Pergamon

Biochemical Pharmacology, Vol. 50, No. 9, pp. 1485–1493, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02061-G

NARINGENIN: A WEAKLY ESTROGENIC BIOFLAVONOID THAT EXHIBITS ANTIESTROGENIC ACTIVITY

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(*Received* 20 *February* 1995; *accepted* 2 *June* 1995)

Abstract—Treatment of immature 21-day-old female Sprague–Dawley rats with 17β-estradiol (E2) (0.5 μg/rat) caused a significant increase in uterine wet weight, DNA synthesis, progesterone receptor (PR) binding, and peroxidase activity. At doses as high as 40 mg/rat, the bioflavonoid naringenin did not cause a significant increase in any of these E2-induced responses. However, in rats cotreated with E2 (0.5 μg/rat) plus naringenin (30 mg/rat), there was a significant decrease in E2-induced uterine wet weight, DNA synthesis, PR binding, and peroxidase activity, indicating that naringenin exhibits antiestrogenic activity in the immature rodent uterus. The binding of uterine nuclear extracts to a ^{32}P-labeled estrogen responsive element (ERE) or progesterone responsive element (PRE) was determined using gel electrophoretic band shift assays. Incubation of [^{32}P]ERE with uterine nuclear extracts from rats treated with naringenin or E2 resulted in the formation of estrogen receptor (ER):ERE complexes; a higher mobility complex was prominent in the extracts from E2-treated rats, whereas a lower mobility complex was observed using nuclear extracts from naringenin-treated animals. There was a significant decrease in the intensity of the E2-induced complex using nuclear extracts from rats treated with E2 plus naringenin. In contrast, transformed cytosol from control rats gave an intense ER:ERE complex, whereas the intensity of the band was decreased markedly using transformed uterine cytosol from treated rats. Formation of a PR:PRE complex was also determined using transformed uterine cytosol. Cytosol from E2-treated rats gave an intense retarded band, whereas only weak bands were observed using cytosols from DMSO- (solvent), naringenin-, or naringenin plus E2-treated cells. The results of *in vitro* studies showed that 1 nM E2 increased (3- to 4-fold) the growth of MCF-7 human breast cancer cells, whereas 1–1000 nM naringenin had no effect on cell proliferation. In cells cotreated with 1 nM E2 plus 1000 nM naringenin, there was a significant decrease in E2-induced cell growth. In MCF-7 cells transiently transfected with a *pS2* promoter-regulated luciferase reporter gene, naringenin exhibited weak estrogenic activity. In cells cotreated with 0.1 or 1.0 μM naringenin plus 1 nM E2, naringenin inhibited E2-induced luciferase activity. The results of these studies confirmed that naringenin is a weak estrogen that also exhibits partial antiestrogenic activity in the female rat uterus and MCF-7 human breast cancer cells.

Key words: estradiol; naringenin; estrogenicity; antiestrogenicity

Several studies indicate that there is a decreased risk for different types of cancer among vegetarians [1–8], and this observation has spurred research on various structural classes of phytochemicals that may contribute to the observed anticarcinogenic effects. Bioflavonoids are major constituents of plants and vegetables [9], and several of these compounds elicit responses that may provide some protective effects [10]. In areas, such as Japan and China where dietary consumption of bioflavonoids is high, there is a decreased incidence of breast cancer in women, and this corresponds to the inhibition of mammary cancer cell or tumor growth by some bioflavonoids in both *in vitro* and *in vitro* models [11–17]. It has also been reported that different structural classes of bioflavonoids bind to the ER[‖] and induce a diverse spectrum of estrogen-induced responses [12, 18–28]. For example, bioflavonoids, such as flavone, apigenin, daidzen, kaempferol, and quercetin, induce proliferation of estrogen-responsive MCF-7 human breast cancer cells and induce estrogen-inducible genes and constructs derived from estrogen-induced genes [26, 27]. Moreover, it has been suggested "that dietary flavonoids have the potential to contribute to the growth of estrogen-dependent tumors in postmenopausal women, where 17β-estradiol is limiting" [27].

However, it has also been shown that most bioflavonoids are weak ER agonists, and therefore a sub-effective (estrogenic) dose of these phytoestrogens may exhibit partial antiestrogenic activity. Markaverich *et al.* [12] have reported previously that the bioflavonoids quercetin and luteolin inhibit E2-induced proliferation of MCF-7 human breast cancer cells and uterine wet weight in immature 21-day-old female rats. This study utilizes naringenin, a plant bioflavonoid, as a model for investigating the estrogenic activity of bioflavonoids in the female rat uterus and MCF-7 human breast cancer cell line. Since naringenin is reported to be a relatively weak estrogen, we hypothesize that this compound may also exhibit partial antiestrogenic activity. The results of cotreatment studies (E2 plus naringenin) demonstrated that naringenin inhibits several estrogen-induced responses in the rat uterus and in MCF-7 cells in culture.

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‖ Abbreviations: CAT, chloramphenicol acetyl transferase; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen responsive element; PR, progesterone receptor; and PRE, progesterone responsive element.

MATERIALS AND METHODS

Chemicals

Naringenin (4′,5,7-trihydroxyflavanone) and E2 were purchased from the Sigma Chemical Co. (St. Louis, MO). The ERE oligonucleotides were synthesized by the DNA Technologies Laboratory, Texas A&M University. The sequences of the oligonucleotides were wild-type ERE (5′-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3′) and mutated ERE (5′-GTCCAAAGTCAGGACACAGTGTCCTGATCAAAGTT-3′); the PRE consensus oligonucleotide was purchased from Promega (Madison, WI). Complementary strands of these oligonucleotides were also synthesized, and the annealed duplexes were used for the gel mobility shift assays. T4 polynucleotide kinase was purchased from Stratagene (La Jolla, CA), and [^{3}H]thymidine (85 Ci/mmol) was purchased from Amersham Life Sciences (Arlington Heights, IL). All other chemicals and biochemicals were of the highest quality available from commercial sources.

Animal treatment and uterotropic effects

Female Sprague–Dawley rats, 19 days old, were obtained from Harlan Sprague–Dawley (Houston, TX) and housed four to a cage with *ad lib* access to food and water. Dosing was initiated on day 21. Animals were dosed (4 per treatment group) intraperitoneally with naringenin (a total dose of 15, 20, 30 or 40 mg/rat), E2 (0.5 μg/rat), or naringenin plus E2 (cotreatment) in DMSO (0.1 mL) daily for 3 days. Control animals received DMSO alone. The animals were killed 24 hr after the last injection, and their uteri were dissected free of fat and connective tissue, excised, blotted, and weighed.

Uterine peroxidase activity

Uteri from each treatment group were homogenized with a tissue grinder apparatus in 12 mL polycarbonate tubes in ice-cold TEGD buffer (50 mM Tris–HCl, 1.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, pH 7.4) and centrifuged at 39,000 *g* at 2° for 45 min. The resultant pellet was washed and resuspended in 10 mM Tris–HCl buffer containing 0.5 M $CaCl_2$ and homogenized. The resulting homogenate was centrifuged for 45 min at 39,000 *g* at 2°, and uterine peroxidase activity in the supernatant was determined as described by Lyttle and DeSombre [29]. The assay mixture (3.0 mL total) contained 13 mM guaiacol and 0.3 mM H_2O_2 in the extraction buffer. The reaction was started by the addition of 1.0 mL of the uterine extract. The initial rate (1 min) of guaiacol oxidation was monitored spectrophotometrically at 470 nm. An enzyme unit was defined as the amount of enzyme required to produce an increase of one absorbance unit per minute under the assay conditions described. Protein concentrations were determined by the method of Bradford [30]. Enzyme activity is expressed as units per milligram of protein.

Progesterone receptor measurement

Uteri were placed in an ice-cold TESHMo (10 mM Tris–HCl, pH 7.4, 1.5 mM EDTA, 15 mM thioglycerol, 10 mM sodium molybdate) buffer, 1 mL/50 mg tissue. Uteri were homogenized with 3 × 8 sec bursts, using a tissue grinder. Samples were then centrifuged for 45 min at 105,000 *g*, and the clear supernatant, constituting the "cytosol" for this experiment, was decanted carefully and used immediately for receptor assays. PR levels were determined with competitive binding assays as previously described [31] and modified [32]. The cytosolic fractions described above were incubated with 20 nM [^{3}H]R5020 ± 2 μM unlabeled progesterone at 4° for 16–18 hr and then were treated with 0.1 vol. dextran-coated charcoal suspension (0.5% dextran:5% charcoal, v/v) for 10 min. Samples were then centrifuged at 5000 *g* for 10 min, and the radioactivity of the entire supernatant was measured by liquid scintillation counting.

[^{3}H]Thymidine incorporation assay

Two hours prior to killing the rats, [^{3}H] thymidine in saline was injected i.p. at a dose of 250 μCi/kg body weight. The pooled uteri were digested in a buffer containing 100 nM NaCl, 10 mM Tris–Cl, pH 8, 25 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.1 mg/mL proteinase K, and the DNA was isolated by extraction with phenol/chloroform. The radioactivity of the purified DNA in TE buffer (10 mM Tris–Cl, 25 mM EDTA, pH 8.0) was determined by liquid scintillation counting. DNA was determined by UV absorbance at 260 nm, and the data were expressed as disintegrations per minute per milligram of DNA.

Gel electrophoretic mobility shift assay

Uteri (3 per group) were homogenized in 1 mL of ice-cold TEGD buffer [10 mM Tris] using a motor-driven Teflon/glass homogenizer on ice. The homogenizer was rinsed with 0.5 mL TEGD buffer, and the combined homogenate was centrifuged at 10,000 *g* for 5 min. The supernatant was then centrifuged at 105,000 *g* for 45 min to obtain the cytosolic fraction. The low speed nuclear pellet was washed two times with 10 mL TED buffer (TEGD without glycerol). KCl (0.6 M in TEGD) was added to the pellet so that the final concentration was 0.4 M KCl. The pellet was incubated at 4° for 1 hr with frequent vortexing and then centrifuged at 105,000 *g* for 30 min to obtain the nuclear extract. The synthetic ERE or PRE oligonucleotide duplex was ^{32}P-labeled at the 5′-end using T4-polynucleotide kinase and [α-^{32}P]ATP as previously described [33]. Ligand-induced binding of nuclear or transformed cytosolic fraction to [^{32}P]ERE or [^{32}P]PRE was measured using a gel retardation assay. Nuclear extracts (9 μg protein) or cytosolic (10 μg protein for ERE binding and 40 μg protein for PRE binding) fractions from each treatment group (E2, 0.5 μg/rat; naringenin, 30 mg/rat; and their combination) were initially incubated in TEGD buffer with poly[d(I-C)] (400 ng) for 15 min at 20°. ^{32}P-Labeled oligonucleotide was added, and the mixtures were incubated for an additional 15 min at 20°. This temperature induced transformation of the cytosolic fractions. Reaction mixtures were loaded onto a 6% polyacrylamide gel (acrylamide:bisacrylamide, 30:0.8) and electrophoresed at 120 V in 0.9 M Tris-borate and 2 mM EDTA, pH 8.0. The gels were dried, and protein–DNA interactions were initially visualized by scanning on a Betascope 603 blot analyzer imaging system followed by autoradiography as previously described [34, 35].

Antibody interaction with ER

Antiserum ER 715 prepared against the hinge region of the rat ER was provided by Dr. Koji Yoshinaga (National Hormone and Pituitary Program, NIH). Antisera

(100 ng) was incubated with nuclear extracts for 2 hr at 4° prior to analysis by gel electrophoretic mobility shift assays.

Cell proliferation assay

MCF-7 cells were seeded at 50,000 cells/well in 6-well plates with 2 mL of DME/F12 (without phenol red) supplemented with 2.2 g/L sodium bicarbonate, 10 mg/L apo-transferrin, 200 μg/mL BSA and 5% fetal bovine serum treated with dextran-coated charcoal (DCC-FBS). The cells were allowed to stabilize and attach for 24 hr, at which time the medium was changed and the appropriate hormones or chemicals were added. The cells were then treated for 11 days and the same medium with test compound was changed every other day. At the end of the treatment, the cells were trypsinized, washed once with the medium, resuspended in 1 mL of the medium, and counted using a Coulter counter. All determinations were carried out in triplicate, and results are expressed as means ± SD.

Cell culture and transient transfection

MCF-7 cells were maintained in phenol red-free Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, sodium bicarbonate, glucose, HEPES, nonessential amino acids, vitamin supplement solution, sodium pyruvate, lipoic acid, vitamin B_{12}, zinc sulfate and glutamine. The medium was also augmented with gentamycin, penicillin/streptomycin and amphotericin B. Cells were grown at 37° in a 5% CO_2 humidified environment. Cells were plated on 60 mm dishes at approximately 50% confluency in medium supplemented with 5% dextran-coated charcoal-treated fetal bovine serum. After attachment and growth for 6 hr, the cells were transfected with 4 μg pCH110 (β-galactosidase expression vector, Pharmacia), 7.5 μg pS2-LUC (luciferase reporter vector) [36], 1 μg of HEGO (ER expression vector, provided by Dr. P. Chambon [37]) and 2.5 μg pBS (carrier DNA, Stratagene) per dish using the calcium phosphate co-precipitation method as described by Sambrook *et al.* [38]. Twenty hours following transfection, the plates were washed, the medium was replaced, and the cells were treated as indicated. The cells were harvested 48 hr following treatment, and the luciferase assay was performed as described in Brasier *et al.* [39]. The reference plasmid, pCH110, was cotransfected as an internal control in order to correct for variations in transfection efficiencies and was measured using standard protocols [38]. Statistical significance was determined by performing Student's *t*-test.

Statistics

Statistical significance was determined using one-way ANOVA at $P < 0.05$ and Student's *t*-test. Data are expressed as means ± SEM or SD as indicated for the various responses.

RESULTS

Treatment of immature rats with E2 (0.5 μg/rat for 3 days) resulted in a 2- to 3-fold increase in uterine wet weight, whereas naringenin (15, 20, 30 or 40 mg/rat for 3 days) did not affect uterine weights significantly compared with control animals (data not shown). The effects of different doses of naringenin on E2-induced uterine wet weight are summarized in Fig. 1. The 15 mg dose of

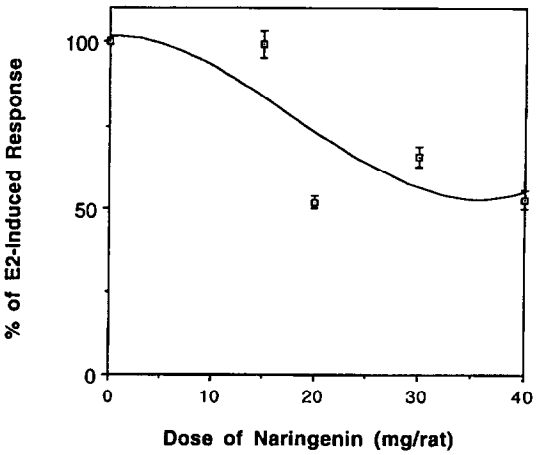

Fig. 1. Inhibition of E2-induced uterine hypertrophy by different doses of naringenin. Animals were treated with DMSO (vehicle), E2 (0.5 μg/animal), and different doses of naringenin or their combination as described in Materials and Methods, and the effects on uterine wet weights were measured. The results are expressed as means ± SEM for at least 4 animals per treatment group. In all experiments, E2 alone caused a 2- to 3-fold increase in uterine wet weights, whereas treatment with naringenin alone (15, 20, 30 and 40 mg/rat) did not cause a significant increase in uterine wet weight (data not shown). In animals cotreated with E2 plus naringenin, there was a significant ($P < 0.05$) decrease in E2-induced uterine hypertrophy at doses of 20, 30 and 40 mg/rat.

naringenin did not inhibit E2-induced uterine hypertrophy, whereas 20, 30 or 40 mg naringenin significantly inhibited E2-induced uterine wet weight increase; however, the latter dose was toxic to some of the animals. The *in vivo* interaction experiments with E2 plus naringenin (30 mg/rat) were repeated using a daily dosing regimen in which naringenin was administered 8 hr prior to E2 over the 3-day period. The uterine wet weights in the control, E2, naringenin, and naringenin plus E2-treated animals were 41 ± 7.1, 199 ± 30, 47 ± 5.0, and 91 ± 24 mg, respectively. E2 significantly induced uterine wet weights and naringenin significantly inhibited the E2-induced response ($P < 0.05$). Similar results were obtained for uterine PR and uterine peroxidase activity (data not shown). These results show that the antiestrogenic activity of naringenin was observed in animals in which naringenin plus E2 were co-administered (Fig. 1) or in animals in which the two compounds were administered at different time points. In a separate experiment, the effects of naringenin (30 mg/rat) on E2-induced uterine peroxidase, PR binding, and [^{3}H]thymidine uptake were determined (Table 1). All three responses were increased significantly after treatment with E2; naringenin alone did not induce PR binding but caused a significant decrease in uterine peroxidase activity and [^{3}H]thymidine uptake. In the animals cotreated with E2 plus naringenin, there was a significant 36, 40, and 63% decrease in uterine PR levels, peroxidase activity, and [^{3}H]thymidine uptake, respectively, compared with rats treated with E2 alone. Thus, naringenin inhibited four E2-induced responses in the rat uterus.

The effects of these various treatments on the cytosolic and nuclear ER and PR were also investigated by determining their interaction with [^{32}P]oligonucleotides in a gel electrophoretic mobility shift assay. The objectives of this study were to determine the subcellular lo-

Table 1. Effects of E2, naringenin, and naringenin plus E2 on rat uterine PR binding, peroxidase activity, and [^{3}H]thymidine uptake*

Treatment (dose)	PR (fmol/uterus)	Peroxidase activity (units/g protein)	[^{3}H]Thymidine uptake (dpm/mg DNA × 10^{-4})
Control (DMSO)	1182 ± 115	0.62 ± 0.06	14.8 ± 0.62
E2 (0.5 μg/rat)	3836 ± 432†	3.38 ± 0.04†	37.3 ± 2.02†
Naringenin (30 mg/rat)	1268 ± 19	0.24 ± 0.05‡	9.3 ± 0.78‡
E2 plus naringenin	2464 ± 216§	2.02 ± 0.004§	13.9 ± 1.14§

* Uteri from 3–4 animals per treatment group were pooled, and uterine peroxidase activity was determined in triplicate as described [29]. PR and [^{3}H]thymidine incorporation were determined with individual uteri from 4 animals per treatment group; results are expressed as means ± SEM.

† Significantly higher ($P < 0.05$) than observed in control animals.

‡ Significantly lower ($P < 0.05$) than observed in control animals.

§ Significantly lower ($P < 0.05$) than observed for animals treated with E2 alone.

calization and DNA binding activity of both the ER and PR using synthetic [^{32}P]ERE and [^{32}P]PRE oligonucleotide duplexes and to compare the results with standard bioassays used to measure estrogenic responses (e.g. Table 1).

The results summarized in Fig. 2 illustrate the ERE binding of nuclear extracts from female rats treated with E2 (0.5 μg/rat), naringenin (30 mg/rat) and their combination. In extracts from untreated (control) animals, a less mobile specifically bound band (upper band) was observed, whereas nuclear extracts from E2-treated rats gave two specifically bound bands in which a more mobile retarded band (lower band) was dominant (Fig. 2). Both bands were also observed using nuclear extracts from naringenin-treated rats; however, the upper retarded band was more intense. Electrophoretic mobility shift assays of uterine nuclear extracts from animals cotreated with E2 plus naringenin induced only formation of the upper band. Incubation of extracts from E2-treated animals with a 1000-fold excess of unlabeled ERE resulted in decreased band intensities in both retarded complexes, whereas incubation with a 1000-fold excess of mutant unlabeled ERE did not affect significantly band intensities of the retarded bands (data not shown), confirming the specificity of the protein–DNA complex. In parallel studies, nuclear extracts from E2- and naringenin-treated rats were treated with ER antibodies (Fig. 3). The results show that both retarded bands were supershifted, confirming that the ER was an integral component of the retarded DNA–protein complex (Fig. 3).

The effects of E2, naringenin and their combination on cytosolic ER and PR were also determined using the gel electrophoretic mobility shift assay procedure. Cytosolic ER and PR exhibited minimal binding affinity for their respective hormone responsive elements; however, after incubation at 20°, the cytosolic receptors underwent transformation. The resulting transformed ER and PR complexes bound with high affinity to their corresponding hormone responsive elements and could be detected as retarded bands in a gel electrophoretic mobility shift assay (Figs. 4 and 5). The cytosolic extracts were derived from the same animals from which the nuclear extracts were prepared (Figs. 2 and 3). Transformed cytosol from control animals exhibited two intense retarded bands; these bands were not detectable in cytosol

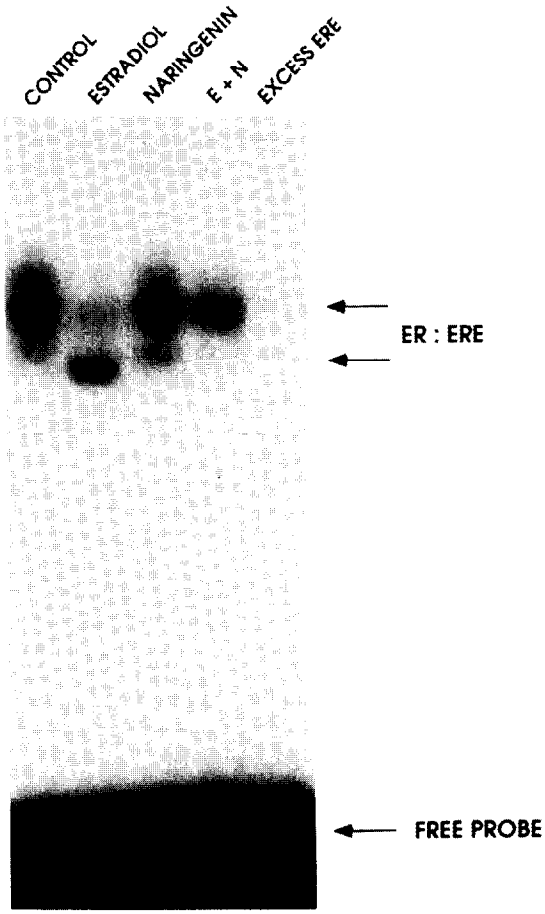

Fig. 2. ERE binding of nuclear uterine extracts from rats treated with vehicle (control), E2(E) (0.5 μg/rat), naringenin (N) (30 mg/rat), or E2 plus naringenin (E + N). Uterine nuclear extracts were isolated from the treated animals, incubated with [^{32}P]ERE, and analyzed by gel electrophoretic mobility shift assay as described in Materials and Methods. Binding specificity was confirmed by incubating nuclear extracts from E2-treated rats with [^{32}P]ERE and a 1000-fold excess of unlabeled ERE (excess ERE lane). In contrast, coincubation of these nuclear extracts with [^{32}P]ERE and a 1000-fold excess of unlabeled mutant ERE did not decrease the intensity of the retarded band (data not shown).

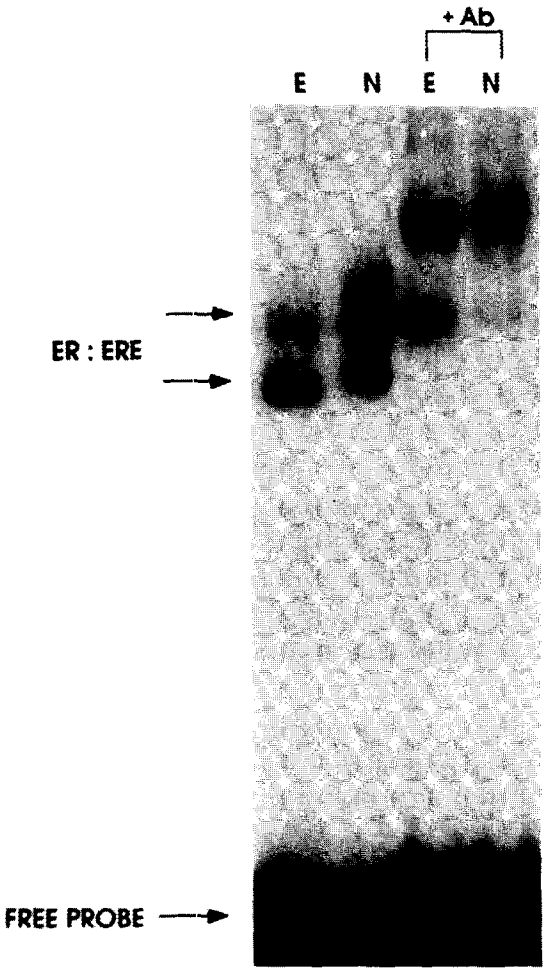

Fig. 3. ER antibody supershift of ER:ERE complexes. Uterine nuclear extracts from E2 (E) or naringenin (N) (30 mg/rat) treated animals (see Fig. 2) were also incubated with ER antibodies (Ab), and both bands were supershifted in the gel electrophoretic mobility shift assay.

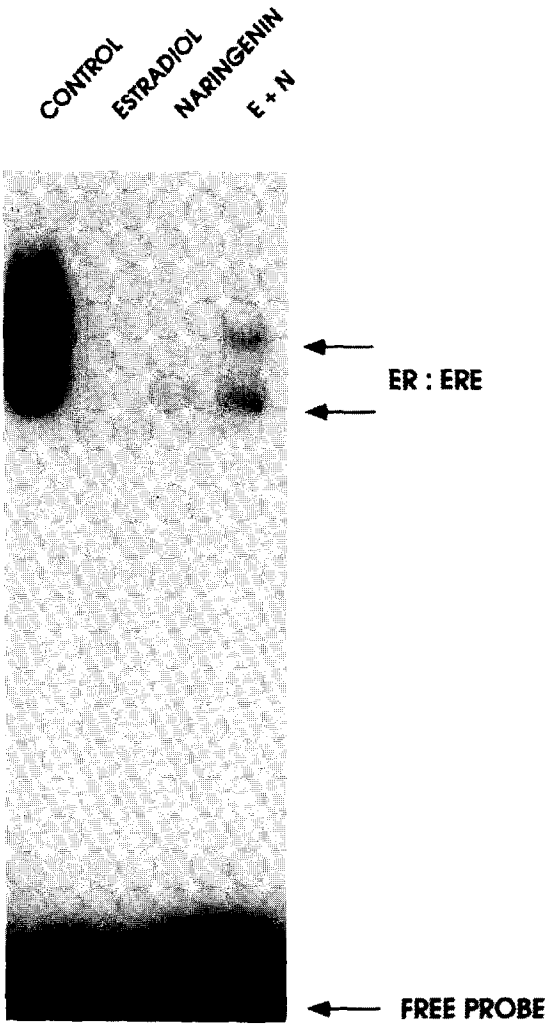

Fig. 4. Depletion of uterine cytosolic ER binding in rats treated with E2, naringenin (30 mg/rat) or their combination. Rat uterine cytosols were isolated from control, E2-, naringenin-, and naringenin plus E2-treated animals, transformed, incubated with [^{32}P]ERE, and analyzed by gel electrophoretic mobility shift assay as described in Materials and Methods. Transformed cytosol from control animals gave intense retarded bands (see arrows), whereas the corresponding bands were decreased markedly in transformed cytosols from the treated animals.

from E2-treated animals, while weaker bands were observed using cytosol from naringenin- and naringenin plus E2-treated rats. The effects of various treatments on the formation of progesterone-induced transformed cytosolic uterine extracts were also determined using [^{32}P]PRE (Fig. 5). Using uterine cytosol from E2-treated rats, there was a marked increase in a PR:PRE retarded band, whereas less intense retarded bands were observed using extracts from control and naringenin-treated animals. The intensity of the PR:PRE retarded band was lower using cytosol from rats treated with E2 plus naringenin compared to with cytosol from animals treated with E2 alone.

The *in vitro* activity of naringenin as a partial ER agonist and as an inhibitor of E2-induced responses was also examined in MCF-7 breast cancer cells. The results, summarized in Fig. 6, illustrate that 1 nM E2 induced a 3- to 4-fold increase in cell proliferation, whereas naringenin at 1–1000 nM did not affect cell growth. In cells, cotreated with 1 nM E2 plus 1–1000 nM naringenin, there was a significant decrease in E2-increased cell growth in cells cotreated with 1000 nM naringenin. In a parallel study, the effects of E2, naringenin and their combination were determined in MCF-7 cells transiently transfected with the pS2-LUC plasmid [39]. The results, summarized in Table 2, demonstrate that 1 nM E2 induced a 7-fold increase in pS2-LUC activity. Cells treated with naringenin alone exhibited a concentration-dependent increase in luciferase activity and a 5.6-fold increase was observed using 10 μM naringenin. Cotreatment of cells with 1 nM E2 plus 1.0 or 0.1 μM naringenin caused a 25.6 and 49.5% decrease in pS2-LUC activity compared with that observed with cells treated with 1 nM E2 alone.

DISCUSSION

A recent study reported that naringenin, a plant bioflavonoid, competitively displaced [^{3}H]E2 from the human ER expressed in COS cells and induced CAT in HeLa cells cotransfected with a human ER expression vector

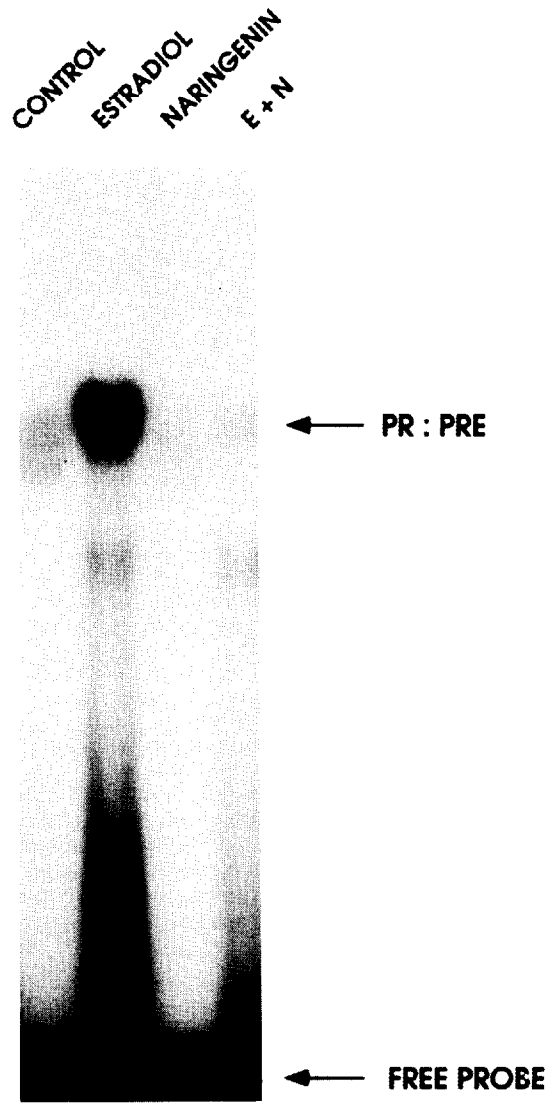

Fig. 5. PRE binding of uterine cytosol from rats treated with vehicle (control), E2, naringenin, or E2 plus naringenin (E + N). Uterine cytosol was isolated from the control, E2-, naringenin-, and naringenin plus E2-treated animals, incubated with [^{32}P]ERE, and analyzed by gel electrophoretic mobility shift assay as described in Materials and Methods. Coincubation of E2-induced nuclear extracts with a 1000-fold excess of unlabeled PRE significantly reduced the radiolabeled retarded band (data not shown). The E2-induced retarded band was decreased or nondetectable using cytosol from animals in the other treatment groups.

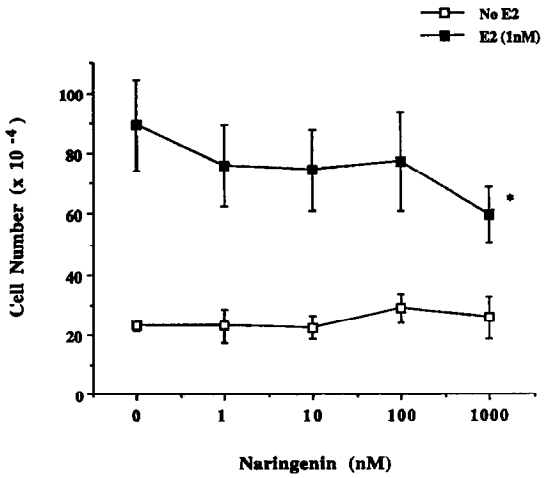

Fig. 6. Effects of naringenin alone or naringenin plus E2 on growth of MCF-7 cells. The cells were grown for 11 days in the presence 1 nM E2 alone (■) (0 concentration of naringenin), 1 nM E2 plus different concentrations of naringenin (■) or naringenin alone (□). The results are means ± SD for 3 separate experiments. Key: (*) E2-induced cell proliferation was inhibited significantly ($P < 0.05$) by 1000 nM naringenin.

and the ERE-tk-CAT reporter plasmid [27]. Their data showed that E2 was at least 1000-fold more potent than naringenin in both binding and induction assays. These results indicate that naringenin is a weak ER agonist in an *in vitro* system, and this is similar to the results of other studies with diverse bioflavonoids. The major objectives of the present study were to compare the estrogenic activity of naringenin in both *in vitro* and *in vivo* models since greatly reduced estrogenic potency may be observed *in vivo* due to pharmacokinetics and metabolism.

Markaverich *et al.* [12] have reported previously that both luteolin and quercetin are also weak estrogens; however, in animals or cells treated with a combination of these bioflavonoids plus E2, there was a significant inhibition of E2-induced responses. Therefore, the present study also investigated the effects of naringenin plus E2 on several *in vitro* and rat uterine estrogen-induced responses to assess the partial antiestrogenic effects of the weak ER agonist.

Naringenin alone did not change rat uterine wet weight significantly at any of the doses (15, 20, 30 and 40 mg/rat); however, at the highest dose (40 mg/rat), naringenin alone was toxic to some of the animals, whereas no adverse effects were observed at lower doses. In the cotreatment studies (E2 plus naringenin), naringenin (20, 30 and 40 mg/rat) significantly inhibited E2-induced uterine wet weight (Fig. 1), and these results were similar to those previously reported for luteolin and quercetin [12]. The effects of E2, naringenin (30 mg/rat), and their combination on rat uterine peroxidase activity, PR levels and [^{3}H]thymidine uptake were also investigated (Table 1). E2 significantly induced these uterine responses, whereas naringenin alone did not affect PR binding but caused a significant decrease in uterine peroxidase activity and [^{3}H]thymidine uptake. The data indicate that the *in vivo* estrogenic activity of naringenin in the female rat uterus was >2.5 × 10^4 times lower than E2, and these results were similar to those previously reported for quercetin [12]. In contrast, the *in vitro* studies show that the estrogenic potency of naringenin relative to E2 was 0.001 to 0.0001 [27] (Table 2). In rats cotreated with naringenin (30 mg/rat) plus E2, the bioflavonoid caused a 36–63% reduction of E2-induced uterine peroxidase activity, PR levels and [^{3}H]thymidine uptake. These results demonstrate that naringenin, like other bioflavonoids which are weak ER agonists, exhibits antiestrogenic activity in the female rat uterus.

In the rat uterus, E2-induced changes in uterine wet weight and uterine peroxidase activity are accompanied by depletion of cytosolic ER levels, increased formation

Table 2. Effect of naringenin on E2-induced pS2-LUC activity in MCF-7 human breast cancer cells*

Treatment (concn)	LUC response (%)
Control	13.7 ± 2.4
Naringenin (10 μM)	77.4 ± 8.3
Naringenin (1.0 μM)	58.2 ± 6.2
Naringenin (0.1 μM)	26.2 ± 2.2
Naringenin (0.01 μM)	21.7 ± 1.6
E2 (1 nM)	100.0 ± 6.9
E2 (1 nM) + naringenin (10 μM)	105.7 ± 6.8
E2 (1 nM) + naringenin (1.0 μM)	74.4 ± 6.5†
E2 (1 nM) + naringenin (0.1 μM)	50.5 ± 7.2†
E2 (1 nM) + naringenin (0.01 μM)	86.6 ± 5.3

* Values are means ± SEM of four measurements for each treatment and are reported as a percentage relative to induction observed with 1 nM E2 (100%). Two samples were taken from each treatment, which were performed in duplicate. The experiment was repeated three times.

† The level of induction of pS2-LUC activity was significantly different from the activity observed following treatment with 1 nM E2 ($P < 0.05$).

of the nuclear ER complex, and modulation of several other responses including induction of the PR [40]. Changes in subcellular hormone receptor levels are routinely determined by radioligand binding and immunoblotting using specific antibodies. In this study, the effects of various treatments on hormone receptor levels (ER and PR) were also investigated by gel electrophoretic mobility shift assays that measure binding of transformed hormone receptor complexes with their cognate [^{32}P]-labeled enhancer sequences. One of the advantages of the latter assay is that hormone receptors bound with hormone or antihormone may form DNA complexes that exhibit different gel electrophoretic mobilities [41–47]. Therefore, this assay system was used to investigate the effects of E2, naringenin and E2 plus naringenin on uterine cytosolic and nuclear ER and cytosolic PR levels.

High salt (0.6 M KCl) nuclear extracts from untreated rats gave one major retarded band (upper) that bound to [^{32}P]ERE in a gel electrophoretic mobility shift assay (Fig. 2). The intensity of the upper retarded band was decreased using extracts from E2-treated rats, and this was accompanied by formation of an intense, more mobile lower band (Fig. 2). The specific enhancement of the lower band was observed only with nuclear extracts from rats treated with E2; moreover, the mobility of the lower band formed with extracts from naringenin-treated rats was different from the band formed with extracts from E2-treated animals. Gel electophoresis of the ERE complex with extracts from rats treated with E2 plus naringenin gave only the top band. These results (Fig. 2) suggest that the E2-induced lower band may represent ERE binding of the transcriptionally active form of the ER and naringenin inhibits formation of the E2-induced complex. That both retarded bands represent ER:ERE binding was confirmed by supershifting both complexes after incubation with ER antibody (Fig. 3). The formation of more than one retarded ER:ERE has been reported previously [41, 42], using nuclear extracts from E2-treated mice. The complexes formed were qualitatively identical with nuclear extracts from mice treated with a variety of estrogenic compounds. However, nuclear extracts obtained from mice treated with an estrogen antagonist (LY117018) formed complexes that migrated more slowly than complexes formed with estrogenic compounds [39].

The effects of E2, naringenin, and E2 plus naringenin on cytosolic estrogen and progesterone receptor binding were also determined by examining heat-induced transformed cytosolic receptor from the treated animals using a gel electrophoretic mobility shift assay. The results presented in Fig. 4 show that the retarded ER:ERE band derived from transformed uterine cytosol from the treated animals was markedly less intense than the corresponding band from control animals. Thus, despite the differences between the estrogenic potency of E2 and naringenin, both compounds caused a depletion of cytosolic ER. These results are somewhat paradoxical since there was not a correlation between chemical-induced depletion of cytosolic ER (Fig. 4) and formation of nuclear ER using the gel electrophoretic mobility shift assay (Fig. 2); the rationale for this discrepancy is being investigated further.

The induction of PR by estrogens is a well-characterized E2-induced response [31, 40], and this was measured by binding of transformed uterine cytosol to a synthetic [^{32}P]PRE followed by the gel electrophoretic mobility shift assay (Fig. 5). E2 induced a single retarded band that corresponded to the PR:PRE complex, whereas extracts from control-, naringenin- or naringenin plus E2-treated rats did not induce formation of this band. Thus, the bioflavonoid significantly inhibited the intensity of the E2-induced PR:PRE complex. However, the failure to detect the PR:PRE retarded band in transformed cytosols from control, naringenin-, and naringenin plus E2-treated animals was unexpected and is being investigated further. In summary, the results obtained from the gel electrophoretic mobility shift assays show that naringenin inhibited E2-induced retarded bands (Figs. 2–5), and these results complement the antiestrogenic effects of naringenin on E2-induced uterine hypertrophy, peroxidase activity, PR levels, and [^{3}H]thymidine uptake.

The second phase of this study investigated the *in vitro* estrogenic activity of naringenin and the partial antiestrogenic effects of this compound in MCF-7 human breast cancer cells. The results summarized in Fig. 6 show that 1–1000 nM naringenin did not induce significantly proliferation of MCF-7 human breast cancer cells, whereas 1 nM E2 caused a 3- to 4-fold increase in cell growth. In the cotreatment studies, 1000 nM naringenin significantly inhibited E2-induced growth. The partial ER agonist/antagonist activity of naringenin was also investigated in MCF-7 cells transfected with a plasmid containing a luciferase reporter gene regulated by the 5′-promoter sequence from the *pS2* gene. E2-induction of *pS2* gene expression is mediated by a palindromic ERE-like sequence that acts as an enhancer for the nuclear ER homodimer [45]. MCF-7 cells treated with naringenin alone caused a concentration-dependent induction of pS2 Luc activity, thus confirming the weak estrogenic activity reported for this compound and other bioflavonoids using similar *in vitro* systems [26, 27]. The results also showed that the fold induction by 10 μM naringenin was significantly lower than that observed for 1 nM E2 and that naringenin was $>10^4$ times less active than E2 as previously reported [27]. Luciferase activity in cells cotreated with 1 nM E2 plus different concentrations of naringenin was lower than the expected ad-

ditive responses for all treatment groups and at naringenin concentrations of 0.1 and 1.0 μM, there was a significant inhibition of E2-induced luciferase activity (Table 2). A recent study reported that several dietary estrogens including the bioflavonoids biochanin and genistein did not inhibit E2-induced proliferation of MCF-7 cells [28]. However, the concentration of E2 used in their experiments was 10 pM, and this resulted in only a 20–60% increase in cell growth. In contrast, 1 nM E2 was used in the present study, and this concentration appeared to be more sensitive to the inhibitory effects of weak estrogens.

The results of this study confirm that the weak ER agonist, naringenin, inhibits a broad spectrum of E2-induced responses in the female rat uterus and MCF-7 human breast cancer cells. The antiestrogenic activity of this compound may represent one mechanism associated with the activities of bioflavonoids as inhibitors of mammary cancer in rodent studies [13] and the decreased incidence of breast cancer in women living in countries with high consumption of phytoestrogens [1–8]. The potential beneficial or adverse effects of both natural and industrial-derived environmental estrogens will depend on many factors, including the overall dietary mass balance of compounds that exhibit estrogenic and antiestrogenic activities. Current studies in this laboratory are investigating the ER agonist and partial antagonist activities of other phytoestrogens and phenolic industrial compounds and their relative contributions to the human intake of estrogenic chemicals.

Acknowledgements—The financial assistance of the Texas Agricultural Experiment Station and the Medical Research Council of Canada is gratefully acknowledged. S. Safe is a Sid Kyle Professor of Toxicology. T. Zacharewski is supported by a PMAC HRF/MRC Career Award in Medicine.

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