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Article in *Journal of Endocrinology* · December 2002

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The phytochemical lindleyin, isolated from *Rhei rhizoma*, mediates hormonal effects through estrogen receptors

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Abstract

Some plant compounds or herb mixtures are popular alternatives to conventional therapies and contain organic compounds that bind to some nuclear receptors, such as the estrogen receptor (ER), to exert various biological effects. We studied the effect of various herbal extracts on ER α and ER β isoforms. One herbal extract, *Rhei rhizoma* (rhubarb), acts as an agonist to both ER α and ER β . The phytochemical lindleyin, a major component of rhubarb, might contribute to this estrogenic activity through ER α and ER β . 4-Hydroxytamoxifen, an ER antagonist, completely reversed the estrogenic activity of lindleyin.

Lindleyin binds to ER α *in vitro*, as demonstrated using a fluorescent polarization assay. The *in vivo* effect of rhubarb extract was studied using a vitellogenin assay system in the freshwater fish, Japanese medaka (*Oryzias latipes*). There were marked increases in serum vitellogenin levels in male medaka exposed to rhubarb extract. We conclude that lindleyin, a component of some herbal medicines, is a novel phytoestrogen and might trigger many of the biological responses evoked by the physiological estrogens. *Journal of Endocrinology* (2002) **175**, 289–296

Introduction

Phytoestrogens are plant (predominantly legumes and grasses) substances that have structural and functional similarity to estradiol-17 β (Jordan *et al.* 1985). Plant-derived isoflavonoids, coumestans, and ligands compete with estradiol with varying affinities to bind to estrogen receptors (ERs), induce transcription of estrogen-responsive genes (Kuiper *et al.* 1998) and, depending on the outcome measured, either mimic or antagonize the action of steroidal estrogens (Hughes 1996). Humans are exposed to phytoestrogens through their diet, a major source being soy and soy-derived foods, which contain high levels of the isoflavone class of non-steroidal estrogenic compounds, genistein and daidzein (Martin *et al.* 1978, Price & Fenwick 1985, Setchell & Cassidy 1999, de Kleijn *et al.* 2001). The impact of dietary phytoestrogens on normal biological processes was first recognized in sheep (Schinckel 1948). Observations on sheep grazing on fields rich in clover and cheetahs fed high soy diets in zoos suggested that flavonoids and related phytochemicals can affect mammalian health (Bennetts *et al.* 1946, Shutt 1976, Setchell *et al.* 1987). Therefore, the fact that these compounds function as phytoestrogens might be an important dietary factor affecting human health (Adlercreutz 1995, Bingham *et al.* 1998, Cline & Hughes 1998, Humfrey 1998, Murkies *et al.* 1998, Tham *et al.* 1998). Among their

widespread clinical effects, dietary phytoestrogens are purported to reduce the risk of cancer, have antioxidant and free radical scavenger activity, reduce serum cholesterol, induce cellular differentiation, and inhibit angiogenesis (Fotsis *et al.* 1993, Murkies *et al.* 1998, Tham *et al.* 1998). Phytoestrogens can therefore potentially benefit humans, and yet there are probably many more unidentified phytoestrogens in nature.

Herbal therapies are unconventional treatments in wide use for many diseases. They can have important biological activity. For example, saw palmetto inhibits 5 α -reductase, an enzyme involved in testosterone metabolism (Delos *et al.* 1995), and St Johnswort, similar to pharmacologic antidepressants, blocks monoamine oxidase activity (Cott 1997). Recently, DiPaola *et al.* (1998) reported that PC-SPES (Botaniclab, Byea, CA, USA), a commercially available combination of eight herbs used as a non-estrogenic treatment for cancer of the prostate, has potent estrogenic activity both *in vivo* and *in vitro*. In many cases, however, the mechanisms by which these herbs produce their effect remains to be fully elucidated. Little is known about the agonistic or antagonistic effects of various herbs that are used as herbal medicine on nuclear receptors. In the present study, 24 herbs traditionally used by herbalists for treating a variety of health problems were extracted and tested for their interaction with ERs.

Here we report that one of the herbs, *Rhei rhizoma* (rhubarb), has estrogenic activity both *in vivo* and *in vitro*. We also demonstrated that the phytochemical lindleyin, a major component of rhubarb extract, is a major contributor to this estrogenic activity and is a novel phytoestrogen.

Materials and Methods

Materials

Herb powders were kindly provided by Tsumura & Co. (Tokyo, Japan). (–)–Epicatechin 3-*o*-gallate, rhein, sennoside A, and lindleyin were also provided by Tsumura & Co. Naphthalene was purchased from Nacalai Tesque (Kyoto, Japan). The ER agonist estradiol-17 β (E2) was purchased from Nacalai Tesque, and 4-hydroxytamoxifen, the ER antagonist, was purchased from Sigma Chemical Co. (St Louis, MO, USA). Tri-iodothyronine (T3) was purchased from Nacalai Tesque. Troglitazone was provided by Sankyo Pharmaceutical Company (Tokyo, Japan). Daidzein and genistein were purchased from Wako (Kyoto, Japan). TSA201 cells, clones of human embryonic kidney 293 cells (Margolske *et al.* 1993), were used for all transfection experiments.

Preparation of herbal extracts

Stock solutions of herbal extracts were prepared as follows. Herbal powders (100 mg) were suspended in 10 ml water (dilution 1:100, w/v), sonicated for 1 min, and centrifuged at 3500 r.p.m. for 15 min. The supernatants were filtered through a 0.45 μ m filter (DISMIC25CS; Tokyo Roshii, Tokyo, Japan).

The herbs used in this study were numbered as follows: 1 *Cnidii rhizoma*, 2 *Forsythiae fructus*, 3 *Atractylodis rhizoma*, 4 *Ginseng radix*, 5 *Paeoniae radix*, 6 *Cinnamoni cortex*, 7 *Moutan cortex*, 8 *Sinomeni caulis et rhizoma*, 9 *Zingiberis rhizoma*, 10 *Schizonepetae spica*, 11 *Atractylodis lanceae rhizoma*, 12 *Hoelen*, 13 *Schisandrae fructus*, 14 *Pinelliae tuber*, 15 *Gardeniae fructus*, 16 *Zizyphi fructus*, 17 *Bupleuri radix*, 18 *Platyodi radix*, 19 *Alismatis rhizoma*, 20 *Angelicae radix*, 21 *Magnoliae flos*, 22 *Rhei rhizoma* (rhubarb), 23 *Polypous*, and 24 *Ophiopogonis tuber*.

Plasmids

pGAL-ER α , pGAL-ER β , pGAL-TR α , and pGAL-PPAR γ were constructed as follows: the ligand-binding domain of human ER α (amino acid 282–595), ER β (amino acids 240–530), thyroid hormone receptor (TR)- α (amino acids 120–410), and peroxisome proliferator-activated receptor- γ (PPAR γ) (amino acids 204–506) were amplified by polymerase chain reaction (PCR) and subcloned into a pM vector (Clontech, Palo Alto, CA, USA) which carries the DNA-binding domain of GAL4

(GALDBD) in frame to generate GALDBD, a chimeric protein. Expression vector for full-length ER α (pCMXER α , called pER α full) has been described elsewhere (Chien *et al.* 1999). Expression vector for full-length human ER β (pER β full) was generated by PCR and subcloned into pcDNA3.1 (Invitrogen). PG5-luc, which has a firefly luciferase reporter gene under the control of five tandem repeats of the GAL4 recognition site, and pRL-TK, which harbors the *Renilla* luciferase reporter gene driven by thymidine kinase promoter, were obtained from Promega (Madison, WI, USA). The reporter plasmid ERE2-tk109-luc was described previously (Gehm *et al.* 1997).

Transfection

TSA201 cells, a clone of human embryonic kidney 293 cells (Margolske *et al.* 1993), were maintained in phenol red-free Dulbecco's modified Eagle's medium (Nikken Biomedical Laboratory, Kyoto, Japan) containing 10% charcoal-treated fetal bovine serum (ICN Biochemical Inc., Costa Mesa, CA, USA), and 1% penicillin/streptomycin (Gibco-BRL, Grand Island, NY, USA) at 37 °C in 5% CO₂. One day before transfection, cells were seeded at approximately 0.5 to 1 \times 10⁶/well in 12-well plates, and transfection was performed using the calcium phosphate co-precipitation method (Nagaya *et al.* 1992). The amounts of transfected plasmids were as follows: 50 ng for pGAL-derived plasmids or expression vectors for full-length ERs (pER α full and pER β full), 100 ng for pG5-luc or ERE2-tk109-Luc, and 5 ng for pRL-TK plasmids per well. Herbal extracts or various chemicals were added to the medium 8 h after transfection. Forty-eight hours after transfection, cells were harvested and assayed for luciferase activity using the Picagene kit (Promega) following the manufacturer's protocol. The luciferase activity was detected using a Plate Lumino luminometer (Strattec Biomedical Systems, Birkenfeld, Germany). In all experiments, both firefly and *Renilla* luciferase activities were measured to monitor the transfection efficiency and cytotoxicity of the added materials.

ER competitor assay

A fluorescence polarization assay was performed to examine the *in vitro* binding of lindleyin to ER α . ER α was added to a fluorescent estrogen (FluormoneTM ES2, PanVera, Madison, WI, USA) ligand to form an ES2/ER α complex with high fluorescence polarization. The complex was then added to various concentrations of either bisphenol A or lindleyin. These experiments were performed using an ER α competitor assay kit (PanVera) and performed according to the manufacturer's protocol. Polarization values were read using a Becon 2000 fluorescence polarization instrument (PanVera) at 485 nm excitation and 530 nm emission. Each data point in the

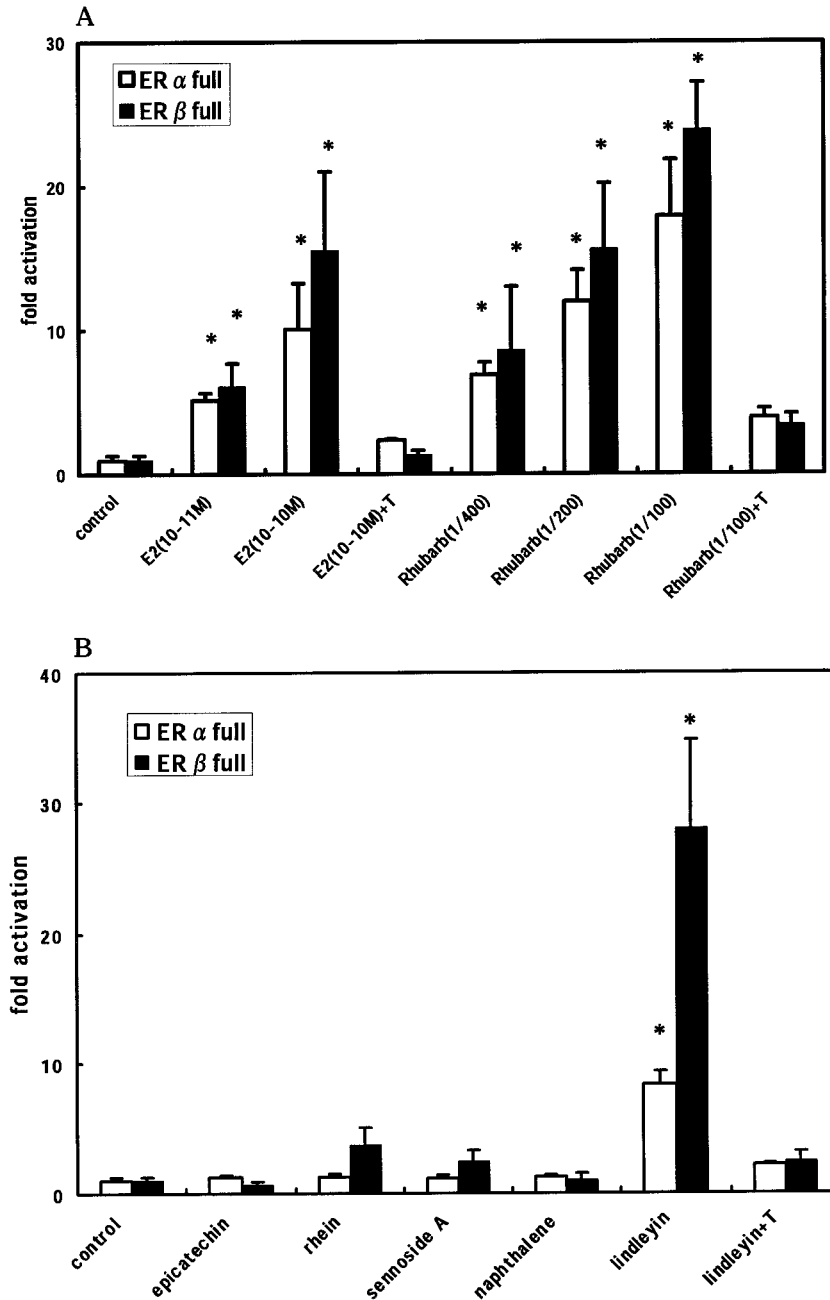


Figure 1 Transcriptional activity of rhubarb extract and five major components of rhubarb for ER α and ER β . TSA201 cells were transfected with 100 ng EREtkLuc, 5 ng pRL-TK plasmid, and 50 ng pER α full (ER α full) or pER β full (ER β full). (A) The cells were harvested 48 h after transfection in the presence of rhubarb extract (1/400, 1/200, and 1/100 dilution of the stock solution) or E2 (10^{-11} and 10^{-10} mol/l). (B) The cells were harvested 48 h after transfection in the presence of five components of rhubarb. The components of rhubarb used in this study were as follows: (–) – epicatechin 3-o-gallate (epicatechin), 10^{-6} mol/l; rhein, 10^{-6} mol/l; sennoside A, 10^{-6} mol/l; naphthalene, 10^{-6} mol/l; lindleyin, 10^{-6} mol/l. The addition of 10^{-6} mol/l 4-hydroxytamoxifen is indicated as +T. Results are indicated as fold activation. The data represented are the means \pm S.D. ($n=4$). * $P<0.001$ vs controls.

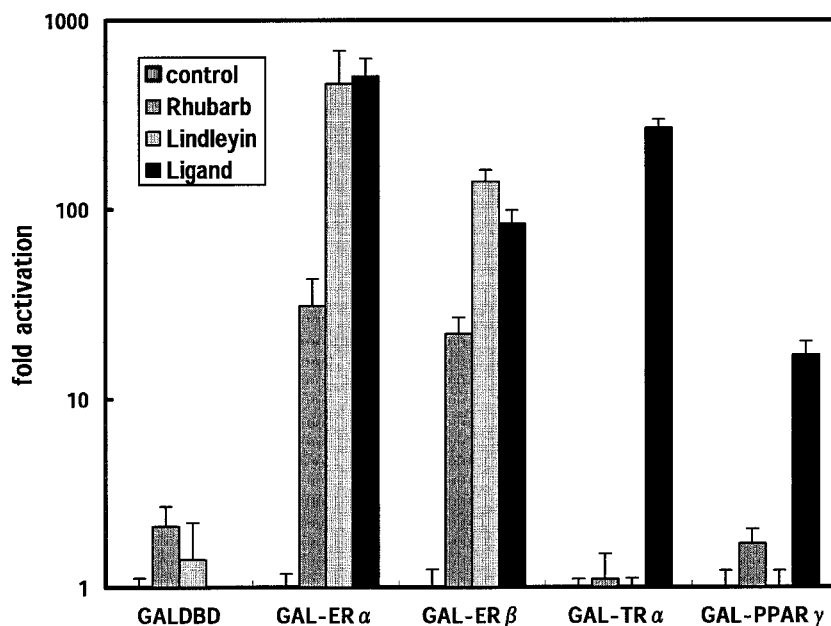


Figure 2 The effects of rhubarb extract, lindleyin and ligands on ER α -, ER β -, TR α -, and PPAR γ -induced reporter gene expression. TSA201 cells were transfected with 100 ng pG5-luc, 5 ng pRL-TK, and 50 ng pM, pGAL-ER α (GAL-ER α), pGAL-ER β (GAL-ER β), pGAL-TR α (GAL-TR α), or pGAL-PPAR γ (GAL-PPAR γ). The cells were harvested 48 h after transfection in the presence of extract (1/100 dilution (w/v) of stock solution), lindleyin (10^{-6} mol/l) and E2 (10^{-10} mol/l) for ERs, T3 (10^{-6} mol/l) for TR, and troglitazone (10^{-6} mol/l) for PPAR γ . Results are indicated as fold activation compared with the pM-transfected cells. The data represented are the means \pm S.D. ($n=4$).

polarization assay was run in triplicate, and the reported data are the means \pm S.D. of three experiments.

Medaka vitellogenin assay

Male Japanese medaka (*Oryzias latipes*, orange-red type) were purchased from a dealer. They were kept in indoor tanks and fed TetraMin flakes (TetraWerke, Melle, Germany). After a week maintained in fresh water, they were divided into three groups: exposure to E2 (3 p.p.b.), exposure to rhubarb (1/400 dilution of rhubarb stock solution), and controls. After 48 h, their blood was collected for vitellogenin assay. Vitellogenin levels were measured using an EnBio vitellogenin medaka enzyme-linked immunosorbent assay (ELISA) System (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) according to the manufacturer's protocol.

Results

The effect of rhubarb and lindleyin on ER α - and ER β -transfected cells

Of the 24 herbs examined in this study, only rhubarb extract showed significant reporter gene activation in

ER-transfected TSA201 cells (data not shown). The effect of rhubarb extract was dose dependent, and the addition of a 1/200 dilution of stock solution to the culture medium was equivalent to approximately 10^{-10} mol/l E2. The rhubarb-induced reporter gene activation was blocked by 4-hydroxytamoxifen (Fig. 1A). To determine which components of rhubarb extract contribute to its estrogenic activity, we studied the effects of five known major components of rhubarb on reporter gene expression. (–)-Epicatechin 3-*o*-gallate, rhein, sennoside A, and naphthalene had no effect on reporter gene expression in pER α full- and pER β full-transfected cells. In contrast, the addition of 10^{-6} mol/l lindleyin markedly increased the reporter gene transcription in both pER α full- and pER β full-transfected cells (Fig. 1B). Moreover, lindleyin-induced reporter gene transcription in pER α full- and pER β full-transfected cells was completely reversed by 4-hydroxytamoxifen. To demonstrate the specificity of the effects of rhubarb extract and lindleyin on ER-mediated reporter gene activation, we studied the effects of rhubarb and lindleyin on pGAL-TR α and pGAL-PPAR γ . As shown in Fig. 2, neither rhubarb extract nor lindleyin had an effect on TR α - or PPAR γ -mediated reporter gene expression, indicating that the interaction of rhubarb extract and lindleyin on ERs are specific. Figure 3

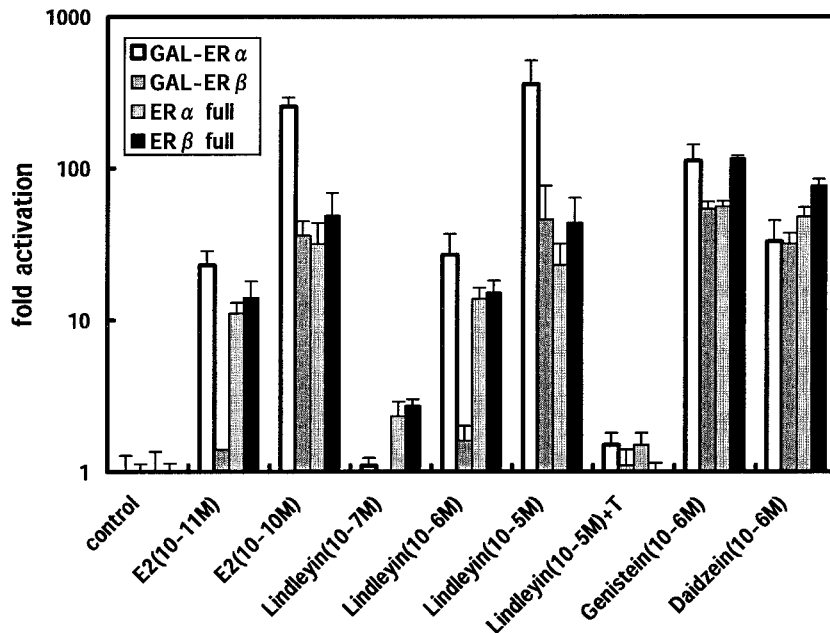


Figure 3 The effect of E2, lindleyin, genistein, daidzein and 4-hydroxytamoxifen on GAL-ER α -, GAL-ER β -, ER α full-, and ER β full-mediated reporter gene expression. TSA201 cells were transfected with 100 ng pG5-luc or ER β -luc, 5 ng pRL-TK plasmid, and 50 ng pGAL-ER α , pGAL-ER β , pER α full, and pER β full. The cells were harvested 48 h after transfection in the presence of E2 (10^{-11} mol/l and 10^{-10} mol/l), lindleyin (10^{-7} , 10^{-6} , and 10^{-5} mol/l), genistein (10^{-6} mol/l), and daidzein (10^{-6} mol/l). Addition of 10^{-6} mol/l 4-hydroxytamoxifen is indicated as +T. Results are indicated as fold activation. The data represented are the means \pm s.d. ($n=4$).

shows the effects of lindleyin, genistein, and daidzein on GAL4-ERs and full-length ERs on reporter gene expression. Lindleyin at 10^{-5} mol/l activated GAL4-ER and full-length-mediated reporter gene expression. The potency of 10^{-5} mol/l lindleyin is equivalent to approximately 10^{-6} mol/l genistein and daidzein.

Lindleyin binds to ER α *in vitro*

A fluorescence polarization assay was performed to confirm the *in vitro* binding of lindleyin to ER α . Lindleyin (10^6 nmol/l) replaced approximately 40% of the labeled estrogen (Fig. 4). The IC_{50} values of E2 and bisphenol A were 13.9 nmol/l and 45 μ mol/l respectively. The IC_{50} of lindleyin was calculated to be 225.2 to 435.8 μ mol/l, which is approximately 0.005% that of E2 and 14% that of bisphenol A.

Exposure to rhubarb extract increased serum vitellogenin levels in male medaka

The *in vivo* effect of rhubarb was studied using a vitellogenin assay in Japanese medaka. Vitellogenin is a yolk protein, which is scarcely present in male medaka, and is markedly induced by environmental estrogenic com-

pounds (Gronen *et al.* 1999, Shioda & Wakabayashi 2000). Exposure to 3 p.p.b. of E2 markedly increased serum vitellogenin levels. Exposure to a 1/400 dilution of stock solution of rhubarb markedly increased serum vitellogenin

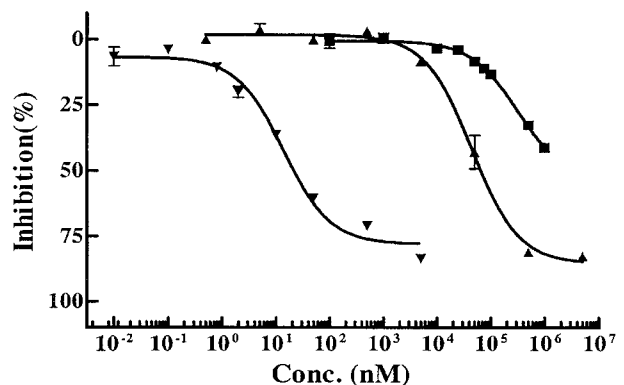


Figure 4 Competition binding curves of E2, bisphenol A, and lindleyin against a human recombinant ER α /fluorescent ligand complex. Increasing concentrations of competitors (E2 (▼), bisphenol A (▲), and lindleyin (■)) were incubated with human ER α and fluorescent estrogen for 60 min at room temperature followed by measurement of fluorescence polarization. Data points and error bars represent the means \pm s.d. ($n=3$).

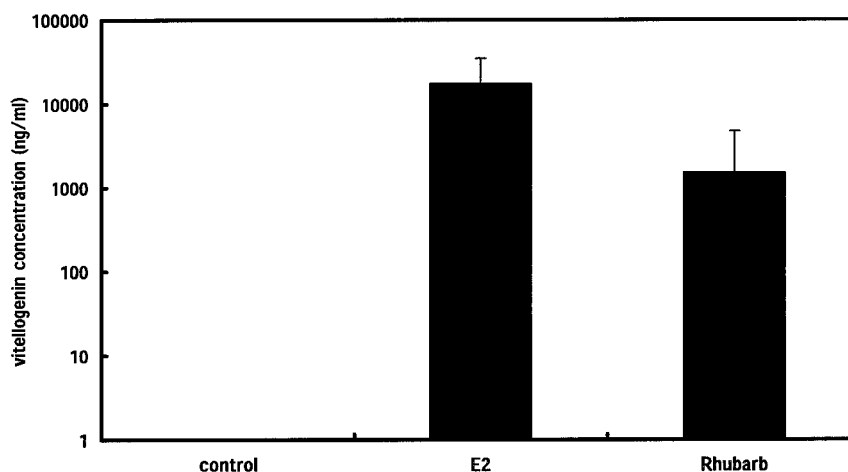


Figure 5 The effect of E2 and rhubarb extract on serum vitellogenin levels in male medaka. Male medaka were divided into three groups, control ($n=8$), exposure to E2 (3 ppb) ($n=5$), and exposure to rhubarb extract (1/400 dilution of stock solution) ($n=8$). After 48 h of exposure, serum vitellogenin levels were assayed using an ELISA as described in the Materials and Methods. The data represented are the means \pm s.d.

levels, whereas none of the control medaka had increased serum vitellogenin concentrations (Fig. 5).

Discussion

Herbal medicine is used as an alternative medicine in most countries. The precise molecular mechanisms of the various biological effects, however, are not known. Some herbal components such as PC-SPES, a blend of eight Chinese medical herbs, have estrogenic activity both *in vitro* and *in vivo*, and improve the tumor marker level in prostate cancer patients (DiPaola *et al.* 1998). Some plants have estrogenic compounds known as phytoestrogens or estrogenic flavonoids. The results of the present study demonstrated that rhubarb extract has profound estrogenic effects via both ER α and ER β . As the major components of rhubarb contain no known phytoestrogens, this herb must contain some unknown phytoestrogens. Rhubarb is traditionally used as an antiphlogistic, cathartic, antipyretic, anticoagulant, and homeostatic prescription in Chinese medicine (Kosuge & Ishida 1985). A 1/200 dilution of the stock solution of rhubarb has estrogenic activity equivalent to 10^{-10} mol/l E2 in ER α -transfected cells. Of the five major components of rhubarb, only lindleyin stimulates reporter gene expression, suggesting that lindleyin might be the major contributor to the ER-mediated estrogenic activity of rhubarb. The fact that the estrogenic activity of rhubarb and lindleyin was completely reversed by adding 4-hydroxytamoxifen indicates that these effects were due to interactions with the ERs. Previous reports have demonstrated greater binding to and activation of ER β vs ER α by phytoestrogens

(Kuiper *et al.* 1997, 1998, Barkhem *et al.* 1998, McInerney *et al.* 1998, An *et al.* 2001). At the concentrations of genistein and daidzein that were used in these previous reports, stronger transcriptional activity was observed on ER β than ER α in our reporter gene assay system (data not shown). Unlike these known phytoestrogens, lindleyin interacts equally with ER α and ER β when the full-length plasmid is used. Both rhubarb extract and lindleyin, however, had no effect on other nuclear receptors such as TR α or PPAR γ , suggesting that the ER interactions are specific. In our reporter gene assay, the estrogenic activity of lindleyin is approximately tenfold less effective at the same concentration of genistein or daidzein.

To confirm that the estrogenic effects of lindleyin occurred through direct receptor interaction, the binding of lindleyin to ER α was analyzed using a fluorescent polarization assay. The binding affinity of lindleyin to ER α was approximately 0.005% that of E2 and 14% that of bisphenol. The binding affinities of genistein and daidzein to ER α were reported to be 4% and 0.1% in solid-phase competition experiments, and 0.7% and 0.2% in solubilized receptor competition experiments (Kuiper *et al.* 1998). The discrepancy between the low binding affinity to the receptor and strong transactivation *in vivo* might be explained by the fact that lindleyin can induce, at least partially, conformational changes involved in the formation of a transcriptionally competent activation function in the ligand-binding domain (Brzozowski *et al.* 1997).

Because of the limited amount of lindleyin available, we studied the *in vivo* effect using rhubarb extract in a medaka vitellogenin assay system. Vitellogenin levels in medaka exposed to rhubarb extract were remarkably higher than in controls, showing that rhubarb contains some estrogenic

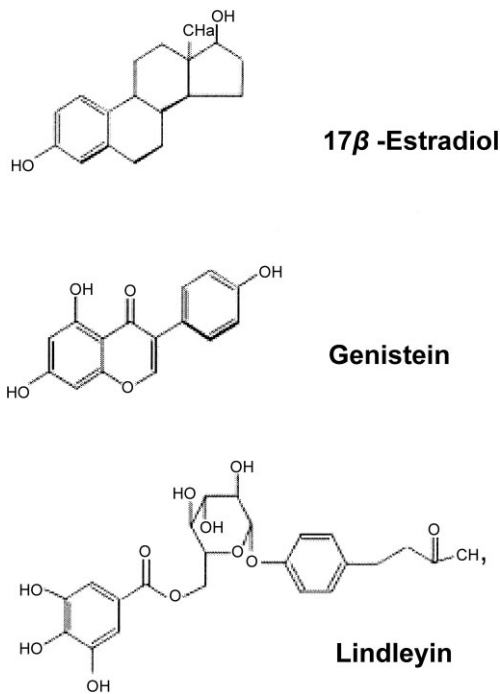


Figure 6 Structures of lindleyin, genistein, and estradiol.

compounds that affect vitellogenin synthesis through ERs expressed in medaka liver.

Lindleyin is a glucoside obtained from *Aeonium lindleyi* (Darias *et al.* 1978), a crassulacea endemic to the Canary Islands. Its formula (4-(4'-hydroxyphenyl)-2-butanone-4'-O-β-D-(6''-O-gallyl)glucopyranoside) is shown in Fig. 6. Like other estrogenic compounds, it has a phenolic backbone. We therefore conclude that lindleyin is a novel phytoestrogen. The potential biological impact of environmental and dietary estrogens on human health has generated considerable interest (Cotton 1994, Safe 1995, Feldman 1997). These agents include phytoestrogens as well as a variety of synthetic compounds. Pharmacological information about lindleyin is limited and there are no reports on the measurement of plasma lindleyin levels in humans. Daidzein and genistein are well-characterized phytoestrogens and their plasma concentrations in Japanese and Finnish populations have been reported (Adlercreutz *et al.* 1993). *In vivo* experiments on lindleyin are needed to address its pharmacological effects on humans. An estrogenic hydroxystilbene was recently reported to occur naturally in wood (Mellanen *et al.* 1996), and hops contain the potential phytoestrogen, 8-prenylnaringenin (Milligan *et al.* 1999). Gehm *et al.* (1998) reported that the phytochemical, resveratrol, present in grapes and wine, is a phytoestrogen which exhibits variable degrees of ER agonism. Bowers *et al.* (2000) reported that resveratrol acts as a mixed agonist/antagonist for ERα and ERβ. Recently, Burow *et al.*

(2001) reported that phytochemical glyceollins mediate antihormonal effects through ERα and ERβ.

The finding that lindleyin is estrogenic not only expands the spectrum of known dietary phytoestrogens but is also useful as a novel tool for examining the action of estrogen. There are no reports on the long-term effect of lindleyin or rhubarb on human health; however, it has a potential benefit as a novel selective ER modulator (Cosman & Lindsay 1999) for the postmenopausal syndrome, atherosclerosis, or osteoporosis. Further studies, however, are required to assess the physiological significance of lindleyin in humans, and a more complete understanding of its estrogenic action is needed to understand its role as a dietary substance.

Acknowledgements

We thank Tsumura & Co. for providing the herbal powders and chemical components of rhubarb. We also thank Takara Shuzo Co. Ltd for performing the fluorescence polarization assay. This work was supported in part by a grant from the Ministry of Health, Labor, and Welfare, Suzuken Memorial Foundation and the Foundation for the Growth of Science in Japan.

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Received 29 May 2002

Accepted 23 July 2002