used. A P value ≤ 0.05 was considered significant.

Results

Since LY117018, a raloxifene analogue, differs from raloxifene at one position with a piperidine ring on the basic side chain instead of a pyrrolidine ring, the efficacy of these two compounds in regards to activation of the classical signalling pathway was determined. Short-term treatment of raloxifene and raloxifene analogue induced similar stimulation of luciferase activation compared with vehicle control in all three organs investigated (Table 1), showing that these two compounds do not differ in their ability to activate classical oestrogen signalling.

Since raloxifene has known antagonistic effects in some specific organs, e.g. uterus, and inhibits oestrogenic effects, luciferase activity after raloxifene treatment was examined in sham-operated animals. In bone and uterus, we did not detect any significant effects on luciferase activation after raloxifene treatment in sham-operated animals compared with vehicle treatment, while a significant upregulation of classical signalling was seen in thymus ($+205\%$ $P < 0.01$ Mann–Whitney, data not shown).

The raloxifene analogue LY117018 and OE_2 have equipotent effects on BMD, and both compounds activate the classical pathway in bone

To compare the activation of the classical oestrogen-signalling pathway with physiological effects mediated via oestrogen and the raloxifene analogue LY117018, mice were given s.c. injections for 3 weeks. Treatment of ovx mice with OE_2 resulted in increased total BMD compared with vehicle treatment, as measured using pQCT ($+130\%$ $P < 0.01$; Fig. 1a and b). A similar increase was seen after treatment with LY117018 ($+128\%$ $P < 0.01$). When determining cortical effects, both OE_2 as well as LY117018 treatment increased BMD (2.8 and 3.2% respectively), bone mineral content (BMC; 10.7 and 16.2% respectively) and cortical

Table 1 Effects of oestradiol, raloxifene or the raloxifene analogue LY117018 on three oestrogen-responsive organs after short- and long-term treatment^a. Results are presented as mean $\pm$ S.D.

	Short-term (10 h)		Long-term (3 weeks)	
	Raloxifene	Raloxifene analogue LY117018	Oestradiol	Raloxifene analogue LY117018
Uterus	30 $\pm$ 20*	20 $\pm$ 13*	120 $\pm$ 53 [†]	30 $\pm$ 12
Bone	9 $\pm$ 4*	9 $\pm$ 3*	1900 $\pm$ 1600 [†]	430 $\pm$ 230*
Thymus	8 $\pm$ 4*	4 $\pm$ 2*	20 $\pm$ 20 [†]	9 $\pm$ 5*

^aLuciferase activation in three oestrogen-responsive organs (uterus, bone and thymus) was analysed and related to protein as a measure of the classical oestrogen signalling. The luciferase activation is presented as fold increase compared with vehicle-treated mice.

* < 0.05 , [†] < 0.01 , versus vehicle, Kruskal–Wallis test followed by a χ^2 post test ($n = 5-8$).

Figure 1 Treatment with the raloxifene analogue LY117018 or oestradiol increases bone mineral density (BMD) and activates classical signalling in total bone, cortical bone and bone marrow. Three-month-old ERE-luciferase mice were ovariectomized and treated with vehicle, raloxifene analogue (LY117018) or oestradiol for 3 weeks. Luciferase activity related to amount of protein is considered a measurement of classical signalling. (a) Representative peripheral quantitative computer tomography images of cross section of the femur showing BMD. The scale indicates the density of the bone, from 0 (black) to 750 mg/cm³ (white). (b) Scatter plots of individual data (bars indicate the mean per group) showing the total BMD. ** $P < 0.01$, Kruskal–Wallis test followed by a χ^2 post test. Scatter plots of individual data (bars indicate the mean per group) showing luciferase activity in (c) total bone, (d) cortical bone and (e) bone marrow. * $P < 0.05$, ** $P < 0.01$, Kruskal–Wallis test followed by a χ^2 post test.

thickness (8.4 and 11.4% respectively) as compared with vehicle treatment (Table 2). Similar effects were also seen regarding trabecular bone, with a 46% significant increase after OE₂ treatment and a 36% increase ($P = 0.052$, Mann–Whitney) after LY117018 treatment as compared with vehicle (Table 2).

Both OE₂ and LY117018 increased luciferase activity significantly as compared with vehicle treatment (Fig. 1c–e). The luciferase activation of OE₂ was two- to four-fold greater compared with LY117018 in the bone preparations; however, these differences did not reach statistical significance.

The raloxifene analogue LY117018 has modest effects on both uterus weight and activation of the classical pathway compared with OE₂

OE₂ treatment of ovx mice resulted in increased uterus weight (tenfold) and luciferase activity (120-fold) compared with vehicle treatment (Fig. 2a and b). Treatment with the

raloxifene analogue LY117018 increased luciferase activity (30-fold) in uterus compared with vehicle treatment (Fig. 2b), and a tendency to increased uterus weight was also seen. However, the effects of LY117018 on both weight and luciferase activation were significantly smaller compared with the effects of OE₂ ($P < 0.05$).

Raloxifene analogue LY117018 does not induce thymic atrophy, but activates the classical pathway in thymus

As expected, treatment with OE₂ induced significant involution of the thymus, as demonstrated by weight reduction (–46%) compared with vehicle treatment (Fig. 3a). In contrast, no significant effect of the raloxifene analogue LY117018 treatment was found on thymus weight compared with vehicle treatment. Interestingly, both OE₂ and LY117018 treatment resulted in significantly increased luciferase activity in thymus (Fig. 3b), and no significant difference was found between the two treatment groups.

Table 2 Effects of oestradiol and the raloxifene analogue LY117018 on bone parameters^a. Result are presented as mean $\pm$ s.d.

	Vehicle	Oestradiol	Raloxifene analogue
Total			
BMD (mg/cm ³)	396 $\pm$ 26	527 $\pm$ 45 [†]	507 $\pm$ 21*
Trabecular			
BMD (mg/cm ³)	200 $\pm$ 32	295 $\pm$ 60*	272 $\pm$ 55
Cortical			
BMD (mg/cm ³)	1200 $\pm$ 21	1234 $\pm$ 21*	1239 $\pm$ 21 [†]
BMC (mg/mm)	1.00 $\pm$ 0.05	1.11 $\pm$ 0.07*	1.16 $\pm$ 0.09 [†]
Thickness (mm)	0.19 $\pm$ 0.01	0.21 $\pm$ 0.01*	0.22 $\pm$ 0.01 [†]

^aThe femur from the long-term treatment (3 weeks) was analysed with peripheral quantitative computed tomography (pQCT) to measure total, trabecular and cortical bone mineral density (BMD), and cortical bone mineral content (BMC) and thickness.

* $P < 0.05$, [†] $P < 0.01$, versus vehicle, Kruskal–Wallis test followed by a χ^2 post test ($n = 5-7$).

OE₂ and raloxifene effects via non-classical pathways

Real-time PCR analyses of homogenates of uterus, bone and thymus was performed from mice treated with OE₂, raloxifene or vehicle 10 h prior to termination (short-term treatment). The non-classical oestrogen pathways were investigated by examining transcription of *Igf1*, reflecting *Ap1* signalling (Umayahara *et al.* 1994) and *c-fos* reflecting transcription activated via phosphorylation cascades (Duan *et al.* 2001).

In uterus, both OE₂ and raloxifene treatment increased IGFI expression compared with vehicle treatment (99-fold, $P < 0.001$ and 16-fold, $P < 0.05$ respectively; Table 3). The expression of *c-fos* (listed as *fos* in the MGI Database) mRNA was significantly higher after OE₂ treatment compared with vehicle treatment (ninefold, $p < 0.05$), while no significant effect of raloxifene was detected (Table 3). In bone, *Igf1* expression was not significantly affected by neither OE₂ nor raloxifene; however, *c-fos* expression was significantly altered (-42% , $P < 0.05$) by OE₂ compared with vehicle, while raloxifene had no effect (Table 3). In thymus, OE₂, but not raloxifene, significantly up-regulated the mRNA levels of *Igf1* compared with vehicle (twofold, $P < 0.05$), while neither OE₂ nor raloxifene had effects on mRNA levels of *c-fos* (Table 3).

Discussion

Oestrogen and oestrogen-like substances are widely used as contraceptives, as hormone replacement for postmenopausal women and as treatment for certain pathological states such as osteoporosis. Since these therapies are afflicted with certain side effects, it is important to carry out mechanistic studies in order to develop better and more potent substances. Most previous studies on how raloxifene exerts its effects have been performed *in vitro*. However, since oestrogen and SERM signalling is found to be cell type and cell context specific, we believe that it is necessary to study the signalling of these compounds *in vivo*. There are two main signalling pathways,

Figure 2 Treatment with the raloxifene analogue LY117018 has modest effects on uterus weight and classical signalling in uterus as compared with oestradiol. ERE-luciferase mice were ovariectomized and treated with vehicle, raloxifene analogue (LY117018) or oestradiol for 3 weeks. Luciferase activity related to amount of protein is considered a measurement of classical signalling. Scatter plots of individual data (bars indicate the mean per group) showing (a) uterus weight and (b) luciferase activity in uterus. * $P < 0.05$, ** $P < 0.01$, Kruskal–Wallis test followed by a χ^2 post test.

Figure 3 The raloxifene analogue LY117018 does not induce thymic atrophy, but activates the classical pathway in thymus. Thymus weight and luciferase activity related to protein in ovariectomized ERE-luciferase mice after treatment with vehicle, oestradiol or raloxifene analogue (LY117018) for 3 weeks. Scatter plots of individual data (bars indicate the mean per group) showing (a) thymus weight and (b) luciferase activity in thymus. * $P < 0.05$, ** $P < 0.01$, Kruskal–Wallis test followed by a χ^2 post test.

by which oestrogen can affect gene transcription, the classical involving binding to EREs and the non-classical, which is referred to as ERE independent.

To compare the activation of the classical oestrogen-signalling pathway with physiological effects mediated by raloxifene, mice were given s.c. injections of the raloxifene analogue LY117018 for 3 weeks.

In this study, long-term treatment with the raloxifene analogue LY117018 potentially increased BMD, and also cortical parameters, such as BMC and thickness to the same

extent as OE₂, as demonstrated earlier (Erlandsson *et al.* 2002). This increase in bone mass after LY117018 treatment was accompanied by a significant increase in luciferase activation, demonstrating that a raloxifene analogue can activate the classical oestrogen-signalling pathway in bone *in vivo*. As previously shown, by our group and others, OE₂ was found to activate the classical signalling pathway in bone (Ciana *et al.* 2003, Windahl *et al.* 2007). These findings suggest that the classical signalling pathway is involved in the bone-protecting effects of both raloxifene and OE₂. It has recently been demonstrated that OE₂ also has effect on bone via non-classical signalling pathway by using transgenic mice where ER α is incapable of binding to EREs (Syed *et al.* 2005, 2007). Furthermore, our finding that c-fos transcription, which is demonstrated to be mediated via phosphorylation cascades, is altered by OE₂ in bone further supports involvement of non-classical oestrogen signalling in the regulation of the skeleton. Thus, both classical as well as non-classical signalling seems to be of importance for the effects of OE₂ on bone. Raloxifene treatment has, in contrast to our present *in vivo* data, in previous *in vitro* studies of cultured osteoblasts, shown no or limited activation of the classical pathway (Yang *et al.* 1996b). Instead, non-classical pathways have been implicated (Yang *et al.* 1996a). In our study, looking at gene expression known to be regulated via non-classical oestrogen-signalling pathways, we did not detect any significant effect of raloxifene treatment on the non-classical signalling pathways we examined. However, our results do not rule out the involvement of other non-classical signalling pathways for the regulation of bone by raloxifene. Thus, our present *in vivo* data, together with these *in vitro* studies, suggest that the mechanism(s) by which raloxifene preserves bone may involve both the classical- and the non-classical signalling pathways. However, the importance of non-classical signalling for the bone protective effects of raloxifene *in vivo* needs to be further analysed.

Table 3 Real-time PCR after short-term treatment of oestradiol and raloxifene^a. Results are presented as mean $\pm$ s.d

	Vehicle	Oestradiol	Raloxifene
Uterus			
IGF1	0.02 $\pm$ 0.01	2.07 $\pm$ 0.77 [†]	0.34 $\pm$ 0.40*
c-fos	0.12 $\pm$ 0.06	1.11 $\pm$ 0.44*	0.40 $\pm$ 0.36
Bone			
IGF1	1.9 $\pm$ 2.9	3.4 $\pm$ 3.5	2.6 $\pm$ 2.5
c-fos	40 $\pm$ 23	23 $\pm$ 8*	27 $\pm$ 17
Thymus			
IGF1	0.51 $\pm$ 0.28	0.96 $\pm$ 0.42*	0.81 $\pm$ 0.42
c-fos	1.12 $\pm$ 0.72	1.20 $\pm$ 1.13	0.71 $\pm$ 0.40

^aTranscription of insulin-like growth factor-1 (*Igf1*), reflecting *Ap1* signalling and *c-fos* expression reflecting transcription activated via phosphorylation cascade, was analysed using real-time PCR in uterus, bone and thymus in a short-term experiment.

* $P < 0.05$, [†] $P < 0.001$, versus vehicle, Kruskal–Wallis test followed by a χ^2 post test ($n = 5-8$).

Raloxifene antagonizes oestrogen-induced stimulation of uterine weight gain via pharmacological competition for the ERs (Bryant *et al.* 1996). Furthermore, raloxifene has been proposed to have antagonistic effects in uterus in sham-operated mice (Erlandsson *et al.* 2002); however, we did not detect any antagonistic effect on classical signalling by raloxifene in uterus. We therefore suggest that antagonistic effects on uterus mediated by raloxifene may be mediated via other non-classical signalling pathways.

As shown in this study and others, raloxifene has mild agonistic effects on uterine weight in oestrogen-deprived states, and the mechanisms behind these agonistic effects are not completely understood (Black *et al.* 1994, Erlandsson *et al.* 2002). We demonstrate here that raloxifene can activate the classical signalling pathway *in vivo* in uterus. These data are contradictory to previous *in vitro* studies where uterine cancer cell lines show lack of raloxifene signalling via the classical pathway (Paech *et al.* 1997, Cheung *et al.* 2003), and OE₂-induced proliferation of uterine epithelial cells is shown to be independent of the classical pathway (O'Brien *et al.* 2006). Our analysis of *Igfl* expression, which is mediated via *Ap1*, supports the involvement of non-classical signalling in the regulation of the uterus by both OE₂ and raloxifene. Thus, both classical and non-classical signalling may be involved in regulation of uterine growth *in vivo*; however, the relative importance of these signalling pathways for the *in vivo* effects in uterus needs further investigation.

In thymus, an organ important for the immune system, OE₂ treatment results in involution (Erlandsson *et al.* 2000). In addition, oestrogenic compounds, including raloxifene, have also been reported to result in variable degree of thymic atrophy (Buelke-Sam *et al.* 1998, Erlandsson *et al.* 2000, Yellayi *et al.* 2002, Garcia-Perez *et al.* 2006). In our study, OE₂ resulted in a dramatic decrease in thymus weight, while no thymic atrophy was detected after treatment with the raloxifene analogue LY117018. The inconsistency between our study and previous studies might be dependent on different doses used and different administration routes. However, when comparing OE₂ and raloxifene analogue effects, a significant difference between the two compounds is found in regards to thymic weight (Erlandsson *et al.* 2000, Garcia-Perez *et al.* 2006), with OE₂ being the most potent compound, supporting our data. Interestingly, when examining the capacity to activate the classical oestrogenic pathway, both OE₂ and raloxifene resulted in a significant increase in luciferase activation. Thus, there is a discrepancy between treatment effects on thymus weight and activation of the classical signalling pathway. Raloxifene is known to affect T cell differentiation and various T cell subpopulation (Erlandsson *et al.* 2000), effects that are not necessarily reflected by thymic weight. Thus, these raloxifene effects may be mediated via classical signalling. These data also indicate that the OE₂ induced thymic involution is not only dependent on the effects of the classical pathway. Instead, the involution of thymus by OE₂ may be, at least partly, mediated via non-classical signalling or nongenomic,

membrane-associated pathways. Supporting this assumption is our finding that OE₂ up-regulated *Igfl* mRNA in thymus. Since oestrogenic effects on *Igfl* transcription are shown to be mediated via *Ap1* (Umayahara *et al.* 1994), our data indicate that non-classical signalling pathway via *Ap1* could be of importance for thymic involution. Furthermore, signalling via GPR30, a putative membrane-bound ER, induces thymic atrophy and apoptosis of a subpopulation of double-negative thymocytes (Wang *et al.* 2007).

Since LY117018 differs from raloxifene at one position with a piperidine ring on the basic side chain instead of a pyrrolidine ring, the efficacy of these two compounds in regards to activation of the classical signalling pathway was determined in a short-term experiment. We showed similar effects of the raloxifene analogue and raloxifene regarding activation of the classical signalling pathway in this short-term experiment. However, we cannot rule out differences in non-ERE-dependent genomic effects initiated by interactions between transcription factors and ligand-bound ERs between raloxifene and raloxifene analogue. Similarly, differences between the raloxifene analogue and OE₂ regarding indirect and non-ERE-dependent genomic effects cannot be ruled out and need further investigation.

In conclusion, since oestrogen has both positive as well as negative physiological effects, it is important to know by which intracellular pathways oestrogen and SERMs, such as raloxifene, mediate their biological effects in various organs. We have demonstrated *in vivo* that raloxifene can activate the classical oestrogen-signalling pathway in the oestrogen-responsive tissues, bone, uterus and thymus.

Declaration of interest

C E, C J, P T van der S, H C and M K L have nothing to declare. J-Å G consults for and has equity interests in Karobio AB.

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