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Resveratrol Blocks Eicosanoid Production and Chemically-induced Cellular Transformation: Implications for Cancer Chemoprevention

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ABSTRACT

*Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a naturally occurring compound shown to inhibit 7,12–dimethylbenz[a]anthracene-induced preneoplastic lesions in mouse mammary organ culture and 12-*O*-tetradecanoylphorbol-13-acetate-promoted mouse skin tumors. It has been postulated that resveratrol may inhibit tumorigenesis in mouse skin through interference with reactive oxidant pathways, and possibly by modulating the expression of c-fos and TGF-ß1. The chemopreventive potential of resveratrol was further examined by investigating its effect on cyclooxygenase (COX) metabolites monitored by HPLC analysis. Resveratrol was found to inhibit the generation of arachidonic acid metabolites catalyzed by both COX-1 and COX-2. In addition, this compound significantly inhibited malignant transformation induced by chemical carcinogens in the mouse C3H10T1/2 cell culture system. These data serve to corroborate the cancer chemopreventive potential of resveratrol.*

INTRODUCTION

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a ubiquitous phytoalexin generated in species such as *Vitis vinifera* L. (Vitaceae) in response to fungal infection (Langcake & Pryce, 1976). Since relatively high quantities are found in grape skins and red wines, resveratrol is routinely incorporated into the diet of humans. As a result, consumption of red wine has been proposed to play a role in reducing coronary arterial disease mortality (Goldberg et al., 1995), due in part to the ability of resveratrol to inhibit platelet aggregation and coagulation (Pace-Asciak et al., 1995), alter eicosanoid production (Dercks & Creasy, 1989; Kimura et al., 1985), and modulate lipoprotein metabolism (Arichi et al., 1982).

In previous studies conducted in our laboratory, the cancer chemopreventive potential of resveratrol was established based on inhibition of 7,12–dimethylbenz[*a*]anthracene (DMBA)-induced preneoplastic lesion formation in mouse mammary organ culture, and reduction of the incidence and multiplicity of DMBA/12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced papillomas in the two-stage mouse skin model (Jang et al., 1997). Although the mechanism by which resveratrol inhibits carcinogenesis is not clear, biological responses are mediated consistent with altering the three major stages of carcinogenesis (initiation, promotion, and progression) (Jang et al., 1997). More recently, in mouse skin studies, we found that resveratrol can significantly inhibit TPA-induced oxidative stress and the expression of certain genes, such as c-*fos* and *TGF-ß1* (Jang and Pezzuto, 1998). These findings indicate that resveratrol may inhibit tumorigenesis in mouse skin through interference with reactive oxidants, and possibly by modulating the activity of c-*fos* and *TGF-ß1*.

In addition to the activities noted above, resveratrol is an established inhibitor of cyclooxygenase (COX) (Jang et al., 1997). There are two isoforms of COX, referred to as COX-1 and COX-2, which is a key enzyme that catalyzes the biosynthesis of eicosanoids such as prostaglandins (PGs). Since these eicosanoids

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affect mitogenesis, cellular adhesion, immune surveillance, and apoptosis, COX metabolites are thought to be important in the pathogenesis of cancer (Lupulescu, 1996). In fact, non-steroidal anti-inflammatory drugs (NSAIDs), a class of compounds which inhibit COX, have been identified as cancer chemopreventive agents through their ability to block PG biosynthesis (Subbaramaiah et al., 1997).

In the current study, we investigated the effect of resveratrol on eicosanoid production assessed by HPLC analysis of COX-1 and COX-2 catalyzed reactions. In addition, we have examined the potential of resveratrol to inhibit chemically-induced neoplastic transformation in the mouse C3H10T1/2 cell system. The morphological changes of these cells correlate well with oncogenic transformation, since a high proportion of cell lines derived from these transformed foci are tumorigenic in irradiated syngeneic mice (Jones et al., 1976; Reznikoff et al., 1973). Taken together, the results of this study serve to corroborate the cancer chemopreventive potential of resveratrol.

MATERIALS AND METHODS

Chemicals and Enzyme Preparations

Resveratrol was supplied by Pharmascience (Montreal) and indomethacin was obtained from Sigma Chemical Co. (St. Louis, MO). NS-398 was purchased from Cayman Chemical Company (Ann Arbor, MI). COX-1 was derived from microsomal fractions of sheep seminal vesicles (Jang & Pezzuto, 1997). COX-2 was derived from microsomal membrane of insect cells transfected with recombinant human COX-2 cDNA (supplied by J.K. Gierse, Monsanto Company, St. Louis, MO) (Gierse et al., 1995).

HPLC Analysis of Cyclooxygenase Metabolites

COX-1 and COX-2 activities were determined in a microliter scale by measuring arachidonic acid metabolites production. The incubation mixture contained COX-1 (3.5 μg protein) or COX-2 (0.45 μg protein), various concentrations of test compounds dissolved in ethanol, 1 mM reduced glutathione (Sigma), 1 mM epinephrine-hydrogentartrate (Sigma), and 0.05 mM sodium-EDTA in 0.1 M Tris buffer (pH 8.0) (Wagner et al., 1986). The reaction was started by addition of 0.4 mM [^{3}H]-arachidonic acid (0.25 μCi) (American Radiolabeled Chemicals Inc., St. Louis, MO) for COX-1, or 2.5 μM [^{3}H]-arachidonic acid (0.25 μCi) for COX-2 (final volume, 100 μl), and incubated for 30 min at 37°C. The reaction was terminated by the addition of 5 μl of 10% formic acid. Samples were extracted with an equal volume of ethyl acetate. After centrifugation, the ethyl acetate layer was evaporated under N_2, and resuspended in 100 μl of acetonitrile for HPLC analysis. An aliquot (10 μl) was applied to a reverse-phase column (RCM Nova-pak C_{18}, 8 × 100 mm, Waters Associates, Milford, MA) in conjunction with a C_{18} guard column (Waters), and eluted with an acetonitrile-water [30:70 (A), 80:20 (B)] gradient containing 1% (v/v) 0.1 N phosphoric acid. Elution conditions were as follows: Gradient from 100% A to 100% B in 18 min, then 100% B for 10 min, with a flow rate 1 ml/min (Wagner et al., 1986). The separated arachidonic acid and its metabolites were monitored with an on-line radiochemical detector (β-Ram®IN/US System Inc., Tampa, FL), and the peaks identified by co-chromatography with non-labeled reference compounds, which were commercially available (PGE_2 and 15S-HETE were from Sigma; PGD_2, $PGF_{2\alpha}$, and 12S-HHT were from Cayman Chemical).

Cells and Cell Culture

C3H10T1/2 CL8 mouse embryo fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Eagle's basal medium with Earle's salts (EBME) (Gibco, Grand Island, NY) containing 10% (v/v) fetal bovine serum (Gibco) without antibiotics, and sodium bicarbonate (2.2 g/l). Cells were seeded at 5×10^4 per 75-cm^2 flask and grown in a humidified incubator containing 5% CO_2 in air at 37°C. Cells used in all these experiments were between passage 10–13.

Transformation Assay

Two-stage transformation assays with C3H10T1/2 cells were performed by a modification of the procedure described by Mondal et al. (1976). Briefly, 1000 cells obtained from freshly confluent flasks were seeded per well in 12-well plates containing 2 ml of medium (12 wells per experimental point). After incubation at 37°C for 3 days, 3-methylcholanthrene (MCA) (Sigma) or DMBA (Sigma) were added to give a final concentration of 0.25 μg/ml. Carcinogen-containing medium was removed from the growing cultures after 24 h and fresh culture medium supplemented with 10% fetal bovine serum and gentamycin (25 μg/ml) (Gibco) was added to the cells. Following 5 days of further incubation without carcinogen, medium was changed to fresh medium containing TPA (0.1 μg/ml) (Sigma) or TPA and various concentrations of resveratrol. TPA and test compounds

were added to the medium each time the medium was changed (twice weekly). After confluency was reached, the fetal bovine serum concentration was reduced to 5%, and amphotericin B (fungizone, 1.5 μg/ml) (Gibco) was added to the medium. Subsequent medium changes were performed weekly. Cultures were fixed with methanol and stained with Giemsa (Sigma) at the 7th week. Type II and III transformed foci were scored according to the guidelines of Landolph (1985).

RESULTS

Arachidonic Acid Metabolites Monitored by HPLC

^{3}H-Labeled arachidonic acid and metabolites were analyzed by HPLC using a reversed-phase C_{18} column and a radiochemical monitor. In the reaction catalyzed by COX-1 (derived from sheep seminal vesicles), PGE_2 was the predominant metabolite (Fig. 1A) which was confirmed by co-elution on HPLC with authentic unlabeled-PGE_2 (Sigma). When this reaction mixture contained COX-1 inhibitors, such as indomethacin, PGE_2 concentrations were dramatically decreased, compared with control groups without inhibitors. As shown in Fig. 1B, resveratrol significantly blocks PGE_2 production. Reduction of PGE_2 production was proportional to the concentration of resveratrol added to the reaction mixture, with an IC_{50} value of 10.2 μM. A similar inhibitory curve was observed on addition of indomethacin, with an IC_{50} value of 3.2 μM (Fig. 2). These results are consistent with the effects of these inhibitors on COX-1 activity as judged by an oxygen consumption assay (Jang et al., 1997).

The radiolabeled arachidonic acid metabolites generated by COX-2 (recombinant human COX-2) were also assessed using HPLC analysis. As shown in Fig. 3A, COX-2 generated a more complicated profile of metabolites, compared with that generated by COX-1. In general, prostaglandins such as PGE_2, PGD_2, and $PGF_{2\alpha}$, were still the major products. Some minor products included 12S-hydroxyheptadecatrienoic acid (12S-HHT) and 15S-hydroxyeicosatetraenoic acid (15S-HETE), with uncertain biological activity. When the reaction mixture contained NS-398, a selective COX-2 inhibitor, or indomethacin, each of the arachidonic acid metabolites were markedly decreased. In addition, resveratrol, which showed no inhibitory activity on COX-2 based on the oxygen consumption assay (Jang et al., 1997), significantly blocked arachidonic acid metabolite production in the COX-2 catalyzed reaction (Fig. 3B). The inhibitory effect of resveratrol noted with COX-2 was less intense than that of NS-398 (IC_{50} value, 3.2 μM) or indomethacin (IC_{50} value, 1.9 μM), but nonetheless, significant inhibition of prostaglandin production was observed, with an IC_{50} value of 32.2 μM (Fig. 4).

Inhibitory Effects of Resveratrol on Chemical-Induced Cell Transformation

To further investigate the chemopreventive activity of resveratrol, we utilized two-stage transformation experiments in the C3H10T1/2 system, with 3-methylcholanthrene (MCA) or DMBA as inducer, and TPA as promoter. As shown in Table 1, when cells were treated with DMSO alone or a low dose of MCA (0.25 μg/ml), no subsequent transformation was observed. However, repeated treatment with TPA after exposure to the low dose of MCA caused an increase in focus formation with 58% of the wells containing at least one Type II or Type III transformed focus. This enhancement of transformation by repeated exposure to TPA was significantly reduced in the presence of resveratrol (Table 1). In the MCA-induced experiments, co-treatment with 2.5, 5.0, or 10 μM resveratrol resulted in 70, 70, or 86% inhibition, respectively. In the DMBA/TPA-induced transformation system, resveratrol also exhibited similar dose-dependent inhibition, with approximately 32, 65, or 65% reduction at 2.5, 5.0 or 10 μM resveratrol, respectively.

DISCUSSION

A general strategy for cancer chemoprevention is to block the different stages of carcinogenic process (Ito & Imaida, 1992). As demonstrated previously, resveratrol inhibits cellular events associated with tumor initiation, promotion, and progression (Jang et al., 1997). Furthermore, this compound was found to mediate significant chemopreventive activities in mouse mammary organ culture and the two-stage mouse skin tumorigenesis models (Jang et al., 1997). In this report, we have demonstrated the effect of resveratrol on the production of COX metabolites and chemical-induced transformation of mouse fibroblasts.

Both isoforms of COX, COX-1 and COX-2, catalyze cyclooxygenase and peroxidase reactions. As previously reported, resveratrol inhibited COX-1, but not COX-2, when oxygen uptake was assessed (Jang et al., 1997). However, this compound exhibited considerable inhibition of the peroxidase activity of both COX isoforms. This observation may account for the effect of

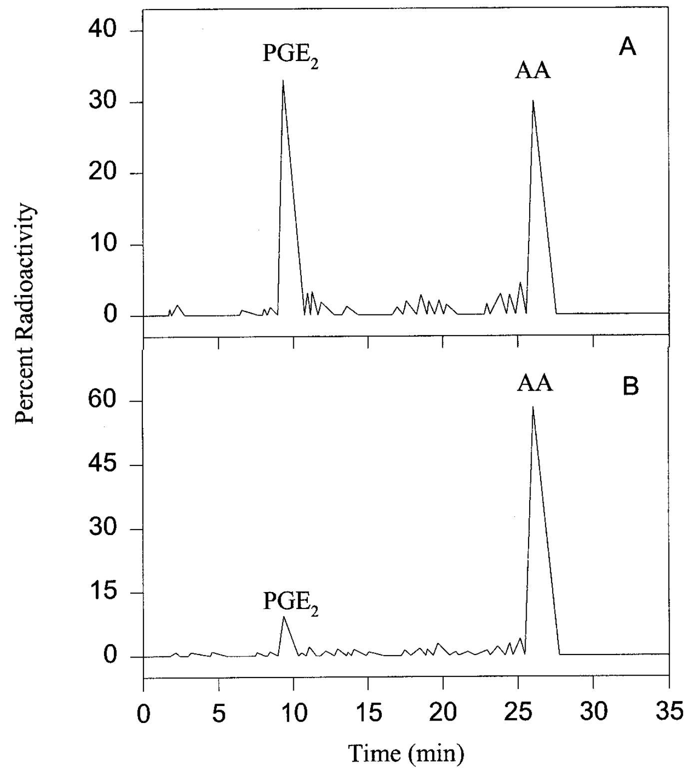

Fig. 1. HPLC analysis of [^{3}H]arachidonic acid metabolism catalyzed by COX-1. [^{3}H]Arachidonic acid (0.25 μCi) was incubated for 30 min at 37°C with microsomes (3.5 μg of protein) derived from sheep seminal vesicles as a crude source of COX-1, in a reaction mixture that contained 0.1 M Tris-HCl (pH 8.0), 0.05 mM EDTA, 1 mM reduced glutathione and 1 mM epinephrine-hydrogenetartrate, in the absence (A) or presence (B) of 50 μM resveratrol. Metabolites were extracted with ethyl acetate and analyzed by HPLC as described in "Materials and Methods."

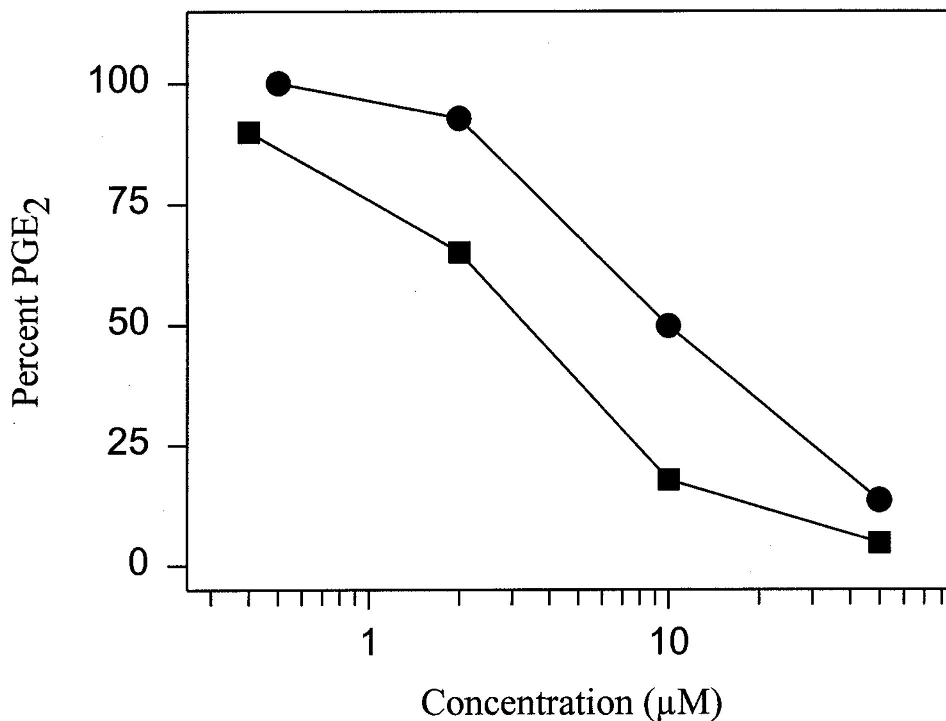

Fig. 2. Dose-dependent effect of resveratrol (●) or indomethacin (■) on COX-1 measured by HPLC analysis. The percent activity was determined by comparing PGE_2 levels of control incubations with levels observed in the presence of the indicated concentrations of test compounds. For additional details, see the legend for Fig. 1.

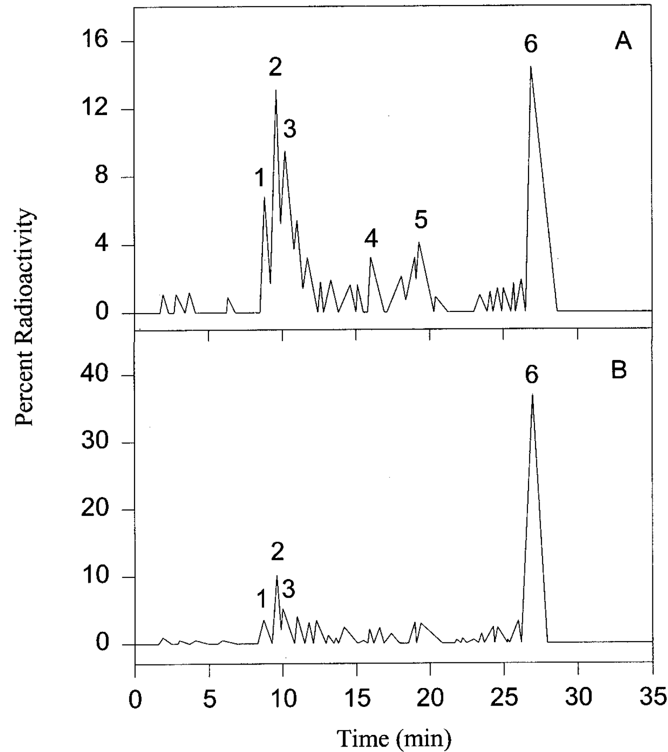

Fig. 3. HPLC analysis of [^{3}H]arachidonic acid metabolism catalyzed by COX-2. [^{3}H]Arachidonic acid (0.25 μCi) was incubated for 30 min at 37°C with COX-2 (0.45 μg of protein) derived from microsomal membrane of insect cells transfected with recombinant human COX-2 cDNA, in a reaction mixture that contained 0.1 M Tris-HCl (pH 8.0), 0.05 mM EDTA, 1 mM reduced glutathione and 1 mM epinephrine-hydrogenetartrate, in the absence (A) or presence (B) of 50 μM resveratrol. HPLC analysis was performed as described in "Materials and Methods." Peak 1, $PGF_{2\alpha}$; peak 2, PGE_2; peak 3, PGD_2; peak 4, 12S-HHT; peak 5, 15S-HETE; peak 6, arachidonic acid.

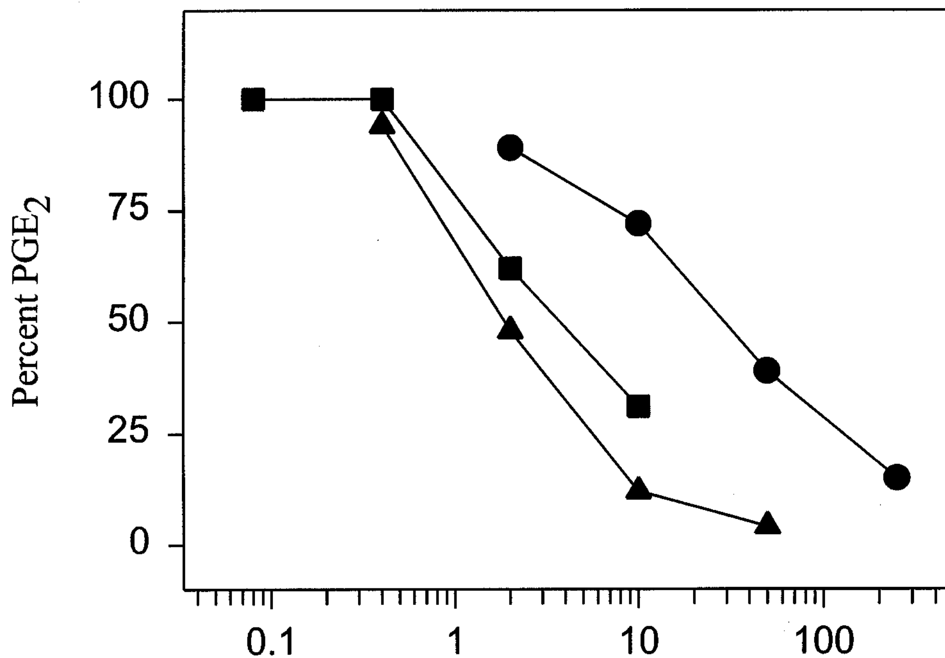

Fig. 4. Dose-dependent effects of resveratrol (●), NS-398 (■) and indomethacin (▲) on COX-2 measured by HPLC analysis. The percent activity was determined by comparing levels of PGE_2 formation observed in control incubations with levels observed in incubation mixtures containing the indicated concentrations of test compounds. For additional details, see the legend for Fig. 3.

Table 1. Inhibitory effects of resveratrol on two-stage transformation of C3H/10T1/2 cells.

Treatment	Transformed foci no.			Total foci/ total wells	Wells with foci/ total wells	Wells with foci (%)
	Type II	Type III	Total			
0.5% DMSO control	0	0	0	0/12	0/12	0
MCA (0.25)*	0	0	0	0/12	0/12	0
MCA (0.25); TPA (0.1)	8	0	8	8/12	7/12	58
+ Resveratrol 2.5 μM	3	0	3	3/12	2/12	17
+ Resveratrol 5.0 μM	3	1	4	4/12	2/12	17
+ Resveratrol 10 μM	1	0	1	1/12	1/12	8.3
DMBA (0.25)	1	0	1	1/12	1/12	8.3
DMBA (0.25); TPA (0.1)	4	0	4	4/12	4/12	33
+ Resveratrol 2.5 μM	3	0	3	3/12	3/12	25
+ Resveratrol 5.0 μM	1	1	1	2/12	2/12	17
+ Resveratrol 10 μM	1	1	1	2/12	2/12	17

* Numbers in parentheses, concentration (μg/ml).

resveratrol on COX metabolite production observed by HPLC analysis. Using ^{3}H-labeled arachidonic acid as substrate, resveratrol significantly inhibited both COX-1 and COX-2 radiolabeled metabolite production. Since COX metabolites such as prostaglandins are known to stimulate tumor cell growth and suppress immune surveillance, the inhibitory effect of resveratrol on the production of these metabolites implies an important role of this compound in cancer prevention. On the other hand, resveratrol did not alter the expression of COX-1 or COX-2 induced by TPA treatment of mouse skin (Jang & Pezzuto, 1998), or stimulated by lipopolysaccharide (LPS) treatment of mouse macrophages in culture (Jang & Pezzuto, unpublished observations). This may be species or test system specific, since it is known that induction of COX-2 is inhibited by resveratrol in other model systems (Subbaramaiah et al., 1998). However, resveratrol was able to inhibit the induction of inducible nitric oxide synthase (iNOS) in LPS-stimulated mouse macrophages (Jang & Pezzuto, unpublished observations). Since iNOS mediates the synthesis of nitric oxide that modulates a range of physiological responses, including inflammation and carcinogenesis, the inhibitory effect of resveratrol on iNOS induction may also contribute to its cancer chemopreventive potential.

In addition, resveratrol was found to significantly inhibit chemically-induced neoplastic transformation of cultured C3H10T1/2 embryonic mouse fibroblasts. The response of this cell line to certain chemopreventive agents such as retinoids appears to mirror closely that of the whole animal (Bertram, 1985). In the two-stage cell transformation assay, with MCA as inducer and TPA as promoter, resveratrol inhibited the promotion of morphological transformation. The mechanism by which resveratrol blocked the expression of neoplastic transformation in this cell system is not known. However, in recently studies, we found that resveratrol interferes with reactive oxygen species-generating pathways and modulates antioxidant defense systems through negating events associated with TPA-induced oxidative stress in mouse skin (Jang & Pezzuto, 1998). Since active oxygen species promote the transformation of mouse C3H10T1/2 cells (Zimmerman & Cerutti, 1984), it can be postulated that resveratrol blocks transformation by interfering with oxidative stress induced by TPA. Moreover, a cellular state of oxidative stress has been demonstrated to regulate gene expression in C3H10T1/2 cells (Parfett & Pilon, 1995), and this is believed to be an important mediator of two-stage chemical carcinogenesis in rodent tissues (Cerutti & Trump, 1991). Accordingly, the ability of resveratrol to block the generation of cellular pro-oxidant states may contribute to its inhibitory effect on certain genes that are expressed in TPA-stimulated mouse skin, such as c-*fos* proto-oncogene and *TGF-ß1* (Jang & Pezzuto, 1998). These types of activities are clearly relevant to chemopreventive activity.

In summary, the current report demonstrates resveratrol is capable of inhibiting eicosanoid production catalyzed by both COX-1 and COX-2, and transformation of chemically-initiated 10T1/2 cells, particularly by suppressing the TPA-promoting effect in this system. These results are consistent with previous studies that demonstrated the cancer chemopreventive potential of resveratrol, and provide additional mechanistic rational for the anti-inflammatory and preventative actions of this compound.

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