Dietary administration of an extract from rosemary leaves enhances the liver microsomal metabolism of endogenous estrogens and decreases their uterotropic action in CD-1 mice

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We evaluated the effects of a methanol extract from the leaves of the plant Rosmarinus officinalis L. (rosemary) on the metabolism and action of estradiol and estrone. Treatment of female CD-1 mice with 2% rosemary in AIN-76A diet for 3 weeks increased the liver microsomal 2-hydroxylation of estradiol and estrone by ~150%, increased their 6-hydroxylation by ~30% and inhibited the 16α-hydroxylation of estradiol by ~50%. Treatment of female CD-1 mice with 2% rosemary diet for 3 weeks also stimulated the liver microsomal glucuronidation of estradiol and estrone by 54–67% and 37–56%, respectively. In additional studies, feeding 2% rosemary diet to ovariec-to-mized CD-1 mice for 3 weeks inhibited the uterotropic action of estradiol and estrone by 35–50% compared with animals fed a control diet. The results of this study showed that feeding female mice a 2% rosemary diet increased the liver microsomal oxidation and glucuronidation of estradiol and estrone and inhibited their uterotropic action.

Materials and methods

Chemicals

Estrene, estradiol, NADPH, UDPGA triammonium salt and ascorbic acid were obtained from Sigma (St Louis, MO). [6,7-3H]Estradiol or [6,7-3H]estrone (specific activities of 51 or 58 Ci/mol, respectively) and [4-14C]estradiol and [4-14C]estrone (specific activities of 55.1 or 56.4 mCi/mmol, respectively) were all purchased from Du Pont New England Nuclear Research Products (Boston, MA). All solvents (HPLC grade) were obtained from Fisher Scientific (Springfield, NJ).

Rosemary extract-supplemented AIN-76A diet

A methanol extract from the leaves of the plant Rosmarinus officinalis L. (rosemary) was prepared as described previously (12,17). The control AIN-76A diet and 2% rosemary diet (AIN-76A diet supplemented with a methanol extract from rosemary leaves; 2% concentration by weight) were prepared by Research Diets (New Brunswick, NJ).

Oral administration of rosemary extract appeared to be well tolerated in animals. A pilot study on the toxicity of rosemary extract in female CD-1 mice showed that feeding animals 2% rosemary in AIN-76A diet for 3 weeks caused little or no change in body weight. In the present study, this non-toxic, high dose level of rosemary extract was chosen for evaluation of its maximal activity in inducing phase I and II enzymes for metabolizing estrogens.

Preparation of liver microsomes

Six-week-old female CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA). The animals had free access to food and water throughout the experiment. The mice (initially fed the control AIN-76A diet for 4 days after arrival at the laboratory) were either continued on the same diet (control group) or fed a 2% rosemary diet. Animals on both diets were killed by euthanasia in a CO2 chamber 3 weeks later and their livers were immediately removed. Microsomes were prepared by differential centrifugation as previously described (19). Aliquots (~0.5 ml) of microsomal suspension in 0.25 M sucrose were stored at ~80°C. The content of protein was determined with the Bio-Rad protein assay using bovine serum albumin (BSA) as a standard, and the content of cytochrome P450 in microsomal preparations was determined by the method of Omura and Sato (20).

NADPH-dependent microsomal metabolism of estradiol and estrone

The reaction mixture consisted of liver microsomal protein (0.5–1 mg/ml), [4-14C]-labeled estradiol or estrone (dissolved in 5 μl ethanol), 2 mM NADPH and 5 mM ascorbic acid in a final volume of 1.0 ml Tris–HCl (0.1 M) pH 7.4. The mixture was then incubated in a water bath for 10 min at 37°C with constant mild shaking. The reaction was terminated by placing tubes on ice followed immediately by two extractions with 7 ml of 50% aqueous methanol, and a 50 µl aliquot was analyzed for metabolite composition by HPLC.

The HPLC analysis of estradiol and estrone metabolites was performed as

Introduction

A recent study from our laboratory showed that chronic treatment of animals with 2(3)-tert-butyl-4-hydroxyanisole (BHA), a dietary antioxidant (synthetic food additive), preferentially stimulated liver microsomal glucuronidation (a Phase II metabolic pathway) of estradiol and estrone, lowered the circulating levels of these two hormones and inhibited their uterotropic activity in vivo (1). This antiestrogenic effect of dietary BHA (mainly through induction of conjugative estrogen metabolism) has recently been suggested (1) to contribute to its inhibitory effect on estradiol-induced kidney tumors in Syrian hamsters (2) and 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors in female Sprague–Dawley rats (3). Whether dietary rosemary is similar to BHA in modulating estrogen metabolism and action, however, is not known. This question has been examined in the present study.

Abbreviations:

BHA: 2(3)-tert-butyl-4-hydroxyanisole; BHT, 3,5-di-tert-butyl-4-hydroxyanisole; BHT, 3,5-di-tert-butyl-4-hydroxyanisole; BSA, bovine serum albumin; DMBA, 7,12-dimethylbenz[a]anthracene; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; NADPH, β-nicotinamide adenine dinucleotide phosphate (reduced form); NMU, N-nitrosomethylurea; UDPGA, uridine 5′-diphosphogluconic acid.

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Chromatographic profiles for NADPH-dependent metabolism of [4-14C]estradiol by liver microsomes from control or 2% rosemary-treated mice. Female CD-1 mice were fed AIN-76A diet or 2% rosemary diet for 3 weeks and the animals were then killed for preparation of liver microsomes. Incubation mixtures consisted of liver microsomes (0.5 mg protein), 20 or 50 µM estradiol (containing 0.5 µCi [4-14C]estradiol), 2.0 mM NADPH and 5 mM ascorbic acid in a final volume of 1.0 ml Tris–HCl (0.1 M)/HEPES (0.05 M) buffer, pH 7.4. Incubations were for 15 min at 37°C, and the formation of metabolites was determined by HPLC analysis as described in Materials and methods.

The conditions for microsomal glucuronidation of estrogens were as described previously (24). The solvent system for separation of estradiol, estrone and their metabolites consisted of acetone/triton (solvent A), 0.1% acetic acid in water (solvent B) and 0.1% acetic acid in methanol (solvent C). The modified solvent gradient (solvent A/solvent B/solvent C) used for eluting estrogen metabolites from the column was as follows: 8 min of isocratic at 16/68/16; 7 min of concave gradient (curve number 9) to 18/64/18, 13 min of concave gradient (curve number 8) to 20/59/21, 10 min of convex gradient (curve number 2) to 22/57/21, 13 min of concave gradient (curve number 8) to 58/21/21, followed by a 0.1 min step to 92/5/3 and 5 min of isocratic at 92/5/3. The gradient was then returned to the initial condition (16/68/16) and held for 15 min before analysis of the next sample. HPLC metabolite profiles were obtained by radioactivity measurements and radioactive peaks were also detected by UV absorbance at 280 nm. Thus HPLC system separates estradiol, estrone and most of the 27 hydroxylated and keto derivatives of estrogens that were studied (24).

Confirmation of the structural identities of major estradiol and estrone metabolite peaks collected off the HPLC column was achieved by gas chromatography/mass spectrometry (GC/MS) analysis of their trimethylsilyl derivatives as described in detail in our recent study (1).

UDPGA-dependent microsomal glucuronidation of estradiol and estrone

The results for microsomal glucuronidation of estradiol were as described previously (1,25,26). The reaction was initiated by addition of microsomal protein and lasted for 15 min at 37°C. The reaction was arrested by placing the test tubes on ice and adding 0.5 ml ice-cold 50 mM Tris–HCl solution, pH 8.75, followed immediately by two extractions with 7 ml toluene to separate unconjugated estrogen from water-soluble estrogen glucuronides. The toluene-phase, which contains the unconjugated estrogen, was removed. Portions (200 µl) of the aqueous phase, which contained glucuronlated estrogen metabolites, were precisely removed and assayed for radioactivity in a Beckman liquid scintillation spectrometer. The blank values, obtained from incubations in the absence of UDPGA, were determined in each individual assay and subtracted.

The microsomal glucuronidation of 4-nitrophenol was determined according to a previously described method (27,28).

Uterosteric activity of estradiol and estrone

Six-week-old ovariectomized CD-1 mice, obtained from Charles River, were fed a control diet for 4 days upon arrival. These mice were then randomly divided into two groups: one group was kept on the control diet, and the other group was changed to a 2% rosemary diet. Animals were maintained on these two diets for 3 weeks and then received an i.p. injection of estradiol or estrone (45 or 100 ng/mouse, in 50 µl corn oil) once daily for 3 days. Control animals received injections of corn oil alone. At 18 h after the last injection, the animals were killed by euthanasia in a CO2 chamber and their uteri were immediately removed, the surrounding fat tissues trimmed and the wet weights measured.

Results

Effect of rosemary extracts on NADPH-dependent microsomal metabolism of estradiol and estrone

NADPH-dependent metabolism of [4-14C]estradiol by liver microsomes from female CD-1 mice fed the control or 2% rosemary diet for 3 weeks is shown in Figure 1. In both control and rosemary-treated microsomes, 2-hydroxyestradiol was the major metabolite, and several other metabolites were also formed in significant amounts (summarized in Table I). The conversion of estradiol to 2-hydroxyestradiol plus 2-hydroxyestrone (products of estrogen 2-hydroxylation pathway) by rosemary-treated liver microsomes was increased by 140–180% over the controls (Figure 2, upper panel). The combined formation of 6α-hydroxyestradiol, 6β-hydroxyestradiol and 6β-hydroxyestrone (products of estrogen 6-hydroxylation pathway) by rosemary-treated liver microsomes was only slightly increased (~30%) over the controls (Table I). The formation of 4-hydroxyestradiol was not significantly altered by treatment with 2% rosemary diet for 3 weeks (Table I), whereas the formation of 16α-hydroxyestradiol was substantially decreased (50–63%) by the rosemary treatment (Table I).

NADPH-dependent metabolism of 50 µM [4,14C]estrone by liver microsomes from female CD-1 mice fed the control or 2% rosemary diet is summarized in Table I. In both control and rosemary-treated microsomes, 2-hydroxyestrone and 2-hydroxyestradiol were the major metabolites, and several other metabolites were also formed in significant quantities (Table I). The conversion of estrone to 2-hydroxyestrone plus 2-hydroxyestradiol by rosemary-treated
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**Table I.** Stimulatory effect of rosemary extracts on NADPH-dependent metabolism of estradiol and estrone by female CD-1 mouse liver microsomes

<table>
<thead>
<tr>
<th>Diet</th>
<th>Substrate</th>
<th>Estrogen metabolites formed (pmol/mg microsomal protein per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6α-OH-E₂</td>
</tr>
<tr>
<td>Control</td>
<td>20 μM E₂</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>2% rosemary</td>
<td></td>
<td>(74%)</td>
</tr>
<tr>
<td>Control</td>
<td>50 μM E₂</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>2% rosemary</td>
<td></td>
<td>(76%)</td>
</tr>
<tr>
<td>Control</td>
<td>50 μM E₁</td>
<td>0.04 ± 0.00</td>
</tr>
<tr>
<td>2% rosemary</td>
<td></td>
<td>(75%)</td>
</tr>
</tbody>
</table>

Adult female CD-1 mice were fed a control AIN-76A diet or a 2% rosemary diet for 3 weeks and were then killed for preparation of liver microsomes. Incubation mixtures consisted of liver microsomes (0.5 mg protein), 20 or 50 μM [4-14C]estradiol or [4-14C]estrone (0.5 μCi, in 5 ml ethanol), 2.0 mM NADPH and 5 mM ascorbic acid in a final volume of 1.0 ml Tris–HCl/HEPES buffer, pH 7.4. Incubations with estradiol and 50 μM estrone were carried out at 37°C for 20 min. The formation of estrogen metabolites was determined by HPLC analysis as described in Materials and methods. The overall extent of metabolism of the input estrogen is <50% when 20 μM estradiol was used as substrate, and <30% when 50 μM of estradiol or estrone was used as substrate. Each value is the mean ± SD of triplicate determinations. Numbers in parentheses represent the percent increase (↑) or decrease (↓) in estrogen metabolite formation as compared with the control. E₁, estrone; E₂, 17β-estradiol; UKs, non-polar unknown peaks. *P < 0.05 and **P < 0.01, respectively, in comparison with their corresponding controls (Student's t-test).
liver microsomes was increased by 150–175% over the controls (Figure 2, bottom panel). The formation of 6-hydroxylated metabolites of estradiol or estrone by rosemary-treated liver microsomes was slightly increased over the controls (Table I). Little or no conversion of estrone to 4-hydroxyestrone, 4-hydroxyestradiol, 16α-hydroxyestradiol and 6α-hydroxysteradiol was detected in liver microsomes from rosemary-treated mice. Incubation mixtures consisted of liver microsomes (0.5 mg protein), 50 μM estradiol or estrone (containing 0.5 μCi [4-14C]estradiol or [4-14C]estrone), 2.0 mM NADPH, and 5 mM ascorbic acid in a final volume of 1.0 ml Tris–HCl/HEPES buffer, pH 7.4. Incubations were carried out at 37°C for various times as indicated, and the formation of 2-hydroxyestradiol (2-OH-E2) plus 2-hydroxyestrone (2-OH-E1) was determined by HPLC analysis as described in Materials and methods. Each value is the mean of duplicate determinations of a representative microsome preparation.

The identities of 2-hydroxyestradiol, 2-hydroxyestrone, 6α-hydroxyestradiol, 6β-hydroxyestradiol and 6β-hydroxyestrone formed by liver microsomes from rosemary-treated mice were confirmed by comparing their retention times with those of authentic reference compounds (Table II). Because of the relatively minor quantities of some estrogen metabolites (e.g. 4-hydroxyestradiol, 14α-hydroxyestradiol, 16α-hydroxyestradiol) formed, they were only identified by comparing their HPLC retention times with authentic reference compounds.

Effect of rosemary extracts on UDPGA-dependent microsomal glucuronidation of estradiol and estrone
UDPGA-dependent glucuronidation of estradiol and estrone in vitro was proportional to microsomal protein concentration (from 0.3 to 1.0 mg microsomal protein/ml) and also proportional to the incubation time for at least 20 min (data not shown). In this study, we used a final microsomal protein concentration of 1 mg/ml and an incubation time of 15 min for assaying the rates of estradiol or estrone glucuronidation by liver microsomes from control and rosemary-treated mice.

At pH 7.4 (the physiological pH) and pH 8.75 [the optimal pH for estrogen glucuronidation (19)], the rates of estradiol glucuronidation by control liver microsomes were 0.58 ± 0.09 and 0.97 ± 0.14 nmol/mg protein/min, respectively, and the rates of estrone glucuronidation were 0.34 ± 0.05 and 0.86 ± 0.11 nmol/mg protein/min, respectively (Figure 3). Treatment of female CD-1 mice with 2% rosemary diet for 3 weeks increased the rate of liver microsomal glucuronidation of estradiol (at pH 7.4 and 8.75) by 60–75% over the control values (P < 0.01), and increased the rate of estrone glucuronidation by 30–40% over the controls (Figure 3). The UDPGA-dependent microsomal glucuronidation of 4-nitrophenol (a commonly used non-steroid substrate) was increased by <25% (Figure 3).

Effect of dietary rosemary on the uterotrophic action of estradiol and estrone
We evaluated the in vivo effect of chronic administration of 2% rosemary diet on the uterotrophic activity of estradiol and estrone in ovariectomized CD-1 mice. Intraperitoneal injection for 3 days of estradiol and estrone (45 or 100 ng/mouse, once daily) to ovariectomized mice fed the control diet increased the uterine wet weight in a dose-dependent manner (Figure 4). However, the uterotrophic response to estradiol or estrone (45 or 100 ng/mouse, once daily for 3 days) in ovariectomized animals pre-treated with 2% rosemary diet for 3 weeks was inhibited by ~35–50% (P < 0.05; Figure 4).

Discussion
The results of our present study showed that treatment of female CD-1 mice with a diet supplemented with 2% rosemary extracts stimulated the NADPH-dependent liver microsomal 2-hydroxylation of estradiol and estrone (the major metabolic pathway) by ~150% over the controls, and the 6-hydroxylation

| Table II. Identification of estradiol and estrone metabolites |
|------------------|------------------|------------------|
| Estrogen metabolites | Retention times on HPLC (min) | Characteristic mass fragments (m/z) of the trimethylsilyl derivatatives of estrogen metabolites |
| Estradiol (E2) | 50.0 |  |
| Estrone (E1) | 52.5 |  |
| 2-Hydroxyestradiol | 40.2 | 504 (base peak) |
| 2-Hydroxyestrone | 44.0 | 430 (base peak) |
| 4-Hydroxyestradiol | 38.0 |  |
| 6α-Hydroxyestradiol | 11.2 | 504, 489, 414 (base peak), 324, 283, 229 |
| 6β-Hydroxyestradiol | 21.0 | 504 (base peak), 414, 324, 283, 229 |
| 6α-Hydroxyestrone | 23.0 |  |
| 6β-Hydroxyestrone | 25.5 | 430 (base peak), 415, 340, 242, 218 |
| 14α-Hydroxyestradiol | 12.3 |  |
| 16α-Hydroxyestradiol | 15.7 |  |

Liver microsomes from 2% rosemary-treated female CD-1 mice were incubated with estradiol or estrone under conditions described in Materials and methods. Each peak was collected from the HPLC, derivatized with bis(trimethylsilyl)trifluoroaceticamide (BSTFA), and then analyzed by GC/MS as described in detail in our recent study (1). Major fragment (base peak) for each metabolite is underlined. The data presented here for the estrone metabolites are in agreement with those obtained for authentic reference standards.
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Fig. 3. Stimulatory effect of dietary rosemary on the glucuronidation of estradiol, estrone and 4-nitrophenol by female mouse liver microsomes. Female CD-1 mice were fed AIN-76A diet or 2% rosemary diet for 3 weeks and the mice were then killed for the preparation of liver microsomes. Liver microsomes (0.5 mg protein/ml) were incubated with 2 mM UDPGA and 100 µM estradiol, estrone or 4-nitrophenol for 15 min at 37°C, and the glucuronidation of each substrate was determined as described in Materials and methods. Each value is the mean ± SD of triplicate determinations. **Significantly different from the corresponding controls (P < 0.01 by Student’s t-test).

Fig. 4. Inhibitory effect of dietary rosemary on the uterotropic action of estradiol and estrone in ovariectomized mice. Six- to eight-week-old ovariectomized CD-1 mice were fed AIN-76A diet or 2% rosemary in AIN-76A diet for 18 days. While the feeding of these two diets were continued, the animals received an i.p. injection of estradiol or estrone (45 or 100 ng/mouse) once daily for 3 days. At 18 h after the last injection, the animals were killed, the uteri were removed and their wet weights were determined. Each value is the mean ± SD of 7–8 animals. Significantly different from the control value, *P < 0.05 by Student’s t-test, **P < 0.01 by Student’s t-test.

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of estradiol and estrone was also increased but to a smaller extent. Although the liver microsomal 4-hydroxylation of estradiol was not significantly changed by treatment with 2% rosemary diet, the 16α-hydroxylation of estradiol was decreased by ~50%. In addition, chronic treatment with 2% rosemary increased the microsomal glucuronidation of estradiol and estrone by 40–70% over controls. The stimulatory effect of rosemary on the liver microsomal metabolism of estradiol and estrone in female CD-1 mice is accompanied by a 35–50% decrease in their uterotropic response to exogenously administered estradiol or estrone.

Previous studies in animals and humans have suggested that selectively stimulating certain pathways of estrogen metabolism may be beneficial for the prevention of estrogen-dependent tumors in the target organs (reviewed in ref. 29). Chronic administration of indole-3-carbinol stimulates the 2-hydroxylation of estradiol in animals and inhibits the development of spontaneous mammary tumors in C3H/OuJ mice (30) and spontaneous uterine tumors in oncomice (31) and Donryu rats (32). The development of DMBA- or N-nitrosomethylurea (NMU)-induced mammary tumors [both of which are highly dependent on endogenous estrogens (33)] in female Sprague–Dawley rats was also inhibited by chronic treatment with indole-3-carbinol (34). Recent studies from our laboratory also showed that chronic treatment of female C3H/OuJ mice with 0.05% sodium phenobarbital (a prototype P450 enzyme inducer) strongly inhibits the formation of spontaneous mammary tumors, and this tumor-inhibitory effect is accompanied by a many-fold increase of liver microsomal 2-hydroxylation of estradiol with little or no increase of estradiol 4- and 16α-hydroxylations (B.T.Zhu and A.H.Conney, unpublished data). In humans, female smokers have enhanced 2-hydroxylation of estradiol (35,36) and lower serum and urinary levels of estradiol and estrone (37–39), and these individuals have a lower risk of endometrial cancer along with an increased risk of osteoporosis (40–42).

It is noteworthy that rosemary extracts and the dietary antioxidants BHA and BHT each inhibited the DMBA-induced
mammary tumors in female Sprague–Dawley rats (3–6,16). In addition, an earlier study showed that chronic administration of dietary BHA inhibited estradiol-induced renal tumorigenesis in Syrian hamsters (2), a commonly used animal model for studying estrogen carcinogenesis (43,44). Although multiple mechanisms may be involved in BHA inhibition of estrogen-associated cancers, our recent findings that dietary BHA inhibits the hormonal action of estrogens by stimulating preferentially their conjugative metabolism could be an important underlying mechanism. The results of our present study showed that 2% rosemary diet exerted an antiestrogenic efficacy in ovariectomized CD-1 mice similar to that of 0.75% BHA diet. However, rosemary extracts showed a greater stimulatory effect on the NADPH-dependent oxidative metabolism (mainly the 2-hydroxylation pathway) of estradiol and estrone than on their conjugative metabolism.

In summary, the results of this study showed that chronic treatment of female CD-1 mice with rosemary extracts stimulates the 2- and 6-hydroxylations of estradiol and estrone as well as their conjugation metabolism. The stimulatory effect of rosemary extracts on the liver microsomal metabolism of estradiol and estrone is accompanied by a decreased uterotrophic action of these estrogens in vivo. The antiestrogenic effect of dietary rosemary described here may contribute to its inhibitory effect on DMBA-induced mammary tumors in experimental animals (16).

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