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Diet-Derived Polyphenol Metabolite Enterolactone Is a Tissue-Specific Estrogen Receptor Activator

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Numerous dietary compounds can modify gene expression by binding to the members of the nuclear receptor superfamily of transcription factors. For example, dietary polyphenols, such as soy isoflavones genistein and daidzein, modulate the activity of the estrogen receptors (ERs)-α and ERβ. An additional class of dietary polyphenols that modulate cellular signaling pathways are lignans, compounds that are common constituents of Western diets. In this study, we show that a metabolite of dietary lignans, enterolactone, at physiological concentrations, activates ER-mediated transcription *in vitro* with preference for ERα. The effects of enterolactone are mediated by the ER ligand binding domain and are susceptible to antiestrogen treatment. Furthermore, the affinity of enterolactone toward ERα, measured by a novel ligand binding assay, is augmented in cell culture conditions. Moreover, our results demonstrate for the first time that enterolactone has estrogenic activity *in vivo*. In transgenic estrogen-sensitive reporter mice, enterolactone induces tissue-specific estrogen-responsive reporter gene expression as well as promotes uterine stromal edema and expression of estrogen-responsive endogenous genes (CyclinD1 and Ki67). Taken together, our data show that enterolactone is a selective ER agonist inducing ER-mediated transcription both *in vitro* in different cell lines and *in vivo* in the mouse uterus. (*Endocrinology* 148: 4875–4886, 2007)

COMPOUNDS CAPABLE OF interacting with members of the nuclear receptor superfamily are regular constituents of many dietary products and are thus ingested daily. For instance, vitamin D exerts its cellular effects via vitamin D receptor, and some dietary lipids bind to peroxisome proliferator-activated receptors. The interactions between dietary compounds and nuclear receptors are often essential to the organism and may influence physiological functions as well as disease risk and development.

The link between diet and cancer is highlighted in immigrant studies, in which people moving from lower disease-risk countries in Asia to Western higher-risk areas soon adopt to the local risk level (1). Asian and Western diets have therefore been compared in order to understand whether these observations are due to differences in dietary habits. A particular subgroup of dietary phytoestrogens, isoflavones, has been suggested to account for the health promoting effects of Asian diet. Similar polyphenolic compounds are present in Western diets. For example, lignans are an important group of such compounds, and the richest sources of lignans are fiber-rich food items like cereals, berries, vegetables, and some beverages (2, 3). Because lignans are found mainly bound to dietary fiber, their intake correlates positively with fiber consumption. Interestingly, many health effects, such as lower risk for breast cancer, have been linked to high intake of dietary fiber. Currently the fiber intake in many Western countries is lower than recommended.

In mammals, the ingested dietary plant lignans are fermented by gut microbiota into compounds commonly known as enterolignans or mammalian lignans (4). Enterolactone (ENL) is an enterolignan absorbed from the gut. It circulates in the blood, enters the enterohepatic circulation, and is excreted in urine (5). The inter- and intraindividual variations in serum ENL levels in humans are large, but typically the concentration in serum is in the lower nanomolar range (0–100 nM) in the general population (6). In contrast, vegetarians and individuals consuming a high quantity of products rich in lignans, such as flaxseed and sesame seed, can reach micromolar concentrations of ENL in their serum (7).

Lignans, and in particular their putative end metabolite ENL, are suggested to account for the ability of dietary fiber to lower the risk for breast cancer. Indeed, epidemiological studies show an inverse correlation between both lignan intake and breast cancer (8) and serum ENL and breast cancer (9–11). However, the results are inconsistent and the mechanisms of ENL's activity remain to be elucidated.

The two estrogen receptors (ERs)-α and ERβ are members

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Abbreviations: DAPI, 4′,6-Diamidino-2-phenylindole; E_2, 17β-estradiol; ENL, enterolactone; EP, 17β-estradiol dipropionate; ER, estrogen receptor; ERE, estrogen-responsive element; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; ICI, ICI 182,780; LBD, ligand binding domain; 4OHT, 4-hydroxy-tamoxifen; RIfS, reflectometric interference spectroscopy.

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of the nuclear receptor superfamily and characterized by a conserved domain arrangement. The N-terminal A/B domain harbors a strong tissue-specific transcriptional activation function. The C-domain mediates sequence-specific DNA binding activity, and finally the ligand binding domain (LBD) of the ERs is located at the C terminus. The activity of ERs is modulated by the female sex hormone, 17β-estradiol (E_2). In the presence of agonists, ERs modulate gene transcription by binding to DNA enhancer elements [estrogen responsive elements (EREs)] and subsequently recruiting cofactors and histone modifying enzymes (12).

Some dietary polyphenols, such as isoflavones, interact with and modulate the activity of the ERs (13). Also lignans, in particular ENL, has been suggested to modulate ER activity. Indeed, some studies show that high doses of ENL can induce a weak proliferative effect in estrogen-dependent cell lines (MCF-7, T-47D) (14–17) and weakly activate expression of E_2-dependent genes such as pS2, progesterone receptor, or transfected ERE-driven reporter constructs (14, 17, 18). These results are obtained at rather high concentrations of ENL (micromolar level). Furthermore, the binding affinity of ENL toward both ERs is low (18). In addition, *in vivo* no uterotrophic activity was observed in animals fed diets rich in plant lignans or treated with ENL (19–21).

In this study we demonstrate that exposure to ENL, at a concentration that is physiologically relevant in general population, leads to robust activation of ER-mediated events, both *in vitro* and *in vivo*.

Materials and Methods

Plasmids and reagents

Details of the plasmids used in this study [pSG5-ERα, pSG5-ERβ, 3xERE-TATA-Luc, pCMV-βGal, Gal4-ERα-LBD, Gal4-ERβ-LBD, 5xGal4-TATA-Luc, and pE-green fluorescent protein (GFP)-ERα)] can be obtained from the authors on request. ENL was purchased from VTT (Technical Research Centre of Finland, Helsinki, Finland), and E_2, 17β-estradiol dipropionate (EP), and 4-OH-tamoxifen (4OHT) from Sigma (St. Louis, MO). ICI 182,780 (ICI) was obtained from AstraZeneca (Södertälje, Sweden).

Cell culture and transfections

Stable 3xERE-Luc mouse mammary epithelial (HC11-ERE) cells and human cervix adenocarcinoma (HeLa) cells were routinely maintained as described previously (22, 23) with minor modifications. Medium, penicillin/streptomycin, gentamicin, and fetal bovine serum were purchased from Life Technologies, Inc. (Paisley, Scotland), and insulin and epidermal growth factor were obtained from Sigma. For reporter gene assays, cells were seeded 40,000/well in 12-well plates and used for transfections (HeLa) or treatments (HC11-ERE) the following day. HeLa cells were transfected with 5 ng/well of pSG5-ERα or pSG5-ERβ expression vectors together with 200 ng 3xERE-TATA-Luc reporter gene and 50 ng pCMV-βGal or with 10 ng/well of Gal4-ERα-LBD or Gal4-ERβ-LBD together with 100 ng 5xGal4-TATA-Luc and 50 ng pCMV-βGal. Transfections were performed in medium without phenol red with Lipofectamine reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Ligands were added to cells 4 h after transfection in fresh white medium containing 5% charcoal-dextran stripped fetal bovine serum (HyClone, Logan, UT) and antibiotics. All ligands were dissolved in ethanol, except from 4OHT, which was in dimethylsulfoxide. All treatment mediums contained 0.1% ethanol. For assays in which 4OHT was used, a control containing 0.1% dimethylsulfoxide was also used.

Luciferase activity was measured after 48 h treatment with BioThema's luciferase assay kit (Haninge, Sweden) and β-Gal expression with Tropix's Galacto-Light Plus kit (PE Biosystems, Bedford, MA) according to the manufacturer's instructions. Produced light was recorded with Lucy 3 microplate luminometer (Anthos Labtech Instruments, Salzburg, Austria). Luciferase activity was normalized against β-Gal expression (HeLa) or protein concentration measured with the Bradford method (HC11-ERE) in each well. All the treatments were performed in triplicate in one assay, and each assay was performed three times. Results are shown as averages of three independent assays, and the positive control (E_2) is set to 100%.

Receptor localization assay

HeLa cells were grown on glass coverslips, treated with poly-L-lysin (Sigma), in 6-well plates, and transfected with 250 ng pE-GFP-ERα as above. After ligand treatment the cells were fixed with 3.7% paraformaldehyde, DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) according to the manufacturer's instructions, and glass plates were mounted to object glasses with FluorSave reagent (Calbiochem, Darmstadt, Germany). Results were viewed with Leica's AS MDW multidimensional imaging workstation (Wetzlar, Germany) equipped with CoolSNAP HQ charge-coupled device camera (Roper Scientific, Duluth, GA). The filter used for excitation of GFP-tag was 495 nm (bandwidth 12) and emission was collected at 530 nm (bandwidth 30). For DAPI a 420-nm (bandwidth 30) filter was used for excitation and emission was collected at 465 nm (bandwidth 20). Z-stacks of cells' nuclei were created by capturing images at 0.3-μm intervals throughout the nuclei. Image restoration of the z-stacks was achieved by the three-dimensional blind deconvolution procedure using AutoQuant program (Troy, NY). Results were interpreted visually.

Receptor mobility assay

For fluorescence recovery after photobleaching (FRAP) assays, HeLa cells were seeded in 35-mm plates and transfected as above. Subsequently cells were used for FRAP assays using Leica's laser scanning confocal microscope (Heidelberg, Germany). For imaging and bleaching, the GFP tags were excited using the 488-nm laser line of an Ar-Kr laser and emitted light was captured between 500 and 550 nm. From each plate 10 cells were selected, and an area less than 10% of the nucleus was point bleached for 4 sec with full laser power. To avoid artifacts, cells expressing high levels of GFP were excluded. Images were recorded after the bleaching every 0.5 sec for a total of 20 sec. Fluorescence recovery curves were created for the bleached point and an unbleached area in all cells. The observed general drop in fluorescence due to the bleaching was monitored with the help of background quantification, and it was used to normalize the fluorescence recovery curves. The assay was repeated three times with similar results. Results are shown for a representative assay.

Receptor binding assays

The radioligand displacement assay was performed at KaroBio (Huddinge, Sweden) as described previously (24).

For the reflectometric interference spectroscopy (RIfS) assay, cell culture supernatant was collected from a routine transfection assay and buffered with HEPES to pH 7.0. RIfS transducer chips of 1 mm D 263 glass with layers of 10 nm Nb_2O_5 and 330 nm SiO_2 (Unaxis Balzers AG, Balzers, Liechtenstein) were first cleaned for functionalization in 1 M NaOH for 2 min, washed with tap water, and cleaned and mechanically dried with KIMTECH tissues (Kimberly-Clark, Reigate, UK). Then the transducer chips were treated with freshly prepared Piranha solution [mixture of 30% hydrogen peroxide and concentrated sulfuric acid at a ratio of 2:3 (vol/vol)] for 30 min in an ultrasonic bath. After rinsing with Milli-Q water and drying in a nitrogen stream, the surface was immediately activated for polymer functionalization by incubation with (3-glycidyloxylpropyl)trimethylsiloxan (Fluka Chemie GmbH, Buchs, Switzerland) for 1 h. The surface was subsequently cleaned with water-free acetone and dried in a nitrogen stream.

RIfS transducers were modified with two layers of polymer (diaminopolyethylene glycol (mean molecular mass of 2 kDa; Rapp Polymere, Tübingen, Germany), as described (25) and subsequent immobilization of aminodextran (100 kDa) (Innovent, Jena, Germany) to reduce nonspecific binding of sample components to the sensor and provide bind-

ing sites for the diethylstilbestrol-derivative. Carboxyl groups were introduced by letting the sensor react with glutaric anhydride (Sigma-Aldrich, Deisenhofen, Germany) at 70 C for 2 h (26).

The ER ligand diethylstilbestrol (Acros Organics, Geel, Belgium) was modified in a two-step synthesis to introduce a spacer termed by a primary amino group. This diethylstilbestrol derivative was covalently bound to the sensor using standard peptide chemistry to provide specific binding sites for ER. Before assembly into the flow cell, the transducers were rinsed with Milli-Q water and thoroughly dried in a nitrogen stream. Eighteen microliters of each sample were preincubated with the same volume of an ERα solution (PanVera, Inc., Madison, WI) (final concentration 200 nM) in HEPES (pH 7.4) for 90 min at room temperature and then stored on ice until the measurement. The sample was guided to the sensor using a syringe-driven flow system (Hamilton, Bonaduz, Switzerland) optimized for small sample volumes. The duration of a single measurement was 865 sec. As a reference, medium (DMEM with ethanol, no incubation with cells) was incubated with HEPES (pH 7.4) without ER to check for nonspecific binding of the sample to the sensor. The resulting signal was used to correct the other binding curves to obtain only specific ER binding signals.

Maximum binding was determined and percentual inhibition factors calculated as follows:

$$\text{inhibition (percent)} = \frac{(N-S)}{(N-P)} \times 100$$

where N is the signal of the blank sample (medium containing ethanol, negative control), which was set to 0% inhibition (full binding), and P the signal of medium containing E_2, which was set to 100% inhibition (media nonincubated with cells), and S the ER binding signal of the sample measured.

Reporter mouse study

All animal work was performed at the Karolinska Institute animal department and approved by the local animal authorities. Animals were housed in standard conditions and had free access to tap water and feed throughout the experiment.

Forty female C57BL/6J mice bearing 3xERE-TATA-Luc transgene with chicken β-globin insulators (27) were used in this study. The females were ovariectomized at 11–13 wk of age and allowed to recover for 2 wk. Animals were changed to C1000 semisynthetic low-estrogen basal diet (Altromin, Lage, Germany) 2 wk before treatments. Subsequently mice were divided into five treatment groups and injected ip with either 1 mg/kg EP or 10 mg/kg ENL dissolved in rape seed oil. Stocks were made directly into the oil and dissolved by sonication. Tissues (uterus, vagina, and tibia) were collected at two time points: 12 and 24 h after injections. Control animals received rape seed oil only, and control tissues were collected 12 h after injections. At the time the animals were killed, one animal in the ENL 12-h group was excluded from the study due to an incomplete ovariectomy. Dissected tissues were immediately snap frozen in liquid nitrogen and stored at −86 C until analysis. One uterine horn from each animal was collected in formalin and stored in ethanol.

Luciferase activity measurement

Reporter gene activity was measured in tissues homogenized under liquid nitrogen and dissolved in sample buffer [25 mM Tris acetate (pH 7.8), 1.5 mM EDTA, 10% glycerol, 1% Triton X-100] containing freshly added 2 mM dithiothreitol and Complete Mini-proteinase inhibitor tablets (Roche Diagnostics GmbH, Penzberg, Germany). Supernatant of the tissue homogenates was used for luciferase activity measurement using BioThema's Luciferase assay kit and Victor^2 Multilabel Counter (PerkinElmer, Turku, Finland) and for protein concentration measurements with the Bradford method. Results were calculated as relative light units per milligram protein in the sample.

Immunohistochemical and morphological examination of the uteri

Morphology of the uteri was controlled with a standard hematoxylin and eosin staining. Expression of luciferase was monitored with immunohistochemical staining using polyclonal rabbit antiluciferase antibodies (Sigma). Uterine horns stored in ethanol were routinely processed into 5-μm-thick tissue sections, deparaffinized in UltraClear (J. T. Baker, Deventer, Holland) and ethanol, and antigens were retrieved with a microwave oven treatment in citric acid buffer (pH 6.0). Endogenous peroxidase activity was blocked with H_2O_2 before addition of 1:2000 dilution of primary antibodies. The next day secondary antibodies were added, and antibody complexes were visualized with diaminobenzidine (rabbit EnVision system-horseradish peroxidase; DakoCytomation, Carpinteria, CA). The background was stained with Mayer's hematoxylin, and sections were dehydrated with ethanol and UltraClear and mounted with UltraKitt (J.T. Baker, Deventer, Holland). In addition, cyclin D1 and Ki67 stainings were performed on the uterine tissue sections with virtually the same protocol as above using 1:100 dilution of monoclonal rabbit anticyclin D1 antibodies (NeoMarkers, Fremont, CA) and 1:200 dilutions of polyclonal rat anti-Ki67 antibodies (Dako, Glostrup, Denmark). Before the addition of anti-Ki67 antibodies, the sections were blocked for 1 h in 10% rabbit serum. For the luciferase and cyclin D1 staining, DakoCytomation's rabbit EnVision system-horseradish peroxidase kit and for the Ki67 staining 1:100 diluted peroxidase conjugated rabbit antirat antibodies (Rockland, Gilbertsville, PA) were used for secondary antibodies.

The evaluation of the stromal response (presence of stromal edema) and luciferase staining results was performed by evaluating photographs of the sections. The samples showing similar response were grouped by two persons blinded to the codes. With this method the uteri fell into three separate groups: class 1, no edema; class 2, moderate edema; and class 3, profound edema. Similarly, the luciferase stained uteri fell into four groups: class 1, only epithelial staining; class 2, epithelial and stromal staining; class 3, moderate staining in all compartments (epithelium, stroma and myometrium); and class 4, intense staining in majority of cell in all compartments. Cyclin D1 and Ki67 expression in stained uteri was evaluated by calculating the number of the positive cells in stroma and on luminal and glandular epithelium using the ImageJ cell counter program (http://rsb.info.nih.gov/ij/).

Statistical analysis

The data analysis was performed with Statistica 7.0 software (StatSoft, Tulsa, OK). All the data sets were analyzed with Kruskall-Wallis ANOVA for their statistical significance. Pair wise comparisons with Mann Whitney *U* test were performed to assess which treatment groups differ from the control group. In all figures Bonferroni-corrected *P* values are presented. Difference between groups was considered significant if the corrected $P < 0.05$.

Results

ENL induces ERα/β transcriptional activation that is sensitive to antiestrogens

Due to its structure, ENL (Fig. 1A) is suspected to possess estrogenic properties. We tested the effects of ENL on ER transcriptional activation in the noncarcinoma cell line HC11 stably transfected with a 3xERE-Luc reporter gene (HC11-ERE). HC11-ERE cells express both ERα and ERβ and are derived from mouse mammary gland epithelial cells (23). The cells were treated with E_2 or increasing concentrations of ENL, and the effects were determined by monitoring luciferase activity. We observed a dose-dependent activation of reporter gene expression starting at 10^{-6} M concentration (Fig. 1B), suggesting that ENL can directly enhance ER-dependent transcription.

Next we investigated which ER subtype contributed to the observed activation of the reporter gene. For this purpose, we transiently cotransfected HeLa cells with expression vectors for ERα or ERβ and β-gal together with 3xERE-Luc reporter gene. Again, ENL dose-dependently induced reporter gene expression via both ERα (Fig. 1C, *left panel*) and ERβ (Fig. 1C,

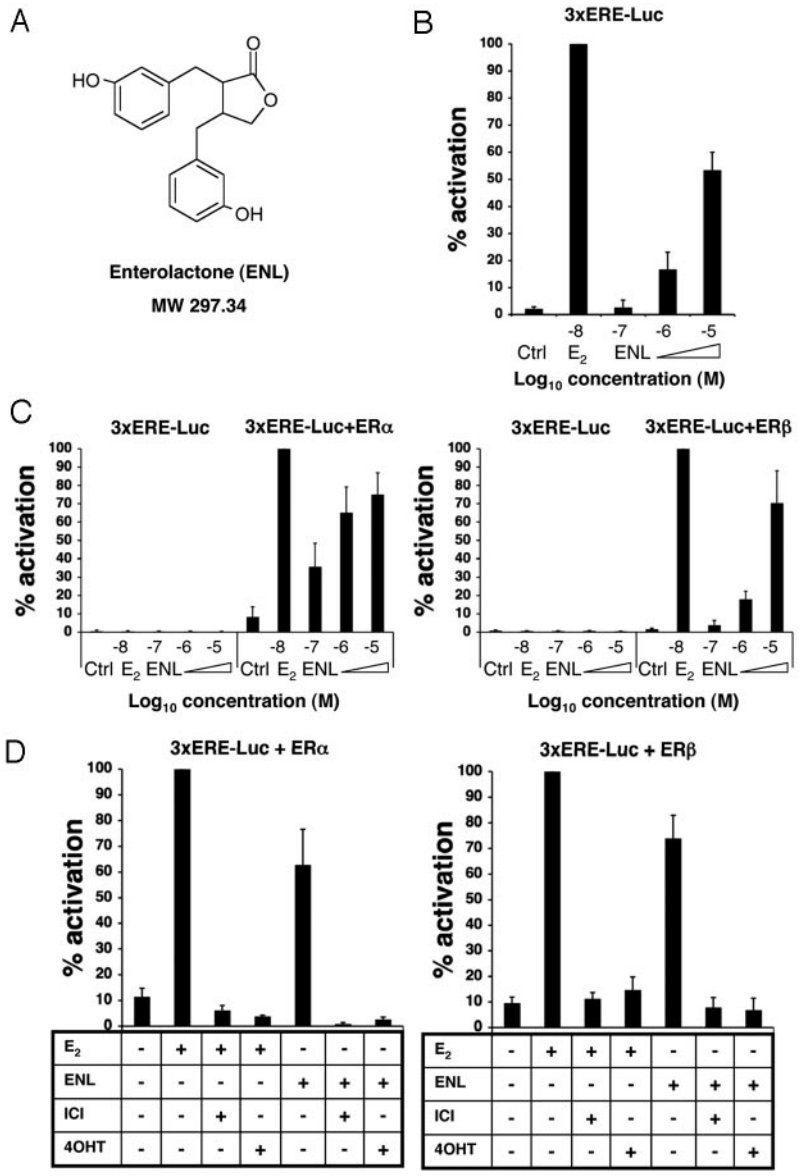


FIG. 1. ENL activates ERE-driven transcription through both ERs. A, The structure of ENL. ENL activates the expression a 3xERE-Luc reporter gene in stably transfected HC11-ERE cells (B) and transiently transfected HeLa cells (C). HeLa cells were transfected with the ERE-Luc reporter gene construct and expression vectors for ERα (C, *left panel*) or ERβ (C, *right panel*). Both cell lines were treated with vehicle alone, E_2 (10^{-8} M), or increasing concentrations of ENL (10^{-7}, 10^{-6}, and 10^{-5} M). D, HeLa cells were transfected as above and treated with vehicle, E_2, and ENL (10^{-5} M) in combination with ICI (10^{-7} M) and 4-OHT (10^{-6} M). In all figures the activation obtained with E_2 is set to 100%. *Bars* represent average of luciferase activity values, normalized against protein concentration (HC11-ERE) or β-gal expression from at least three independent assays performed in triplicate + SD. Ctrl, Control.

right panel). Interestingly, we observed a marked difference in the concentration of ENL required to activate the ER subtypes. The ERα isoform was activated at 10^{-7} M concentration, which is physiologically relevant for ENL, but activation through ERβ required a higher concentration (10^{-6} M) (Fig. 1C). The effects were observed only in the presence of the transfected receptors (Fig. 1C, *right sides of the panels*), demonstrating that the ERs are required. Furthermore, the full antiestrogen ICI and partial antiestrogen 4OHT blocked the ENL-induced reporter gene activation (Fig. 1D), again suggesting that ERs are the mediators of ENL activity.

Taken together these results suggest that ENL is a potential ligand for both ERα and ERβ *in vitro* in HC11-ERE and HeLa cells.

The LBD of the ERs is sufficient for ENL activity

To dissect the ENL-ER interaction further, we used expression vectors coding for fusion proteins consisting of GAL4 fused to the ERα or ERβ LBD, and a 5xGal4-Luc reporter gene. A dose-dependent activation of the reporter gene was detected both via ERα (Fig. 2A) and ERβ (Fig. 2B) LBD. The pattern of the response was similar to the full-length receptors (Fig. 1C), in that transcription via ERα-LBD was activated at a lower concentration of ENL, compared with the ERβ-LBD. The effects were observed only in the presence of the receptor constructs (Fig. 2, *right sides of the panels*), again suggesting that the ERs are the mediators of the observed effects.

Taken together, these results confirm that ENL can activate the transcriptional activity of the ERs through direct binding to the LBDs of the receptors.

ENL affects the mobility and subnuclear localization of ERα similar to E_2

The results presented above (Figs. 1 and 2) strongly suggest that ENL activates ERE-dependent transcription at rel-

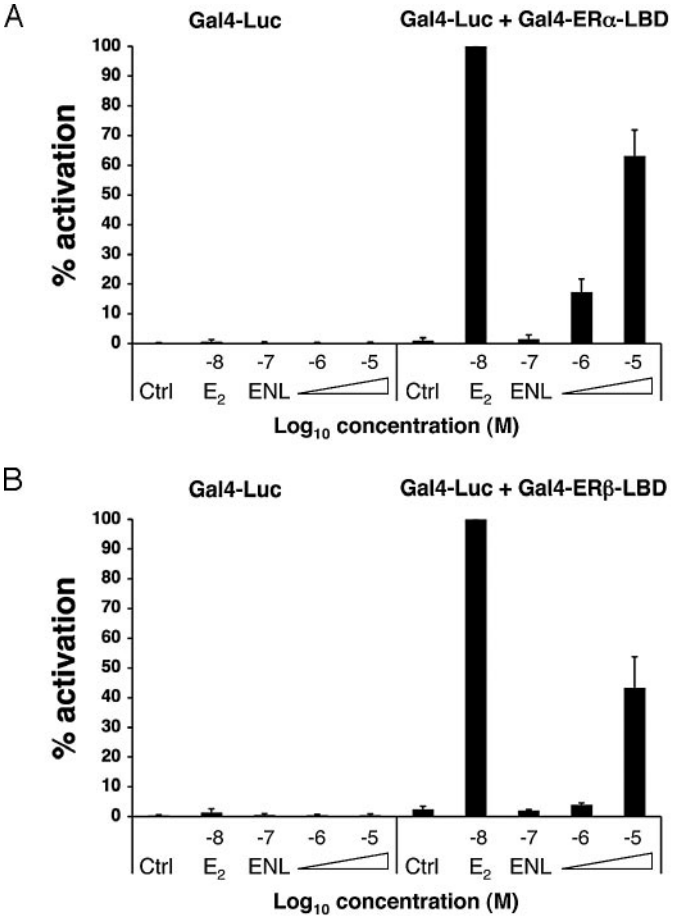


FIG. 2. The LBD of ERs is sufficient for ENL's activity. HeLa cells were cotransfected with Gal4-regulated luciferase reporter construct and expression vectors encoding a Gal4-ERα-LBD (A) or Gal4-ERβ-LBD fusion protein (B). Cells were exposed to increasing concentrations of ENL (10^{-7}, 10^{-6}, and 10^{-5} M) and luciferase activity was determined. The activation obtained with 10^{-8} M E_2 is set to 100%. *Bars* represent an average of at least three independent assays performed in triplicate +SD. Ctrl, Control.

atively low concentrations and that both ERα and ERβ mediate these effects. However, because only activation of ERα occurs at a concentration that is physiologically relevant in general population (nanomolar), we focused our attention on this ER isoform.

Ligands for ERα induce changes in the intranuclear localization of the receptor (28, 29). We used a GFP-tagged ERα construct to study the subnuclear localization pattern after treatment with ENL. The transcriptional activity of the fusion proteins was first evaluated in transient transfections and was found to retain the normal ER activity (data not shown). HeLa cells transfected with the GFP-ERα construct were treated with E_2, ENL, or vehicle only. The ERs were present in the nuclear compartment of the cell both in the presence and absence of ligands, as reported previously by others (28, 29), which was confirmed by an overlap with DAPI-stained DNA (Fig. 3A, *merge panel*). Redistribution of ERα into bright speckles in the nucleus was observed after treatments with E_2 and ENL but not in the vehicle treated control cells (Fig. 3A), supporting the idea of ENL as an ERα ligand.

In the presence of ligands, the mobility of ERα-GFP is inhibited (30). Therefore, to further evaluate the interactions between ENL and ERα, we performed a FRAP assay with ENL-treated cells. The results show that ENL slows down the mobility of the ERα-GFP to the same extent as E_2 (Fig. 3B), adding proof to a direct interaction between ENL and ERα.

These results prompted us to perform a ligand binding assay with ENL. The affinity of ENL toward ERs was assessed in a radioligand displacement assay. The resultant poor affinity of ENL toward the ERs (Fig. 3C) displays a considerable discrepancy to our other *in vitro* data. Altogether, the results from our assays show that ENL interacts with the ERs in a manner similar to the classical estrogen E_2 in HeLa and HC-11 cells but not in a ligand binding assay.

ENL's ER-binding properties are enhanced in cell culture

The disagreement between our transcriptional activation data (Figs. 1 and 2) and ligand binding data (Fig. 3C) led us to hypothesize that the cell context is important for ENL's estrogenic activity. To assess the contribution of the cell system, we set up a binding inhibition assay based on RIfS, which measures the binding of ERα to a sensor surface modified with a specific ER ligand, and the inhibition of this binding if ER ligands are present in the sample (Fig. 4A). RIfS is a label-free, time-resolved sensing method to measure biomolecular interactions, which has been applied to various applications (31–33). The RIfS set-up is described in detail in supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online Web site at http://endo.endojournals.org, and in the work of Schmitt *et al.* (34).

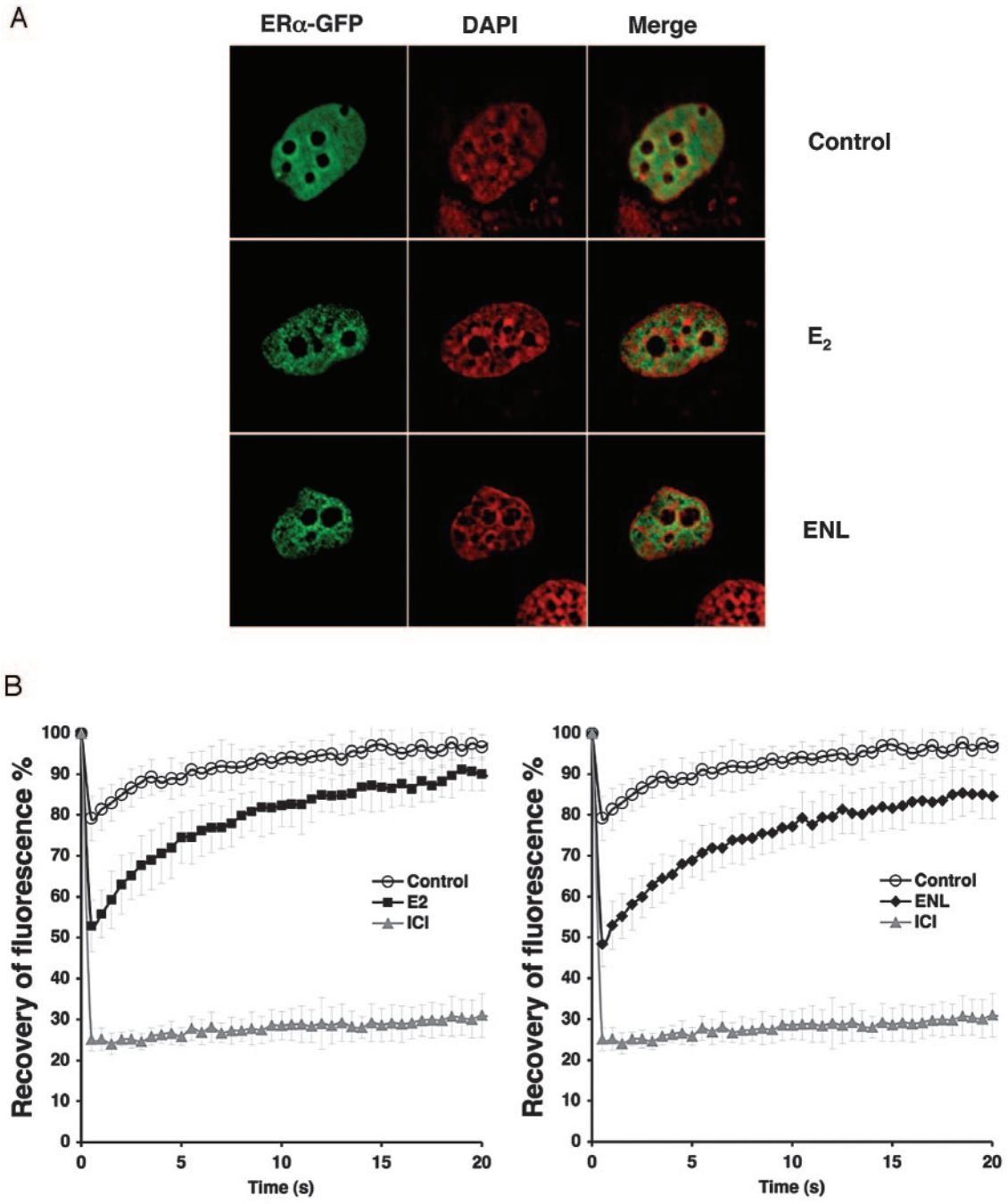
Estrogenic activity of cell culture medium before and after contact with HeLa cells was studied with RIfS. For this purpose the medium of transiently transfected cells (Fig. 1C) was collected. The medium was preincubated with ERα and tested on the optical surface. As a control, aliquots of medium containing ENL were incubated in wells devoid of cells for the same amount of time to exclude the possible formation of non-cell-mediated breakdown products. The specificity of the sensor surface was controlled with dexamethasone-containing cell culture medium.

The inhibition of binding exerted by the cell culture medium containing E_2 was not changed by the presence of HeLa cells during the incubation (Fig. 4B). In contrast, the inhibition of binding exerted by ENL-containing cell culture medium was potentiated by the presence of HeLa cells (Fig. 4B).

Taken together, these results prompted us to hypothesize that in HeLa cell culture ENL is converted into compound(s) possessing higher estrogenic activity than the parental ENL compound.

ENL activates estrogen-responsive reporter gene expression in vivo in mice

Our results suggest that in certain cell types, ENL may exhibit stronger estrogenic activities than previously thought. This raises the possibility that the effects of ENL might be tissue specific. We decided to study the tissue-selective properties of ENL *in vivo* using transgenic estrogen sensitive reporter mice bearing a luciferase reporter gene under the control of three consensus EREs and a minimal



C

IC_{50}	ENL
ERα	130 *10^{-6} M
ERβ	99 *10^{-6} M

FIG. 3. ENL affects ERα-GFP subnuclear localization and mobility in a manner similar to E_2. A, HeLa cells were transfected with ERα-GFP expression vectors and treated overnight with ENL (10^{-5} M), E_2 (10^{-8} M), or vehicle only. The intracellular localization of the receptors was monitored on a deconvolution microscope. The *first column* shows the ERα-GFP localization, the *second column* shows DNA visualized with DAPI staining, and the *third column* is a merge of the two first ones. B, HeLa were transfected and treated as in A and used for a receptor mobility assay (FRAP). In addition to E_2, ICI (10^{-5} M) was also used as a control. *Lines* represent average of 10 cells ± SD. Assay was repeated three times with similar results. C, The binding affinity of ENL toward the full-length ERs as determined in a radio ligand displacement assay performed with bacterially expressed ERs.

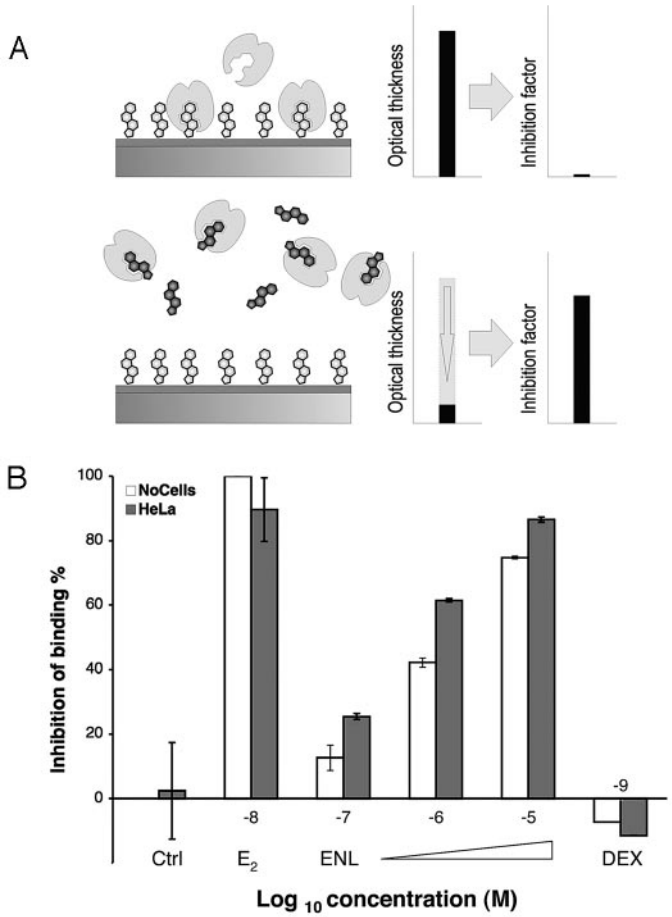


FIG. 4. ENL's receptor binding properties are enhanced in cell culture. A, Description of the RIfS assay. The assay is based on a RIfS transducer onto which a derivative of the specific ER ligand diethylstilbestrol is immobilized covalently to provide specific binding sites. The sample is preincubated with ER and then directed onto the sensor. In case of no other ligands present in the sample, ER binds to the sensor. If the sample contains ER ligands, they bind to ER during the incubation phase and will thus block the binding pocket of ER. In this case, the sensogram shows a reduced binding of ER to the sensor. B, Results of the measurements using crude cell culture supernatant as sample. Three concentrations of ENL (10^{-7}, 10^{-6}, and 10^{-5} M) were tested. The signal of the blank sample [medium containing ethanol, negative control (Ctrl)] was set to 0% inhibition (full binding), and medium containing E_2 (10^{-8} M) was set to 100% inhibition. *Columns* represent average of two independent experiments ± SD. Dexamethasone (DEX) treatment (10^{-9} M) was used as a non-ER ligand control and was performed once only.

TATA-box (27). Adult ovariectomized mice were treated with a single dose of ENL (10 mg/kg), EP (1 mg/kg), or vehicle only. Classical estrogen target tissues (uterus, vagina, and bone) were collected at 12 and 24 h after treatment, and luciferase activity was measured from tissue homogenates. In addition, one uterine horn was used for determining the cell type-specific expression of luciferase with immunostainings.

Luciferase activity was significantly increased at both time points after EP treatment in all the tissues collected (Fig. 5). In contrast, ENL-induced significant luciferase expression only in the uterus and vagina (Fig. 5). In bone, ENL caused no detectable activation of the reporter gene (Fig. 5) The uterine horns immunostained with antiluciferase antibody

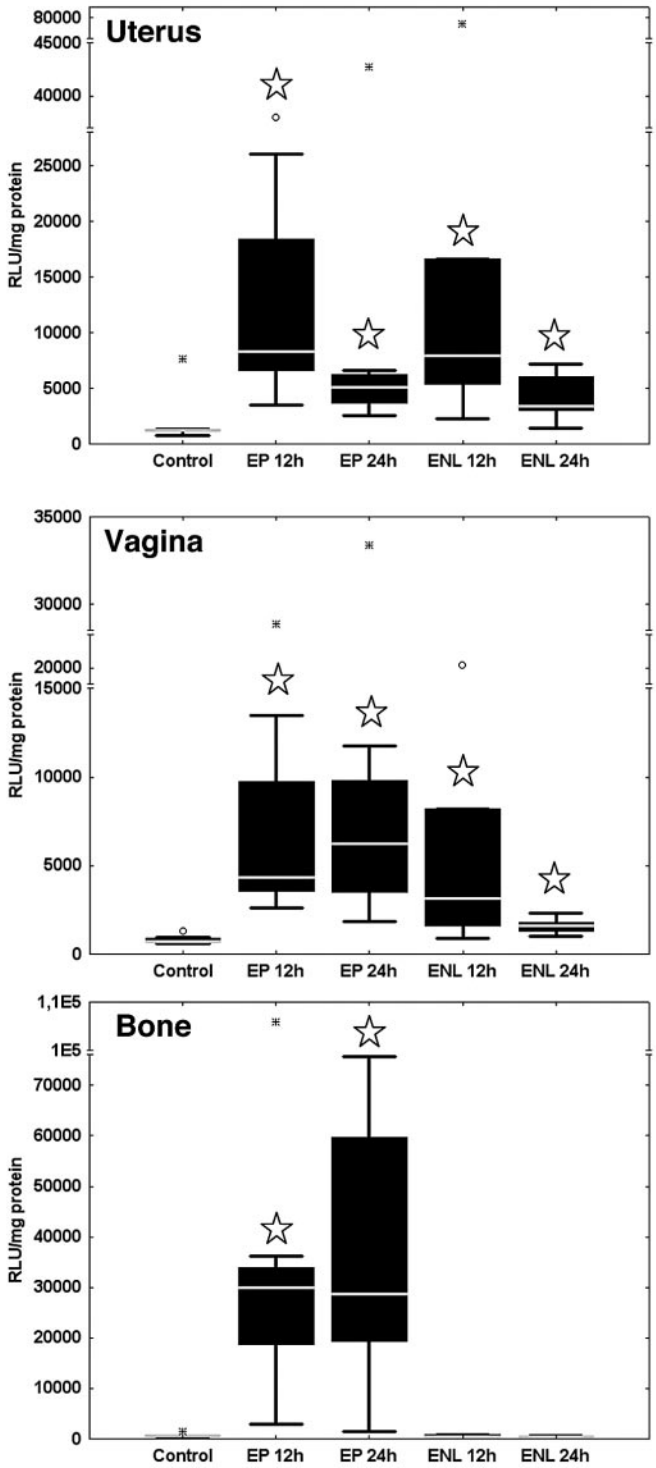


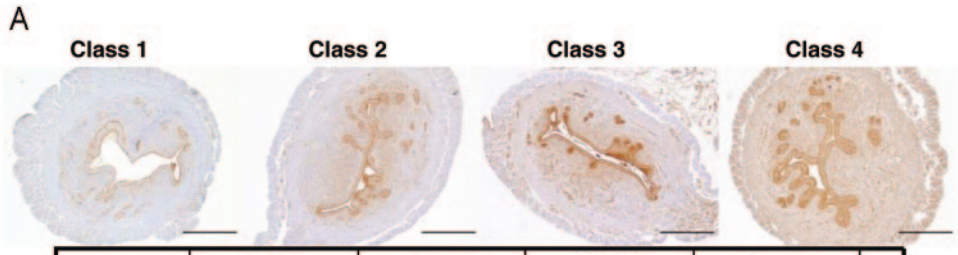
FIG. 5. ENL induces estrogen-sensitive 3xERE-Luc reporter gene expression *in vivo* in mice. Transgenic ovariectomized C57BLmice were treated with a single dose of ENL (10 mg/kg) or EP (1 mg/kg), and tissues were collected at 12 and 24 h. The luciferase activity was normalized against protein concentration in each sample, and results are presented as *boxplots*, in which median is depicted with a *line*, the *box* represents the upper and lower quartiles, and the *whiskers* show the nonoutlier range. Outliers were defined as values differing over one coefficient from the median (depicted with *open circles*), and extremes as those differing over 2.5 coefficients (depicted with *asterisks*). *Stars* indicate statistically significant difference, compared with the control at $P < 0.05$.

were categorized into four different classes according to the extent of positive staining (Fig. 6A), as described in *Materials and Methods*. The control uteri fell into classes 1 (25%) and 2 (75%). After EP 12 h treatment, the uteri were distributed into classes 2 (13%) and 3 (87%), and after 24 h all the EP-treated uteri were in class 4 (Fig. 6A). The corresponding distribution for the ENL 12-h group was similar to that of the EP 12-h group (14 and 86% for classes 2 and 3, respectively) (Fig. 6A). However, after 24 h the ENL group differed clearly from the EP 24-h group. In contrast to the EP group with all uteri in class 4, the uteri in the ENL group started returning back to the baseline (class 2 75% and class 3 25%) (Fig. 6A).

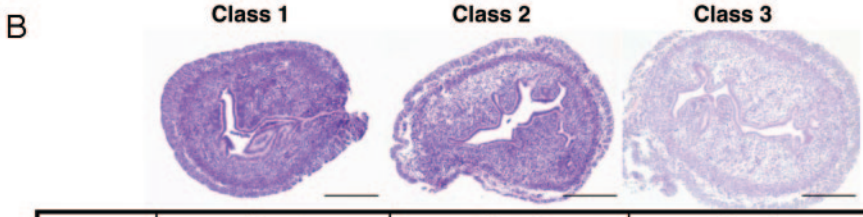
Given the strong effects on the luciferase expression in the mice in this study and the previous reports on ENL's inactivity in uterotrophic assays (19–21), we decided to study the reporter mice's uteri further. Estrogenic markers such as stromal edema, mitotic figures, and epithelial height were monitored and graded visually on hematoxylin and eosin-stained tissue sections. The stromal response was classified into three classes as described in *Materials and Methods*.

The control animal uteri showed dense stroma with no signs of edema (Fig. 6B), the luminal epithelium was flat, and mitotic figures were absent (data not shown). Treatment with EP resulted in classical estrogenic effects on the uterus. At 12 h stromal edema was profound in 75% of the uteri and moderate in rest of the samples (Fig. 6B). The stromal response was similar after 24 h exposure (Fig. 6B), and in addition, mitoses were frequently observed on the luminal and glandular epithelium, and the epithelial height was increased (data not shown). A more modest response was observed in the ENL-treated mice. After 12 h 29% of the animals displayed moderate stromal edema in the uterus, and the rest of the uteri showed no signs of edema (Fig. 6B). At 24 h, no edema, mitotic figures, or epithelial thickening were detected in the ENL group, and the uteri resembled those of the control group (Fig. 6B and data not shown).

We continued the analysis of the uterine sections by staining them for cyclin D1 and Ki67. For both proteins, the number of positive cells was calculated separately on luminal and glandular epithelium and in stroma. Treatment with EP significantly increased the amount of cyclin D1-positive cells at both time points on luminal epithelium and in stroma and after 24 h on glandular epithelium (Fig. 7). In line with this, EP treatment significantly increased Ki67-positive cells 24 h after treatment on luminal epithelium and in stroma but had no effects on glands. The effect of ENL on cyclin D1 expression was very similar to EP, the only difference being on luminal epithelium, in which ENL had effects only 24 h after treatment. Surprisingly, the effect of ENL on Ki67 expression was very different from that of EP. Where EP increased Ki67



	Class 1 % (n)	Class 2 % (n)	Class 3 % (n)	Class 4 % (n)	n
Control	25.0 % (2)	75.0 % (6)	0	0	8
EP 12h	0	12.5 % (1)	87.5 % (7)	0	8
EP 24h	0	0	0	100.0 % (8)	8
ENL 12h	0	14.3 % (1)	85.7 % (6)	0	7
ENL 24h	0	75.0 % (6)	25.0 % (2)	0	8



	Class 1 % (n)	Class 2 % (n)	Class 3 % (n)	n
Control	100.0 % (8)	0	0	8
EP 12h	0	25.0 % (2)	75.0 % (6)	8
EP 24h	12.5 % (1)	25.0 % (2)	62.5 % (5)	8
ENL 12h	71.4 % (5)	28.6 % (2)	0	7
ENL 24h	100.0 % (8)	0	0	8

FIG. 6. ENL causes histological changes in the mouse uterus. A, Luciferase-stained uteri were graded into four classes in regard to the extent of the positively stained cells. B, Classification of hematoxylin and eosin-stained uteri in three classes in regard to their stromal response.

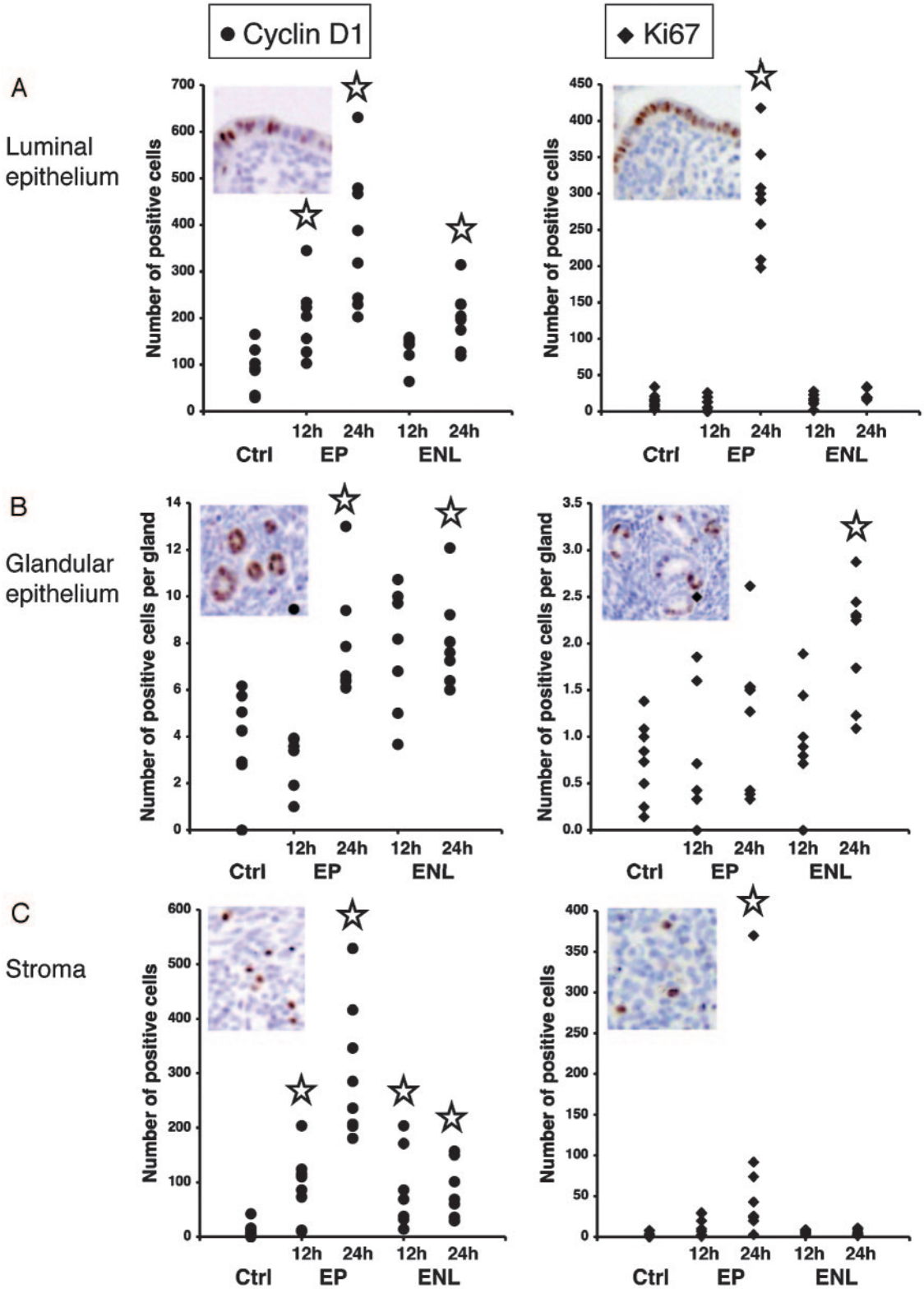


FIG. 7. ENL increases the expression of endogenous estrogen-responsive genes in mouse uterus. Quantification of cyclin D1-positive (*left panel*) and Ki67-positive (*right panel*) cells on luminal epithelium (A), on the glandular epithelium (B), and in stroma (C). Results are presented as *scatter plots*, and examples of positive staining are shown as *small pictures inside the diagrams*. *Stars* indicate statistically significant difference, compared with the control at $P < 0.05$. Ctrl, Control.

expression on luminal epithelium and in stroma (24 h after treatment), ENL significantly induced expression of Ki67 only on glandular epithelium (Fig. 7).

Taken together, the data from the reporter mouse study further support our idea of ENL as a partial ER agonist with tissue and possibly cell type-specific activity.

Discussion

The presence of hormonally active compounds in the diet is well known, and extensive experimental studies have shown that these compounds modulate nuclear receptor signaling pathways. In particular, diet-derived compounds have been shown to activate the transcriptional response of both ER isoforms ERα and ERβ (13). For example, Asian diets are rich in compounds with estrogenic activity and have received considerable attention because certain hormone-related diseases, such as breast cancer, are less common in these countries, compared with Western countries. In this study we investigated the effect of a diet-derived polyphenol metabolite ENL that is typically found in healthy (high fiber) Western diet.

ENL is an enterolignan produced by gut microbiota from plant lignans present in fiber-rich food items, such as cereals, berries, and vegetables. Epidemiological studies have indicated an inverse correlation between serum ENL concentration and breast cancer incidence (9, 11), suggesting that ENL might affect hormone-dependent tumor development and growth. The effects of lignans are widely assumed to be mediated by the putative end metabolite ENL and via the ERs (14, 17, 18). However, results supporting this idea have been obtained with concentrations of ENL that are beyond the physiological range (0–100 nM) in the general population. Furthermore, no classical estrogen-like activity has been observed for lignans *in vivo*, even when high doses of purified ENL are used (19, 20). In this study, we reevaluated the estrogenic properties of ENL by using a combination of *in vitro* and *in vivo* approaches and demonstrate that ENL is a selective modulator of the ER signaling pathway.

A luciferase reporter gene with three vitellogenin ERE sequences and a minimal TATA-box as a promoter is regulated by ENL in HC11-ERE cell line expressing both ERs endogenously (Fig. 1B). In HeLa cells we observe that ENL can activate both ERs but has a marked preference for ERα (Fig. 1C). The LBD of both receptors is sufficient for the activation of the reporter gene (Fig. 2), and the effects of ENL are counteracted by the antiestrogens ICI and 4OHT (Fig. 1D). The interaction between ENL and ER is further demonstrated in FRAP experiments in which the ERα-GFP mobility is inhibited to the same extent by E_2 and ENL (Fig. 3B), a phenomenon typical for ER ligands (30). Treatments with ENL and E_2 also lead to intranuclear rearrangement of ERα-GFP (Fig. 3A), which is another typical response to ER ligands (28, 29). Intriguingly, the binding affinity of ENL toward the ERs is markedly lower (Fig. 3C) than predicted by our cell culture assays.

Reports by others show considerably weaker estrogenic activity for ENL *in vitro* (14–18). For instance, ENL displays substantially lower estrogenic activity in Ishikawa cells on the 3xERE-Luc reporter construct than in our assays (18). Both Ishikawa and HeLa cells are human endometrial adenocarcinoma cell lines lacking endogenous ER expression. Interestingly, Ishikawa cells do not metabolize xenobiotics. To study the contribution of HeLa metabolism to the estrogenic action of ENL, we set up a RIfS-based binding assay (see supplemental Fig. 1 for more details) for comparing the cell medium estrogenicity before and after contact with HeLa cells. The RIfS assay shows that ER binding activity is higher in ENL containing cell culture medium that has been incubated on HeLa, compared with the same medium without contact with cells (Fig. 4). This increased binding activity is presumably due to the conversion of ENL into more estrogenic product(s). This hypothesis is also supported by the lower estrogenic activity of ENL in the metabolically nonactive Ishikawa cells (18).

Not much is known about the metabolic fate of ENL in cell systems or organisms. ENL is known to occur mainly as glucuronides in human serum and urine (7, 35). In HepG2 cells ENL is rapidly converted into sulfate conjugates (36). Furthermore, rat, pig, and human liver microsomes can convert ENL into a variety of aliphatic and aromatic hydroxylated derivatives (37). However, of the identified metabolites only 6-hydroxy-ENL has been assessed for estrogenicity *in vitro* and found to possess weaker activity than ENL (18).

Several of the earlier identified ENL metabolites are also found *in vivo* (rat bile and urine, human urine) (38, 39). We therefore wanted to study ENL *in vivo* in the estrogen-sensitive reporter mouse that allows the easy detection of estrogenic activity in tissues. In this mouse model, ENL displays remarkable tissue specificity and activates the reporter gene transcription in the same order of magnitude as estrogen in uterus and vagina but shows no activation in bone (Fig. 5). The effect on gene regulation in the reporter mice is confirmed by immunostaining for endogenous genes. The ability of ENL to induce cyclin D1 and Ki67 expression in the mouse uterus is a novel observation (Fig. 7).

The differences between estrogen and ENL become even more evident when looking at endogenous responses in the mouse uterus. Despite inducing a strong reporter gene response in the uterus and vagina, ENL displayed no classical uterotrophic effect up to 24 h after exposure (Fig. 6B). Yet a closer examination of the uteri revealed that ENL induced stromal edema, a well-characterized sign of estrogenicity, in 29% of the treated animals at 12 h after treatment. Intriguingly, other morphological signs of estrogenicity such as mitosis and epithelial thickening were not detected at either time point after exposure to ENL. Despite inducing a cyclin that initiates the mammalian cell cycle, ENL treatment does not lead to typical proliferation response in the mouse uterus. As observed with EP in our study, estrogens induce proliferation (measured by immunohistochemical staining for Ki67 in our study) of the uterine epithelium and stroma. Instead, ENL specifically affects the proliferation marker Ki67 expression in the epithelial cells of uterine glands (Fig. 7). Still mitotic figures are not present in these cells on hematoxylin and eosin-stained sections. The reason behind the ability of ENL to activate only certain estrogenic responses is not clear but may depend on at least on two different factors: metabolism and cofactors of estrogen signaling pathway.

Our *in vitro* data suggest that conversion of ENL into more estrogenic metabolites is needed for the full activity in cell culture. The need for metabolic conversion may also dictate the activity *in vivo*. A recent study (40) demonstrates that the uterine luminal and glandular epithelium have distinct molecular signatures and roles in the mouse. Interestingly, the expression of metabolic genes is concentrated on the glandular epithelium (40). In our mouse study, the effects of ENL are more pronounced on the glandular epithelium than on the luminal epithelium (Fig. 7). This suggests that metabolism might indeed dictate ENL's bioactivity *in vivo*.

The effects exerted by ENL on mouse uterus appear to occur on a different time scale, compared with EP. Where EP induces responses at both studied time points, the effects of ENL are more limited in duration. Reporter gene response in uterus and vagina is strongest at 12 h after ENL treatment and starts diminishing thereafter (Figs. 5 and 6A). The same phenomenon is observed with the stromal edema (Fig. 6B). Also, the cascade of events initiated by up-regulated cyclin D1 leading to Ki67 is not completed by ENL. The lack of long-term effects after ENL could also be due to metabolism. The parental compound and its possible estrogenic metabolites may be further converted to inactive breakdown products, which no longer can sustain the estrogenic response in the target cells. Such quick metabolism could also explain the lack of uterotrophic effects in other *in vivo* studies (19–21).

The ER signaling is regulated by the presence of a multitude of transcriptional cofactors that either enhance or repress the target gene expression by ER (for details see recent reviews in Refs. 12, 41). The conformation of the ER is pivotal for cofactor recruitment. The conformation of ER depends on the ligand and has profound effects on the transcriptional activity of the receptor (42). In the mouse uterus, the different morphological changes that occur in response to estrogens are induced by distinct sets of genes activated or repressed in a carefully controlled timely manner (43, 44). The ENL-induced partial estrogen response in the uterus may be due to activation of only a subset of the estrogen-responsive genes. For instance, the ENL bound ER could be selective toward classical ERE-regulated genes and fail to activate nonclassical Sp1 and AP1 regulated estrogen-responsive genes. Studies with the NERKI mice, which lack the classical ERE signaling, have shown that the uterine epithelial proliferation is controlled through the nonclassical (AP1, Sp1) signaling pathways. The uterine stroma does not respond to estrogen treatment in these mice, indicating the ERE dependency of stromal edema. Furthermore, cyclin D1 activation by estrogens is absent in these mice (45). The NERKI mouse studies collectively suggest that the stromal edema, as well as cyclin D1 induction, is an ERE-mediated response in the mouse uterus (45). Therefore, the responses seen in our study after ENL treatment (*i.e.* stromal edema and cyclin D1 induction) could be a result of the selective activation of ERE-regulated genes by ENL-ER.

Based on the results obtained in this study, we propose that ENL represents a novel ER isoform-, tissue-, and cell type-selective agonist of dietary origin. To acquire full transcriptional activity in tissues, ENL probably requires conversion into (a) yet-unidentified compound(s). Furthermore, the response may depend on the cellular cofactor makeup. The ENL selectivity toward certain tissues and cell types could explain the inverse connection between ENL concentration and risk of breast cancer in many epidemiological studies. Instead of driving epithelial cells into proliferation, ENL and/or its metabolites might compete for the receptor with endogenous estrogens and thereby work as an antiestrogen. Alternatively, ENL and ENL metabolites may keep the cells in the differentiated stage because these compounds may not possess the proliferative effects of estrogens. This latter notion is supported by our results, which indicate that despite having strong effects on ERE-driven transgene expression in mouse uterus, especially on epithelial cells, ENL does not promote proliferation of the epithelium.

The antiestrogenic or gene-selective properties of ENL were not evaluated in this work and remain our future task. The connection between the estrogen-like activities of ENL found in this study and possible reduction in risk for breast cancer warrants more studies

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