A Single Dose of Enterolactone Activates Estrogen Signaling and Regulates Expression of Circadian Clock Genes in Mice1–3

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Abstract

Enterolactone (EL) is an enterolignan produced by gut microbiota from dietary plant lignans. Epidemiological and experimental studies suggest that EL and plant lignans may reduce the risk of breast and prostate cancer as well as cardiovascular disease. These effects are thought to at least in part involve modulation of estrogen receptor activity. Surprisingly little is known about the in vivo estrogenicity of EL. In the present study, we investigated the target tissues of EL, the genes affected by EL treatment, and the response kinetics. Following a single dose of EL, luciferase was significantly induced in reproductive and nonreproductive tissues of male and female 3xERE-luciferase mice, indicating estrogen-like activity. Microarray analysis revealed that EL regulated the expression of only 1% of 17β-estradiol target genes in the uterus. The majority of these genes were traditional estrogen target genes, but also members of the circadian signaling pathway were affected. Kinetic analyses showed that EL undergoes rapid phase II metabolism and is efficiently excreted. In vivo imaging demonstrated that the estrogen response followed similar, fast kinetics. We conclude that EL activates estrogen signaling in both male and female mice and that the transient responses may be due to the fast metabolism of the compound. Lastly, EL may represent a link among diet, gut microbiota, and circadian signaling.


Introduction

EL10 is a gut microbe metabolite of dietary plant lignans, polyphenols that are ubiquitously present in the plant kingdom. Plant-derived food items, such as cereal, berries, vegetables, coffee, and tea, are the main contributors to EL exposure (1,2). Daily intake of 4 common plant lignans, secoisolariciresinol, matairesinol, and lariciresinol, all precursors of EL, is ~1 mg in Western countries (1–3). The serum concentration of EL in the general population is typically in the lower nanomolar range, and EL appears to concentrate in some body fluids, such as prostatic and breast cyst fluid (4–6).

Exposure to lignans inversely correlates with the risk of breast, prostate, and colorectal cancer and cardiovascular disease (7,8), and rodents studies further support the anticarcinogenicity of EL in the mammary gland (9). However, not all studies confirm these associations and controversies remain regarding the health effects of lignans. Suggested mechanisms of action for lignans and EL are ER modulation (10,11), aromatase inhibition (12), antioxidant activity (13), and inhibition of angiogenesis (14). Still, information regarding the target tissues and genes of EL is scarce. The documented anticarcinogenic effects suggest bioactivity in the mammary gland (9), and EL promotes estrogen reporter gene expression in uterus and vagina without altering uterine weight (11,12). Regulation of Pgr, Prl, Tff1, Ccnd1, and Mki67 by EL has been suggested in earlier studies (11,15,16).

Here, we have studied target tissues and genes of EL using both conventional and ERE-luc mice. The ERE-luc mice harbor a transgene composed of 3 consensus ERE driving the expression of firefly luciferase, which allows the detection of estrogen activity by light produced by the luciferase enzyme. Our
objectives were to identify EL responsive tissues as well as EL responsive genes. Furthermore, we investigated the kinetics of the responses by studying serum EL concentrations and the time dependency of the luciferase responses.

Materials and Methods

Mice. All mouse work was performed in compliance with institutional guidelines at the Karolinska Institute (study 1) and the University of Turku (studies 2 and 3) with protocols approved by the local committees (license nos. S-109–07, 1592/04, 2008–04702). In studies 1 and 2, ERE-luc C57Bl reporter mice (line IN57) (17) from local colonies were used. Study 3 was carried out in wild-type C57Bl mice purchased from Harlan.

In all experiments, mice were housed under standard conditions of the animal departments with a 12-h-light/-dark cycle and free access to food and tap water throughout the experiments. The mice were killed by CO₂ asphyxiation and cervical dislocation, and tissues collected were snap frozen in liquid N₂ and stored in −70°C until analysis. The 3 separate experiments are described below and illustrated in detail in Supplemental Figure 1.

Study designs. In study 1, 3-mo-old female (n = 40) and 4-mo-old male (n = 30) mice were GNX and a group of 4-mo-old males (n = 20) left intact. Prior to the start of the experiment, all mice were kept for 2 wk on C1000 purified control diet (Altromin) (13655), with corn oil. The mice, weighing 25.3 g (intact males), were randomized into 3 treatment groups: vehicle (Ctrl), EL 1 mg/kg BW, and E2 50 μg/kg BW. With the exception of those groups treated with E2, all mice were treated with diethyl ether. Then the serum was then hydrolyzed overnight at 37°C with β-glucuronidase and sulfatase and then treated again with diethyl ether to extract hydrolyzed (conjugated) EL (19). The extracts were then purified in 3-cm columns of QAE-Sephadex A25 ion exchange gel in acetate form (18). Analytes were eluted with methanol, evaporated, and dissolved in 70% methanol. The concentrations were analyzed with an HPLC using coulometric electrode array detection. For more details, see Supplemental Method 1.

Statistical analysis. All statistical analysis, except the analysis of the microarray data, was conducted with Statistica 8.0. Homogeneity of variance was tested with Levene's test. Luciferase data were log transformed to reduce the variance as before (18). The data were then analyzed with ANOVA followed by Dunnett's 2-tailed post hoc test, where treatments were compared to the Ctrl group. Correlations were tested with Pearson correlation coefficients. Values in the text are mean ± SEM.

The microarray data were normalized using the normalize.quantiles function of Bioconductor from affy package (20) for the R programming software environment version 2.5.1 (21) and further analyzed using the limma package version 2.10.7 (22). Limma uses the hierarchical linear model approach to calculate a moderate t-statistic in which posterior residual SD are used in place of ordinary SD. Associated Benjamini-Hochberg adjusted P values are shown in results as well as B statistics.

Results

EL-responsive tissues (study 1). As expected, EP significantly induced luciferase activity in all studied tissues (Supplemental Fig. 2; data not shown). In contrast, EL promoted luciferase expression in only one-half of the tissues; significant increases were measured in uterus, vagina, white adipose tissue, urinary bladder, pituitary gland, ventral prostate, adrenal gland, liver, and tibia. We also observed gender-specific differences in responsiveness to EL; activity was increased in urinary bladder only in females and in adrenal gland and tibia only in males. Furthermore, in males, the responsiveness differed between intact and GNX mice; in white adipose tissue and ventral prostate, EL induced luciferase expression only in intact males and in pituitary gland, adrenal gland, and tibia, only in GNX males (Supplemental Fig. 2).

When the effect of EL on luciferase activity was evaluated against the positive control EP, the following relative activity was observed: vagina > uterus > white adipose tissue > urinary bladder > pituitary gland > liver in females; pituitary gland > adrenal gland > liver > tibia in GNX males; and ventral prostate > white adipose tissue > liver in intact males (Table 1). EL did not affect luciferase expression in mammary gland, testis, epididymis, brain, heart, lung, spleen, or kidney (Supplemental Fig. 2; data not shown).

EL-responsive genes (studies 2 and 3). We analyzed gene expression in the uterus of mice treated with EL or E2. As expected, E2 significantly regulated a large set of genes: with a cut-off criteria of >1.5-fold change (P < 0.05), 1556 known genes were upregulated and 1195 downregulated (Fig. 1A). Among these, several well-known estrogen-regulated genes were
identified (Supplemental Table 3) (23). Only the higher dose of EL (10 mg/kg BW) had significant effects: 24 known genes were upregulated and 12 downregulated (Fig. 1A; Tables 2 and 3). Most of these genes were also affected by E2 in the same direction (Fig. 1B). The 4 genes specifically regulated by EL were Egr3 (NCBI gene ID 13655), Per3 (18628), Nfil3 (18030), and Arntl (11865).

We studied further the regulation of Egr3, Per3, Nfil3, and Arntl as well as that of Clock (12753) in the uterus and liver of GNX wild-type C57Bl female mice at different time points after treatment. Both EL and E2 significantly modulated these genes in the uterus (Fig. 2). E2 upregulated Clock, Nfil3, Arntl, and Egr3 at the early time points (3 and/or 6 h) and downregulated them at the late time point (24 h) compared to the Ctrl mice at the same points, with the exception of Arntl (Fig. 2). E2 significantly downregulated Per3 compared to Ctrl at all time points. EL upregulated Clock and Arntl at 3 h and downregulated Per3 at 6 h compared to Ctrl in the same time point (Fig. 2). At the late time point (24 h), EL reduced Nfil3 and Egr3 expression compared to Ctrl (Fig. 2). In addition, the expression of Nfil3 tended to increase at 3 h (P = 0.09), that of Per3 decreased at 3 h (P = 0.11), and that of Arntl decreased at 24 h (P = 0.07) in mice treated with EL compared to Ctrl. The treatments did not affect circadian clock gene expression in liver (Supplemental Fig. 3).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Tissue</th>
<th>Time after dosing h</th>
<th>EL-induced luciferase % of EP</th>
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<td></td>
<td>Vagina</td>
<td>12</td>
<td>74**</td>
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<td></td>
<td></td>
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<td>10**</td>
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<td>24</td>
<td>37**</td>
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<td></td>
<td></td>
<td>24</td>
<td>4**</td>
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<tr>
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<td>Pituitary gland</td>
<td>12</td>
<td>17**</td>
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<tr>
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<td>Ventral prostate</td>
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<td>24</td>
<td>0.3**</td>
</tr>
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</table>

1 Values are relative activity of EL compared to 17β-estradiol-dipropionate (EP) (1 mg/kg BW, shown only for tissues where EL induced significant luciferase expression compared to control (Ctrl). 2 Asterisks indicate difference between EL and Ctrl: *P < 0.01; **P < 0.01

Uterine responses in EL-treated mice (studies 2 and 3). Because EL affected estrogen target genes in the uterus, we analyzed the possible morphological consequences. We did not detect any signs of estrogen exposure in the morphology or weights of uteri of EL-treated ERE-luc (study 2) or C57Bl (study 3) mice compared to Ctrl, whereas signs of estrogen exposure, such as uterine weight increase and thickening of luminal epithelia, were present in the uteri of ERE-luc and wild-type C57Bl mice treated with E2 or EP (Supplemental Figs. 4 and 5).

Kinetics of EL-induced estrogen response (study 2). We performed in vivo imaging of luciferase activation in GNX ERE-luc female mice up to 24 h after ligand treatment. The most evident signal was observed with all studied ligands in the upper abdomen (Supplemental Fig. 6) and this area was therefore used for quantification (Fig. 3A). Luciferase induced by the reference estrogens EP and E2 remained significantly elevated compared to Ctrl from the first time point (3 h) until the last time point (24 h). Only the higher dose of EL (10 mg/kg BW) induced luciferase expression and the effect remained significantly higher than Ctrl only up to 12 h postexposure (Fig. 3A). The times of peak activities were 3.5 ± 0.5 h for EL, 6.0 ± 0.8 h for E2, and 19.0 ± 3.3 h for EP. The luciferase signal measured by in vivo imaging was correlated with the luciferase activity in livers ex vivo of the same mice (r = 0.99; P < 0.01) (Fig. 3B).

Concentration of EL in serum after a single dose (study 3). We quantified the concentrations of both unconjugated and conjugated EL in the serum of GNX wild-type C57Bl female mice as a function of time following a single injection. The highest total concentration of EL (7.5 ± 1.6 µmol/L) was detected at the earliest time point (0.5 h) compared to which the concentration declined by 94.0% during the first 6 h and by 99.6% by the last time point (Table 4). Free, unconjugated EL was detected only up to 3 h after injection, and even at the first
time point, when the amount was the highest, the free form represented merely 2.2% of the total concentration (Table 4). In the Ctrl mice, no free EL was detected, but the conjugated form was present in small amounts in 44% of the mice.

### Discussion

The important contribution of environmental factors, such as nutrition, to the development of complex diseases, like cancer and cardiovascular disease, is well recognized. Diet contains a variety of substances, the intake of which correlates with health. Lignans are an example of such components; several epidemiological studies have shown that the intake of lignans, as well as exposure to EL, is inversely correlated with the risk of developing breast and prostate cancer and cardiovascular disease (7,8). Modulation of estrogen signaling is among the suggested mechanisms and EL has indeed been shown to elicit estrogen-like activity in vitro by several independent research groups (10,11,15,16). Still, very little is known about the in vivo estrogenicity of EL. We present here the first screening analysis of the target tissues and genes of EL, and our results show that selective modulation of estrogen signaling could indeed be involved in the biological activity of lignans in vivo in various tissues.

**Tissue-selective activity of EL.** We compared the ERE-luc responses induced by EL to those induced by EP and discovered that EL displays the strongest relative activity in the female reproductive tract. In agreement with earlier uterus growth bioassays (12), we did not detect uterotrophic effects in our studies either despite the strong relative reporter gene response. Hence, it seems that the ERE-luc response is not predictive of a proliferative growth response. In another ER mouse model, similar “uncoupling” of growth response from the ERE response has been observed with tamoxifen and bisphenol A (24). Apparently, mere ERE-driven responses in the uterus are not sufficient to produce the typical estrogen-induced weight response. Our data demonstrate that EL stimulates some ERE responses without promoting growth (proliferation).

Our previous work demonstrated preferential (however, not exclusive) activation of ERα over ERβ by EL in vitro (11). In the present study, we note that the most responsive tissues, uterus and vagina, are typical ERα tissues, suggesting similar receptor selectivity in vivo. However, 2 ERβ-rich tissues responded to EL: ventral prostate and urinary bladder. In ventral prostate, EL was only active in the intact males. Intact prostate expresses predominantly ERβ (25), and EL accumulates in prostatic fluid (4). Hence, the local concentration of EL might have reached a sufficient level to activate ERE-luc through ERβ. Interestingly, activation of ERβ reduces epithelial dysplasia in mouse prostate (26) and EL has been linked to reduced prostate cancer risk in rodent models and humans (7,27). Thus, the role of EL as a possible ERβ ligand in the prostate warrants further studies. Similar to prostate, mouse urinary bladder mucosa predominantly expresses ERβ (28), and ERE-luc activation could be a consequence of high local concentrations of EL in urine (2,12). Although these observations stimulate speculations of ER isoform selectivity in EL in vivo, definite answers to this issue cannot be provided by estrogen reporter mice.

A central question in lignan research has long been the suggested anticarcinogenic effects in the breast (7,8,12). The development of breast cancer is coupled to estrogen exposure. The results from the present study suggest that EL does not activate genomic, ERE-driven estrogen signaling in the healthy mammary gland. Selective ER modulators used in the treatment...
of breast cancer, such as tamoxifen, exhibit antiestrogenic activity in the breast. It remains to be studied whether EL possesses similar antiestrogenic activity in the mammary gland.

We hypothesize that the remarkable tissue selectivity of EL is a sum of at least 3 factors. First, the expression of the ERα and ERβ is clearly a prerequisite for estrogen activity and the relative expression of these receptors might affect ERE-luc inductivity by EL. Second, the local tissue concentration of EL is a likely additional factor contributing to the selectivity. Thus far, few studies have addressed EL in tissues. In male rats, a 4-d administration of 1.5 g/kg BW sesaminol triglucoside (EL precursor) increased EL concentrations to a similar level in plasma and liver, but the concentration in lung, kidney, heart, and brain remained 90% lower (29). Flaxseed administration to male rats led to the highest EL level in liver followed by prostate > testis > lung (30), and in pigs, rye bread feeding increased the EL concentration more in the liver than in the breast (31). Hence, it seems that the tissues that least accumulate EL (lung, kidney, heart, brain, testis, breast) in these studies are tissues that did not respond to EL in the ERE-luc model in our study. Finally, in our earlier study, we discovered that the estrogen-like activity of EL was boosted in cell culture conditions, suggesting metabolic activation in certain cell types (11). This, although unconfirmed in vivo, could further play a role in the tissue-selective activity of EL.

**EL target genes.** The results of our microarray analysis show that EL selectively modulates only a fraction (~1%) of E2 target genes in the uterus. Interestingly, 4 genes in the array were specifically regulated by EL, namely Per3, Egr3, Nfil3, and Arntl, and the regulation of Per3, Nfil3, and Arntl was further confirmed by qRT-PCR. We discovered that the probe for Egr3 in the microarray (probe ID 100870035) does not align with Egr3 cDNA, explaining why this gene behaved differently in the qRT-PCR analysis. Interestingly, the confirmed targets of EL, Per3, Arntl, and Nfil3, are all members of the core circadian signaling pathway. Arntl, as a heterodimer with Clock, is the key positive regulator of circadian genes, such as the negative circadian regulators Cry and Per. Nfil3 is a clock target gene suggested to function as a negative regulator of circadian genes (32). Circadian rhythms are involved, e.g., in sleep-wake behavior, metabolism and obesity, and breast cancer (33–35). We observed significant effects of EL on Clock and Arntl within 3 h after EL administration, suggesting that these might be direct target genes. Although light is an important timekeeper of the central clock in the hypothalamus, circadian rhythms in peripheral tissues, such as liver, can also be set by food (35). Few specific dietary factors capable of directly affecting the components of the clock have been recognized so far (35). With the exciting possibility of EL being a general dietary cue for the circadian clocks in peripheral tissues, we analyzed the effects on clock genes in the liver. Remarkably, we did not detect any significant changes. All in all, these data suggest that EL is a diet-

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**FIGURE 2** Expression of Clock (A), Nfil3 (B), Arntl (C), Per3 (D), and Egr3 (E), in relation to Rip19, in the uterus of gonadectomized C57Bl mice after treatment with a single dose of enterolactone (EL) [10 mg/kg body weight (BW)], 17β-estradiol (E2) (50 μg/kg BW), or vehicle [control (Ctrl)] (study 3). Values are mean + SEM, n = 5. Asterisks indicate different from Ctrl: *P < 0.05; **P < 0.01.

**FIGURE 3** Luciferase induction as a function of time in gonadectomized 3xERE-luciferase female mice after treatment with a single dose of enterolactone (EL) [1 or 10 mg/kg body weight (BW)], 17β-estradiol-dipropionate (EP) [1 mg/kg BW], 17β-estradiol (E2) [50 μg/kg BW], or vehicle [control (Ctrl)] (study 2). (A) Quantification of the in vivo imaged luciferase expression. The data are mean + SEM, n = 4–6. Asterisks indicate different from Ctrl at that time: *P < 0.05. (B) Correlation of the imaged luminescence signal at 24 h to the luciferase activity in livers ex vivo at the end of the experiment. y = 0.98x+0.84; r = 0.98; df = 30; P < 0.01.
absorption than oral administration. The doses we used were high amounts as such and i.p. injection perhaps better mimics intestinal absorption of the gut microbe-produced EL from the large intestine. Nevertheless, EL is not ingested in large amounts as such and i.p. injection perhaps better mimics intestinal absorption than oral administration. The doses we used were high compared to the typical 1-mg/d lignan exposure in human populations (1–3). It is important to note, however, that lignan intake estimates are based on 4 plant lignans only, although hundreds of lignan structures have been identified in plants. Furthermore, certain food items contain tremendous concentrations of lignans; the whole daily dose of 1 mg could be obtained from as little as 0.3 g flaxseed (40). The variation in lignan intake between individuals is significant and intakes as high as 650 mg/d have been reported (3). Consequently, there is a marked variation in serum EL concentrations. In our experiments, the effects of a single dose were studied, but humans are continuously exposed to lignans. We recently demonstrated that a regular consumption of flaxseed can result in over 1 μmol/L EL in human serum in certain individuals (41). In addition, the local concentration of EL in certain tissues is known to exceed that in the serum (4,6).

Conclusions

Our data show that EL regulates estrogen signaling with considerable tissue and gene selectivity. The lack of uterotrophic activity combined with estrogen-like effects in adipose tissue, urinary bladder, and pituitary gland indicate that EL is an interesting selective ER modulator compound of natural origin. Since the discovery of EL, the anticarcinogenicity of lignans has been intensively studied. Estrogen-dependent cancers, such as breast cancer, are treated by antiestrogens like tamoxifen, and dietary compounds possessing estrogenic or antiestrogenic activity are thus of special interest. Our data indicate that EL is not estrogenic in the mammary gland but do not rule out possible antiestrogenic activity, which should be addressed in follow-up studies. Regarding reduction of prostate cancer risk, activation of ERβ is considered an important mechanism. Our results suggest that EL regulates estrogen signaling in the prostate and further studies are warranted to determine if the effect is mediated by ERβ.

Our data further indicate that EL is very rapidly metabolized, which could on one hand reduce the biological activity of this compound but on the other hand allow timely biological responses, which could be essential for instance in the context of circadian regulations.

Finally, we hypothesize that EL represents a link among diet, gut microbiota, and circadian signaling in the reproductive tract.

Acknowledgments

We thank Maarit Airio, Teija Hurmerinta, Liisa Kortela, Yvonne Konikol, Jyni Le, Nina Messner, Erja Mäntyalo, Erica Nyman, Terhi Sivonen, Maria Yli-Hietikälä, Dr. Krista Power, and Dr. Niina Saarinen for their advice and assistance. P.D., T.N., S.M., P.D., and I.P. analyzed data; and P.D. wrote the paper and had primary responsibility for the final content. All authors read and approved the final manuscript.

Literature Cited


TABLE 4 Concentrations of free and conjugated enterolactone (EL) in the serum of gonadectomized C57Bl6 female mice after a single dose of EL (10 mg/kg body weight) or vehicle control (Ctrl) (study 3)1

<table>
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<th>Group</th>
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<th>nmol/L</th>
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1 Values are mean ± SEM, n = 3–5/group.


