Dietary Resveratrol Does Not Affect Intestinal Tumorigenesis in Apc\textsuperscript{Min/+} Mice\textsuperscript{1}

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ABSTRACT To determine its effect on intestinal tumorigenesis and the protumorigenic COX pathway in Apc\textsuperscript{Min/+} mice, resveratrol was administered as a powdered admixture in the diet at 0, 4, 20, or 90 mg/kg body weight for 7 wk. In two separate experiments, resveratrol did not affect intestinal tumor load. It was stable in the diet under experimental conditions, circulated in the plasma as the glucuronide-conjugated form and reached the tumors as evidenced by significant decreases in PGE\textsubscript{2} levels. However, immunohistochemical staining of intestinal tumors revealed no changes in COX-2 expression. This study demonstrates that resveratrol consumed ad libitum in the diet, does not modify tumorigenesis in Apc\textsuperscript{Min/+} mice. J. Nutr. 134: 5–10, 2004.

KEY WORDS: • cancer • resveratrol • Apc • phytochemical • COX-2

Polyphenolic phytochemicals have been shown to be effective antitumorigenic agents in several in vitro models (1–2), and efficacy in vivo has also been demonstrated when these compounds are provided in the diet (3–5). Resveratrol, a resorcinol-containing polyphenol found most commonly in red wine, inhibits the growth of several immortalized human colorectal cancer cell lines (6,7). When provided orally, resveratrol reduced the tumor load in Lewis Lung carcinoma–bearing mice (8) and reduced the number of aberrant crypt foci in an azoxymethane-induced rat model of colorectal cancer (9). Quercetin, curcumin and caffeic acid phenyl ester, chemical analogs of resveratrol, inhibit intestinal tumorigenesis in vitro (10–12) and aberrant crypt foci formation in vivo (5,13). These polyphenolic phytochemicals share a common resorcinol-containing structure, and this structure is believed to be responsible in part for the reported effects (14,15).

Resveratrol’s antitumorigenic effects have been linked in part to inhibition of cyclooxygenase (COX)\textsuperscript{1}–catalyzed reactions (15–18). COX catalyzes the committed step in the conversion of arachidonic acid to protumorigenic eicosanoids, such as prostaglandin (PG)E\textsubscript{2}, which are involved in the maintenance of tumor integrity (19). COX-2 expression is upregulated in 80–85% of human adenocarcinomas and colonic tumors (20,21) and in 80–85% of Apc\textsuperscript{Min/+} mouse adenomas (22). Upregulation was observed in ~60% of the tumors. Resveratrol was shown to inhibit COX-2 promoter activity (15), downregulate COX-2 expression (15,17) and inhibit COX-2 activity in vitro (16,23).

Numerous studies have demonstrated the ability of COX inhibitors to decrease tumor multiplicity in the Apc\textsuperscript{Min/+} mouse, a murine model possessing a germline mutation in the adenomatous polyposis coli (Apc) tumor-suppressor gene (24–27). These mice spontaneously develop a mean of 40 tumors throughout the intestinal tract that are sensitive to COX inhibition. Murine Apc is homologous to human APC (28), and patients with familial adenomatous polyposis (FAP) have an autosomal dominant disorder with a germline mutation in one allele of the APC tumor suppressor gene (29). Adenoma formation results when a somatic mutation is acquired in the remaining functional allele. By the time they reach their twenties, FAP patients typically develop hundreds to thousands of intestinal adenomas, mostly in the colorectal mucosa, which are also sensitive to COX inhibition (30). Similarly, at least one allele of APC is mutated in ~80% of sporadic colorectal cancers, making the Apc\textsuperscript{Min/+} mouse a relevant model for studying the early stages of colorectal cancer (31,32).

The purpose of this study was to evaluate the dose-dependent effects of dietary resveratrol on intestinal tumorigenesis in Apc\textsuperscript{Min/+} mice, and to determine whether tumor COX-2 and/or PGE\textsubscript{2} levels are affected by this treatment.

MATERIALS AND METHODS

Animals. Male C57BL/6J Apc\textsuperscript{Min/+} mice or wild-type littersmates (Jackson Laboratories, Bar Harbor, ME) were obtained after genotyping at 35 d of age and randomly assigned to their respective experimental groups. The mice consumed water and diet ad libitum. Food intake and overall health were monitored daily and body weights were monitored weekly. All animal research procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with the NIH guidelines (33).

Diets. AIN-93G powdered diet (Dyets, Bethlehem, PA) was used as the base diet. Experimental diets were prepared weekly by thoroughly mixing resveratrol (Sigma Chemical, St. Louis, MO) with the base diet to homogeneity. Resveratrol concentrations in the diets...
were adjusted weekly, on the basis of food consumption and body weights to achieve doses of 0, 4, 20 and 90 mg/kg body weight. Diets were prepared under yellow light and stored at −80°C under an atmosphere of nitrogen to minimize degradation of resveratrol. Fresh diet was provided daily.

Experimental design
Pharmacokinetics and absorption of resveratrol. To establish the stability of resveratrol in the diet, dietary samples containing resveratrol at 750 μg/g (dose equivalent to the mice fed 90 mg/kg body weight) (n = 2) were stored under an atmosphere of nitrogen at −80°C (controls), or exposed to experimental conditions (light and room temperature for 24 h). Samples were extracted four times with ethyl acetate (Sigma), evaporated under an atmosphere of nitrogen, resuspended in the reversed-phase HPLC (RP-HPLC) mobile phase and analyzed for resveratrol content via RP-HPLC.

To establish that resveratrol reaches the intestinal tract intact and is subsequently absorbed, 10-ko-old male C57BL/6J mice (n = 15) were administered resveratrol in the diet at a dose of 200 mg/kg body weight. Before killing, the mice were divided into five groups (n = 3/group). After 6 d of consuming the diet, blood was collected postprandially via cardiac puncture (under general anesthesia) at 5, 15, 30, 60 and 90 min, and plasma resveratrol concentration was measured by RP-HPLC. In a follow-up experiment, 10-ko-old C57BL/6J wild-type mice (n = 3) were fed resveratrol at 90 mg/kg body weight. After 6 d of feeding, blood was collected 30 min postprandially and samples were analyzed for resveratrol content via RP-HPLC.

Effects of resveratrol on intestinal tumorigenesis. Male C57BL/6J ApcMin/+ mice (n = 25; 43 d of age) were assigned to four groups (n = 6–7/group) and fed diets supplemented with resveratrol at 0, 4, 20 and 90 mg/kg body weight for 7 wk, until 85 d of age. Tumor number, size and location were determined with the use of a dissecting microscope. Whole blood was collected via cardiac puncture and immediately placed on ice protected from light. Plasma was isolated by centrifugation at 500 g, at 4°C for 15 min. To determine relative levels of resveratrol circulating in the free form, 10-wk-old male C57BL/6J mice (n = 16) were assigned to a control or experimental group (n = 8) and fed resveratrol at a dose of 0 or 90 mg/kg body weight for 7 wk. At 87 d of age, the mice were killed, and excised intestinal tumors were analyzed for COX-2 expression and basal PGE2 levels.

RP-HPLC analysis of trans-resveratrol. Resveratrol levels were analyzed by RP-HPLC using a Hewlett Packard 1090 Series-2 Liquid Chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a 25 cm, 4.6 mm KR100–5C18 Kromasil RP-HPLC column (Akzo Nobel-EKA Chemicals, Bohus, Sweden) with an isocratic mobile phase consisting of 70% CH3CN, 30% methanol, 0.1% glacial acetic acid (Sigma), at a flow rate of 0.3 mL/min. Samples were monitored at 306 nm using a photodiode array detector. Maximum absorption of resveratrol was reported at 306 nm (34). Detection and spectrophotometric identification limits were 2 and 3 ng, respectively.

Extraction of resveratrol from plasma. To determine relative levels of free vs. glucuronidated resveratrol in the plasma, whole blood was collected via cardiac puncture, using EDTA (Life Technologies, Grand Island, NY) at a concentration of 7.5% as an anticoagulant, and immediately placed on ice, protected from light. Plasma was isolated by centrifugation at 500 × g, 4°C for 15 min. To determine relative levels of resveratrol circulating in the free form, 200-μL aliquots of plasma were placed in Eppendorf tubes, diluted 1:10 with methanol, adjusted to a pH of 3.5 with formic acid and subjected to solid-phase extraction using a conditioned C18 cartridge (Burdick and Jackson, MI). Resveratrol was eluted from the column twice with 2 mL of 100% HPLC-grade methanol (Sigma), evaporated under an atmosphere of nitrogen, resuspended in the RP-HPLC mobile phase and analyzed via RP-HPLC.

To determine relative levels of resveratrol circulating as the glucuronide derivative, 200-μL aliquots of plasma were placed in Eppendorf tubes for enzymatic hydrolysis via β-glucuronidase. Powdered H-1 β-glucuronidase (Sigma) was solubilized at 50 g/L in 0.2 mol/L sodium acetate buffer, pH 5.0, at 37°C. This enzyme solution was added to plasma at 5:1, to a final concentration of 0.04 g/mL, and final specific activity of 5 mg/(min · mg protein). Samples were incubated for 2 h at 37°C in a rocking incubator. The reaction was terminated with 10% H2O2, followed by incubation at 37°C with 0.5% formic acid, to a final volume of 10% methanol. Samples were extracted three times with equal volumes of ethyl acetate (Sigma), and pooled organic phases were evaporated under nitrogen, resuspended in 1 mL of a 10% aqueous methanol solution and passed through a 0.45-μm filter ( Pall, Ann Arbor, MI). We do not report specific plasma levels due to lack of an appropriate internal standard. We report an estimated plasma value based on comparison to a standard curve of authentic resveratrol level.

Immunohistochemistry for COX-2 expression. Tissues were formalin-fixed, embedded in paraffin and sectioned at 4 μm. Tissue sections were then heated, deparaffinized in xylene, rehydrated in graded alcohols to PBS (3 times, 5 min each) and pretreated at or just below boiling with 10 mmol/L citrate buffer, pH 6, for 10 min. Endogenous peroxidase activity was quenched by incubation in 3% H2O2, PBS for 15 min at room temperature and incubated for 30 min at room temperature with protein block (Biogenex USA, San Ramon, CA). Slides were then incubated at 20°C for 1 h with affinity-purified rabbit polyclonal anti-mouse COX-2 IgG (1:200) (Cayman Chemical, Ann Arbor, MI), followed by biotinylated anti-rabbit IgG (30 min at room temperature) and streptavidin/biotinhorseradish peroxidase complex (30 min at room temperature), which was localized by reaction with 3,3’-diaminobenzidine tetra-hydrochloride (0.75 g/L; BioRad), 0.05% H2O2 in Tris-HCl, pH 7.6, for 10 min. Slides were lightly counterstained with Mayer’s hematoxylin. Staining for COX-2 protein was evaluated from coded slides and scored on the bases of distribution (extent) and relative staining intensity by a pathologist who was unaware of the treatments.

Relative staining distribution was scored as follows: 0 = < 10 stained cells per high power field (0.2 mm2 at X400 magnification); 1 = 10–20 stained cells per high power field; 2 = 20–30 stained cells per high power field; and 3 = > 30 stained cells per high power field. Both positive and negative tumors (i.e., distribution = 0) were included in calculating mean scores for each group. Relative staining intensities were graded as follows: 0 = no staining; 1 represented perinuclear staining; 2 represented stain extending to the cytoplasm; and 3 indicated a stain that obscured the cell nucleus. Intensity and distribution scores were then added to generate a final value corresponding to COX-2 expression. The reported staining scores represent the sum of intensity and distribution ± SEM.

Basal PGE2 Levels. To determine the effect of resveratrol on PGE2 levels in tumors, intestinal tumors from ApcMin/+ mice fed resveratrol in the diet at 0 or 90 mg/kg body weight were excised on dry ice, snap-frozen in liquid nitrogen and stored at −80°C. At the time of analysis, tumors were pooled from each mouse and immediately homogenized at 4°C using a Polytron handheld homogenizer at 10;1 in ice-cold 0.2 mol/L Tris-HCl buffer, under acidic conditions (pH 3.5) in the presence of metanol (10%) to eliminate any possibility of ex vivo PG synthesis. An 50-μL aliquot from each group was used for protein determination via the Bradford assay (35). PGE2 levels were determined by enzyme immunoassay (ELA) using the Correlate-ELA PGE2 Kit (catalog # 900–201; Assay Designs, Ann Arbor, MI) according to the manufacturer’s instructions.

Statistical analyses. Statistical analyses were conducted using the SAS and JMP software (SAS Institute, Cary, NC). Data were examined for normality of distribution and homogeneity among variances. Differences in body weight, diet intake, tumor number and basal PGE2 levels were analyzed by student’s t test or one-way ANOVA. Differences among groups were detected using Fisher’s least significant difference at P < 0.05. Data are expressed as mean ± SEM.

RESULTS

Food intake did not differ among the treatment groups. During the last week of treatment, weights of mice in the control group were significantly lower compared with the low
dose treatment group (4 mg/kg body weight); the other groups did not differ at that time point (data not shown). The presence of resveratrol was determined by RP-HPLC and identified by its unique UV absorption spectrum (Fig. 1). To determine the general stability characteristics of resveratrol when exposed to UV light, resveratrol in solution was exposed to constant UV light for 18 d and analyzed via RP-HPLC every 2 d for its degradation products. Resveratrol was stable up to 14 d. After 14 d, two additional peaks were observed that corresponded to two resveratrol isomerization products identifiable by distinct UV absorption spectra (36). No isomerization products of resveratrol were detected in the diet when it was exposed to the same conditions present in the feeding study, suggesting that resveratrol remained stable in the diets under these conditions (data not shown).

Free resveratrol was not detected in the plasma of mice fed resveratrol at 200 mg/kg body weight because resveratrol circulates as a glucuronide conjugate. Resveratrol was detected in the plasma of mice after hydrolysis of the glucuronide conjugate. Resveratrol (free or conjugated) was not detected in the plasma of those mice fed a diet devoid of resveratrol. However, in hydrolyzed plasma, plasma resveratrol was detected within 5 min of oral administration; it peaked by 30 min and was maintained for at least 90 min (Fig. 2).

Resveratrol had no effect on intestinal tumor load in the small or large intestine when provided in the diets of APCMin/+/H11001 mice at 0–90 mg/kg body weight (Fig. 3). A second experiment yielded similar results, with no difference in tumor load among mice fed 0 and 90 mg/kg body weight (Table 1). Basal PGE2 levels in tumors of mice fed resveratrol at 90 mg/kg body weight were measured by EIA as an index of relative COX activity compared with the control mice (0 mg resveratrol/kg body weight). PGE2 levels of small intestinal tumors were significantly lower in the resveratrol-treated mice than in controls (Table 1). COX-2 protein levels did not differ between the control mice (0.69 ± 0.20 mg) and those fed resveratrol at 90 mg/kg body weight (1.09 ± 0.24 mg). COX-2 localization was constrained in the stromal cells of the tumor, with no detectable levels in the epithelium (Fig. 4). This confinement of COX-2 expression to stromal cells as opposed to neoplastic epithelium was reported previously (22) and suggests that prostaglandins may exert their protumorigenic effects via paracrine signaling.

**DISCUSSION**

The in vitro chemotherapeutic effects of resveratrol are believed to be related in part to inhibition of COX-2 expression and activity (16), and these same biochemical pathways are linked to intestinal tumorigenesis in APCMin/+/H11001 mice (19,24,37). However, we report that resveratrol (0–90 mg/kg body weight) did not decrease the number of intestinal tumors after 7 wk of treatment.

Analysis of the diets revealed that resveratrol remained stable during the time of feeding, and plasma pharmacokinetic data indicated adequate absorption. Resveratrol and other

<table>
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<th>TABLE 1</th>
<th>Effect of dietary resveratrol administered to APCMin/+/ mice for 7 wk at 0 or 90 mg/kg body weight on small intestine tumor number1</th>
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<td>Resveratrol, mg/kg body weight</td>
<td>Tumors, n</td>
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<td></td>
<td>32.86 ± 5.56</td>
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<td>Prostaglandin E2, pg/mg protein</td>
<td>1.32 ± 0.62</td>
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1 Values are means ± SEM, n = 8. * Different from untreated control.
polyphenols are absorbed in the small intestines where they undergo glucuronidation before appearing in the blood (38). In the circulation, 100% of resveratrol exists as the glucuronide conjugate. However, there is much debate concerning whether this form of resveratrol is biologically active. This is important because in vitro studies utilize the free form of resveratrol, quercetin and catechins display pharmacokinetics similar to the kinetics observed here, with peak plasma levels occurring at ~0.5–1 h postingestion; others confirmed that >95% of all of these compounds circulate in the plasma in their conjugated forms (42–44).

We did not observe any reduction in tumor load with dietary levels of resveratrol that ranged from 4 to 90 mg/kg body weight. These results are in contrast to a previous paper reporting a 74% reduction in tumor frequency in that model when resveratrol was provided in the drinking water dissolved in ethanol at a dose of 12–16 mg/kg body weight (45). We were unable to dissolve resveratrol in the drinking water under experimental conditions similar to those described in that paper. It is possible that a bolus dose (10 mg/kg) of resveratrol could have had the reported effects. We considered whether the presence of ethanol in the drinking water might have had any effect on free resveratrol levels in the plasma. However, it does not appear that the use of ethanol as a vehicle has any influence on altering the availability of free resveratrol (44–49) and therefore cannot explain the disparate results observed between the two studies. We did, however, duplicate our results to recapitulate our earlier findings. We repeated the study using the highest dose (90 mg/kg body weight) and again observed no significant decreases in tumor load with dietary resveratrol.

Furthermore, our results confirm that resveratrol was absorbed and reached the target tissue as evidenced by reductions in PGE2 levels in tumors of resveratrol-fed mice compared with controls. The doses administered were comparable to doses administered in a previous study (45) and the length of the study (7 wk) should have been adequate to observe any decreases in tumor load. Our estimated concentration of 131 μmol/L for total plasma resveratrol (90 mg/kg body weight group) far exceeds the plasma levels (1.5 μmol/L) from a volunteer given a 25-mg oral dose (~0.4 mg/kg body weight for a 70 kg individual) (40). To fully appreciate these oral doses, the amount of resveratrol in a liter of table wine varies from 0.1 to 7.7 mg (36,50). Most wines, however, contain concentrations that are <0.5 μg/L (36).

Disagreement exists concerning resveratrol’s ability to modify tumorigenesis, particularly when comparing results in vitro to those in vivo. Resveratrol inhibited the in vitro growth of 32Dp210 mouse myeloid leukemia cells and resveratrol-pretreated cells implanted subcutaneously; however, when the same cell line was implanted subcutaneously without pretreatment, it was possible that resveratrol orally administered could have had the reported effects. We considered whether the presence of ethanol in the drinking water might have had any effect on free resveratrol levels in the plasma. However, it does not appear that the use of ethanol as a vehicle has any influence on altering the availability of free resveratrol (44–49) and therefore cannot explain the disparate results observed between the two studies. We did, however, duplicate our results to recapitulate our earlier findings. We repeated the study using the highest dose (90 mg/kg body weight) and again observed no significant decreases in tumor load with dietary resveratrol.

**FIGURE 4** The effect of dietary resveratrol on tumor cyclooxygenase (COX)-2 expression in a control APC\(^{Min/+}\) mouse (upper panel) and in a APC\(^{Min/+}\) mouse treated with resveratrol (90 mg/kg body weight) (lower panel). A representative photomicrograph of a immunohistochemically stained small intestinal tumor is shown. Arrows indicate positive (brown) COX-2 expression in stromal cells. Original magnification X400. COX-2 expression in tumors did not differ between resveratrol-treated and control mice.
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genistein orally (56). These conflicting results indicate that it is difficult to predict the in vivo effects of this compound, particularly at human equivalent doses.

We investigated the effect of resveratrol on PGE2 levels and COX-2 expression in intestinal tumors because we and others have established that COX inhibition reduces the growth of intestinal tumors and regresses established tumors in this animal model (as reviewed by Whelan and McEntee (57)). Chemopreventive agents containing a common resorcin moiety (e.g., resveratrol, quercetin, genistein) were shown to suppress COX-2 transcription and activity in a human colon cancer cell line (15), and selective inhibition of COX-2 inhibited tumorigenesis in ApcMin/H11001 mice (26). Resveratrol inhibits COX-2 transcription and directly inhibits COX-2 activity in vitro (16,17,58). We did not observe any difference in COX-2 expression as a result of resveratrol treatment, although we did detect PGE2 levels that were 41% lower in tumors from the resveratrol-fed mice; however, this difference was apparently insufficient to affect tumor number. Inhibition of PGE2, viewed as an isolated event, is not always associated with a reduction in tumors (37,59).

In summary, resveratrol was shown to be stable in the diet, was absorbed intact and reached the target tissue, but had no effect on intestinal tumorigenesis at three doses and in two independent experiments. These results may be explained in part by the fact that resveratrol was ineffective as a modulator of COX-2 expression in tumors, the decreases in PGE2 levels were insufficient to modify tumor integrity and the levels of free resveratrol did not attain a sufficient concentration to duplicate the antitumorogenic effects observed in vitro. Further experimentation is required to investigate the seemingly inconsistent effects of resveratrol using in vivo compared with in vitro models.

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