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Resveratrol Inhibits Cyclooxygenase-2 Transcription and Activity in Phorbol Ester-treated Human Mammary Epithelial Cells*

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We determined whether resveratrol, a phenolic antioxidant found in grapes and other food products, inhibited phorbol ester (PMA)-mediated induction of COX-2 in human mammary and oral epithelial cells. Treatment of cells with PMA induces COX-2 and causes a marked increase in the production of prostaglandin E_2. These effects were inhibited by resveratrol. Resveratrol suppressed PMA-mediated increases in COX-2 mRNA and protein. Nuclear run-offs revealed increased rates of *COX-2* transcription after treatment with PMA, an effect that was inhibited by resveratrol. PMA caused about a 6-fold increase in *COX-2* promoter activity, which was suppressed by resveratrol. Transient transfections utilizing *COX-2* promoter deletion constructs and *COX-2* promoter constructs, in which specific enhancer elements were mutagenized, indicated that the effects of PMA and resveratrol were mediated via a cyclic AMP response element. Resveratrol inhibited PMA-mediated activation of protein kinase C. Overexpressing protein kinase C-α, ERK1, and c-Jun led to 4.7-, 5.1-, and 4-fold increases in *COX-2* promoter activity, respectively. These effects also were inhibited by resveratrol. Resveratrol blocked PMA-dependent activation of AP-1-mediated gene expression. In addition to the above effects on gene expression, we found that resveratrol also directly inhibited the activity of COX-2. These data are likely to be important for understanding the anti-cancer and anti-inflammatory properties of resveratrol.

There are two isoforms of cyclooxygenase (COX)[1] that catalyze the formation of prostaglandins (PGs) from arachidonic acid. *COX*-1 is a housekeeping gene that is expressed constitutively (1). *COX-2* is an immediate, early response gene that is highly inducible by mitogenic and inflammatory stimuli (2–4). The differences in the regulation of *COX-1* and *COX-2* gene expression reflect differences in the regulatory elements in the 5′-flanking regions of the two genes (5).

Considerable evidence has accumulated to suggest that COX-2 is important for tumorigenesis. For example, COX-2 is up-regulated in transformed cells (6–8) and various forms of cancer (9–12), whereas levels of COX-1 remain essentially unchanged. A null mutation for *COX-2* markedly reduced the number and size of intestinal tumors in a murine model of familial adenomatous polyposis, *i.e.* $APC^{\Delta 716}$ knockout mice (13). *COX-2* deficiency also protected against the formation of extraintestinal tumors. Thus, *COX-2* knockout mice developed approximately 75% fewer chemically induced skin papillomas than control mice (14). A selective inhibitor of COX-2 caused nearly complete suppression of azoxymethane-induced colon cancer (15).

There are several possible mechanisms that could account for the link between COX-2 and cancer. Enhanced synthesis of PGs, which occurs in a variety of tumors (16–19), can favor the growth of malignant cells by increasing cell proliferation (20), promoting angiogenesis (21), and inhibiting immune surveillance (22). Overexpression of COX-2 inhibits apoptosis and increases the invasiveness of malignant cells (23, 24); these effects were reversed by the nonsteroidal anti-inflammatory drug, sulindac sulfide. In combination, these studies suggest that targeted inhibition of COX-2 is a promising approach to prevent cancer. Therefore, chemopreventive strategies have focused on inhibitors of COX enzyme activity. An equally important strategy may be to identify compounds that suppress the expression of COX-2 (25, 26).

Resveratrol is a phytoalexin found in grapes and other foods that has anti-cancer and anti-inflammatory effects (27) (Fig. 1). It inhibits the development of preneoplastic lesions in carcinogen-treated mouse mammary glands, for example, and it blocks tumorigenesis in a two-stage model of skin cancer that was promoted by treatment with phorbol ester (27). The anti-inflammatory properties of resveratrol were demonstrated by suppression of carrageenan-induced pedal edema (27), an effect attributed to suppression of PG synthesis via direct, selective inhibition of COX-1. In the current work, we have extended prior observations (27) concerning the effects of resveratrol on PG synthesis by determining if resveratrol modulates the expression of the *COX-2* gene. Our data show that resveratrol suppresses the activation of *COX-2* gene expression by inhibiting the PKC signal transduction pathway. Contrary to prior results (27), we also found that resveratrol directly inhibits the activity of COX-2. These data provide a mechanistic basis for

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[1] The abbreviations used are: COX, cyclooxygenase; CRE, cyclic AMP response element; PGE_2, prostaglandin E_2; PKC, protein kinase C; AP-1, activator protein-1; PMA, phorbol 12-myristate 13-acetate.

the chemopreventive and anti-inflammatory properties of resveratrol.

EXPERIMENTAL PROCEDURES

Materials—Minimal essential medium, PKC assay kits, and LipofectAMINE were from Life Technologies, Inc. Keratinocyte basal and growth media were from Clonetics Corp. (San Diego). Phorbol 12-myristate 13-acetate, sodium arachidonate, resveratrol, PGE_2, indomethacin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (thiazolyl blue), lactate dehydrogenase diagnostic kits, epinephrine-hydrogentartrate, epidermal growth factor, hydrocortisone, and *o*-nitrophenyl-β-D-galactopyranoside were from Sigma. NS398 was from Biomol Research Labs Inc. (Plymouth Meeting, PA). Enzyme immunoassay reagents for PGE_2 assays were from Cayman Co. (Ann Arbor, MI). [^{32}P]CTP was from DuPont NEN. [^{3}H]Arachidonic acid was from American Radiolabeled Chemicals Inc. Random-priming kits were from Boehringer Mannheim. Nitrocellulose membranes were from Schleicher & Schuell. Reagents for the luciferase assay were from Analytical Luminescence (San Diego). The 18 S rRNA cDNA was from Ambion, Inc. Rabbit polyclonal anti-human COX-2 antiserum and goat polyclonal anti-human COX-1 antiserum were from Santa Cruz Biotechnology, Inc. Western blotting detection reagents (ECL) were from Amersham Pharmacia Biotech. Plasmid DNA was prepared using a kit from Promega.

Tissue Culture—The 184B5/HER cell line has been described previously (28). Cells were maintained in minimal essential-keratinocyte basal media mixed in a ratio of 1:1 (basal medium) containing epidermal growth factor (10 ng/ml), hydrocortisone (0.5 μg/ml), transferrin (10 μg/ml), gentamicin (5 μg/ml), and insulin (10 μg/ml) (growth medium). Cells were grown to 60% confluence, trypsinized with 0.05% trypsin, 2 mM EDTA, and plated for experimental use. MSK Leuk1 was established from a dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue in a 46-year-old nonsmoking female (29). Cells were routinely maintained in keratinocyte growth medium and passaged using 0.125% trypsin, 2 mM EDTA. In all experiments, 184B5/HER and MSK Leuk1 cells were grown in basal medium for 24 h prior to treatment. Treatment with vehicle (0.2% Me_2SO), resveratrol, or PMA was always carried out in basal medium. Cellular cytotoxicity was assessed by measurements of cell number, release of lactate dehydrogenase, and the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, which was performed according to the method of Denizot and Lang (30). Lactate dehydrogenase assays were performed according to the manufacturer's instructions. There was no evidence of toxicity in any of our experiments.

PGE_2 Production by Cells—5×10^4 cells/well were plated in 6-well dishes and grown to 60% confluence in growth medium. The cells were then treated as described below. Levels of PGE_2 released by the cells were measured by enzyme immunoassay. Rates of production of PGE_2 were normalized to protein concentrations.

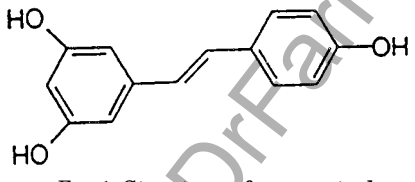

FIG. 1. **Structure of resveratrol.**

Determination of COX-2 Enzyme Activity—The effect of resveratrol, indomethacin, and NS398 on the activity of COX-2 was measured using baculovirus-expressed recombinant human COX-2 enzyme (supplied by J. K. Gierse, Monsanto Co., St. Louis, MO) (31).

The activity of COX-2 was determined in a microliter scale by measuring the synthesis of PGE_2. The incubation mixture contained COX-2 protein (0.45 μg), various concentrations of test compounds dissolved in ethanol, 1 mM reduced glutathione, 1 mM epinephrine-hydrogentartrate, and 0.05 mM sodium-EDTA in 0.1 M Tris buffer (pH 8.0) (32). The reaction was started by addition of 2.5 μM [^{3}H]arachidonic acid (0.25 μCi), in a final volume of 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 high pressure liquid chromatography 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, 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 of 1 ml/min (32). The separated arachidonic acid and PGE_2 were monitored with an on-line radiochemical detector (β-Ram IN/US System Inc., Tempa, FL), and the peaks were identified by cochromatography with unlabeled reference compounds.

Western Blotting—Cell lysates were prepared by treating cells with lysis buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin). Lysates were sonicated for 20 s on ice and centrifuged at 10,000 × *g* for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry *et al.* (33). SDS/PAGE was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (34). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin *et al.* (35). The nitrocellulose membrane was then incubated with a rabbit polyclonal anti-COX-2 antiserum or a polyclonal anti-COX-1 antiserum. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL Western blot detection system according to the manufacturer's instructions.

Northern Blotting—Total cellular RNA was isolated from cell monolayers using an RNA isolation kit from Qiagen Inc. 10 μg of total cellular RNA per lane were electrophoresed in a formaldehyde-containing 1.2% agarose gel and transferred to nylon-supported membranes. After baking, membranes were prehybridized overnight in a solution containing 50% formamide, 5 × sodium chloride-sodium phosphate-EDTA buffer (SSPE), 5 × Denhardt's solution, 0.1% SDS, and 100 μg/ml single-stranded salmon sperm DNA and then hybridized for 12 h at 42 °C with radiolabeled cDNA probes for human *COX-2* cDNA and 18 S rRNA. After hybridization, membranes were washed twice for 20 min at room temperature in 2 × SSPE, 0.1% SDS, twice for 20 min in the same solution at 55 °C and twice for 20 min in 0.1 × SSPE, 0.1% SDS at 55 °C. Washed membranes were then subjected to autoradiography. *COX-2* and 18 S rRNA probes were labeled with [^{32}P]CTP by random priming.

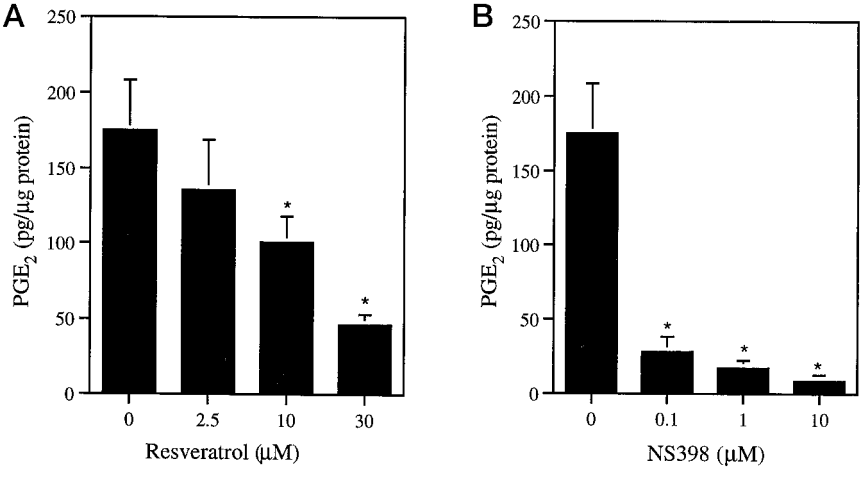

FIG. 2. **Basal COX-2 activity is inhibited by resveratrol.** 184B5/HER cells were treated with resveratrol (0–30 μM, *panel A*) or NS398 (0–10 μM, *panel B*) for 30 min. The medium was then replaced with fresh medium containing 10 μM sodium arachidonate. 30 min later, the medium was collected to determine the rate of synthesis of PGE_2. Production of PGE_2 was determined by enzyme immunoassay. *Columns*, means; *bars*, S.D.; $n = 6$. *, $p < 0.001$ compared with control.

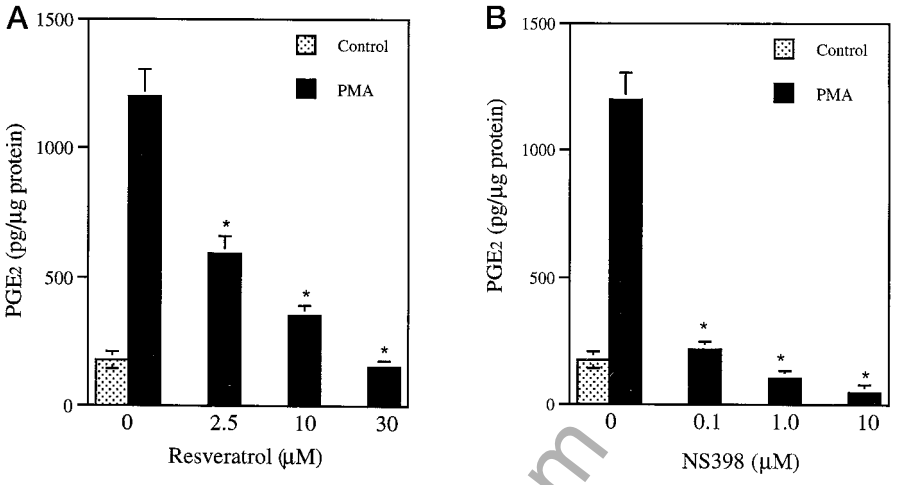

FIG. 3. **Resveratrol directly inhibits PMA-induced COX-2 activity.** 184B5/HER cells were treated with vehicle (*stippled columns*) or PMA (50 ng/ml, *black columns*) for 4.5 h. PMA was given to induce COX-2. Fresh medium containing resveratrol (0–30 μM, *panel A*) or NS398 (0–10 μM, *panel B*) was then added for 30 min. The medium was then replaced with fresh medium containing 10 μM sodium arachidonate. 30 min later, the medium was collected to determine the rate of synthesis of PGE_2. Production of PGE_2 was determined by enzyme immunoassay. *Columns*, means; *bars*, S.D.; $n = 6$. *, $p <$ 0.001 compared with PMA.

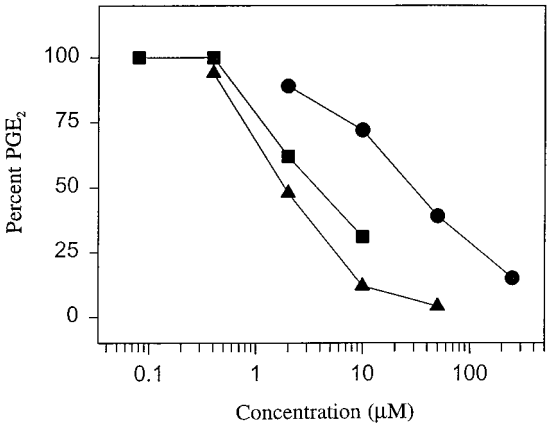

FIG. 4. **Resveratrol causes dose-dependent inhibition of recombinant human COX-2 enzyme activity.** [^{3}H]Arachidonic acid was added to a reaction mixture containing human recombinant COX-2 enzyme, 0.1 M Tris-HCl (pH 8.0), 0.05 mM EDTA, 1 mM reduced glutathione, 1 mM epinephrine-hydrogentartrate, and the indicated concentration of resveratrol (*circles*), NS398 (*squares*), or indomethacin (*triangles*) for 30 min at 37 °C. High pressure liquid chromatography analysis was performed as described under "Experimental Procedures" to assess levels of PGE_2 synthesis. Percent activity was determined by comparing levels of synthesis of PGE_2 in control incubations with levels observed in incubation mixtures containing the indicated concentrations of test compounds.

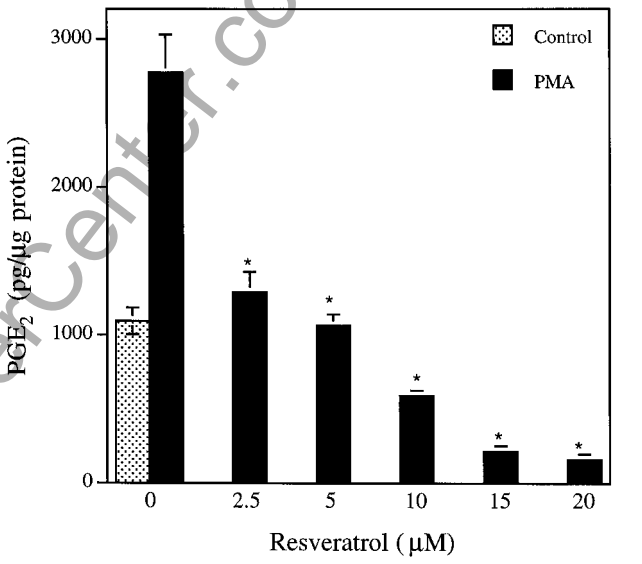

FIG. 5. **Resveratrol suppresses PMA-mediated increases in the production of PGE_2.** 184B5/HER cells were treated with vehicle (*stippled column*), PMA (50 ng/ml, *black columns*), or PMA (50 ng/ml) and resveratrol for 4.5 h. The medium was then replaced with basal medium and 10 μM sodium arachidonate. 30 min later, the medium was collected to determine the rate of synthesis of PGE_2. Production of PGE_2 was determined by enzyme immunoassay. *Columns*, means; *bars*, S.D.; $n =$ 6. *, $p < 0.001$ compared with PMA.

Nuclear Run-off Assay—2.5 × 10^5 cells were plated in four T150 dishes for each condition. Cells were grown in growth medium until approximately 60% confluent. Nuclei were isolated and stored in liquid nitrogen. For the transcription assay, nuclei (1.0 × 10^7) were thawed and incubated in reaction buffer (10 mM Tris (pH 8), 5 mM $MgCl_2$, and 0.3 M KCl) containing 100 μCi of uridine 5′[α-^{32}P]triphosphate and 1 mM unlabeled nucleotides. After 30 min, labeled nascent RNA transcripts were isolated. The human *COX-2*, c-*jun*, c-*myc*, and β-*actin* cDNAs were immobilized onto nitrocellulose and prehybridized overnight in hybridization buffer. Hybridization was carried out at 42 °C for 24 h using equal cpm/ml of labeled nascent RNA transcripts for each treatment group. The membranes were washed twice with 2 × SSC buffer for 1 h at 55 °C and then treated with 10 mg/ml RNase A in 2 × SSC at 37 °C for 30 min, dried, and autoradiographed.

Plasmids—The *COX-2* promoter constructs (−1432/+59, −327/+59, −220/+59, −124/+59, −52/+59, ILM, CRM, and CRM-ILM) have been described previously (5). The human *COX-2* cDNA was generously provided by Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT). Rous sarcoma virus-c-*jun* was a gift from Dr. Tom Curran (Roche Laboratories, Nutley, NJ). The AP-1 reporter plasmid (2 × TRE-luciferase), composed of two copies of the consensus TRE ligated to luciferase, was kindly provided by Dr. Joan Heller Brown (University of California, La Jolla, CA) (36). The ERK1 expression vector was obtained from Dr. Melanie Cobb (Southwestern Medical Center, Dallas, TX). The c-*myc* cDNA was a gift from Dr. Charles Sawyer (University of California, Los Angeles). The PKC-α expression vector was provided by Dr. Geoffrey Cooper (Harvard University, Cambridge, MA). pSV-βgal was obtained from Promega.

Transient Transfection Assays—184B5/HER cells were seeded at a density of 5 × 10^4 cells/well in 6-well dishes and grown to 50–60% confluence. For each well, 2 μg of plasmid DNA were introduced into cells using 8 μg of LipofectAMINE as per the manufacturer's instructions. After 7 h of incubation, the medium was replaced with basal medium. The activities of luciferase and β-galactosidase were measured in cellular extract as described previously (25).

Protein Kinase C Assay—The activity of PKC was measured according to directions from Life Technologies, Inc. Briefly, cells were plated in 10-cm dishes at 10^6 cells/dish and grown to 60% confluence. Cells were then treated with fresh basal medium containing vehicle (0.2% Me_2SO), PMA (50 ng/ml), or PMA (50 ng/ml) plus resveratrol (15 μM) for 30 min. Total PKC activity was measured in cell lysates. To determine cytosolic and membrane-bound PKC activity, cell lysates were centrifuged at 100,000 × *g* for 30 min. The resulting supernatant contains cytosolic PKC; membrane-bound PKC activity is present in the pellet. Subsequently, DEAE-cellulose columns were used to partially purify PKC enzymes. Protein kinase C activity was then measured by incubating partially purified PKC with [γ-^{32}P]ATP (3000–6000 Ci/mmol) and the substrate myelin basic protein for 20 min at room temperature. The activity of PKC is expressed as cpm incorporated/μg protein.

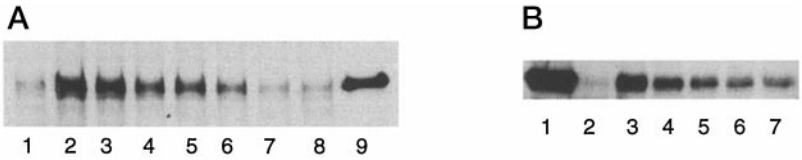

FIG. 6. **Resveratrol causes dose-dependent inhibition of PMA-mediated induction of COX-2.** Cellular lysate protein (25 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed with antibody specific for COX-2. *A*, lysate protein was from 184B5/HER cells treated with vehicle (*lane 1*), PMA (50 ng/ml, *lane 2*), or PMA (50 ng/ml) and resveratrol (2.5, 5, 7.5, 10, 15, 30 μM; *lanes 3–8*) for 4.5 h. *Lane 9* represents an ovine Cox-2 standard. *B*, lysates were from premalignant oral epithelial (MSK Leuk1) cells treated with vehicle (*lane 2*), PMA (50 ng/ml, *lane 3*), or PMA (50 ng/ml) and resveratrol (10, 20, 30, 40 μM; *lanes 4–7*) for 4.5 h. *Lane 1* represents an ovine Cox-2 standard.

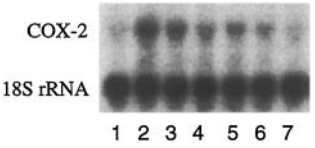

FIG. 7. **PMA-mediated induction of COX-2 mRNA is suppressed by resveratrol.** 184B5/HER cells were treated with vehicle (*lane 1*), PMA (50 ng/ml, *lane 2*), or PMA (50 ng/ml) and resveratrol (2.5, 5, 10, 15, 20 μM; *lanes 3–7*) for 3 h. Total cellular RNA was isolated; 10 μg of RNA was added to each lane. Results of densitometry in arbitrary units: *lane 1*, 18; *lane 2*, 225; *lane 3*, 135; *lane 4*, 72; *lane 5*, 45; *lane 6*, 42; *lane 7*, 9.

Statistics—Comparisons between groups were made by the Student's *t* test. A difference between groups of $p < 0.05$ was considered significant.

RESULTS

Resveratrol Inhibits COX-2 Enzyme Activity—The data in Fig. 2*A* show that resveratrol caused dose-dependent suppression of PGE_2 synthesis in human mammary epithelial cells. To evaluate whether the inhibition of PG synthesis was because of inhibition of COX-2 or COX-1, we compared the effects of resveratrol and a selective inhibitor of COX-2 (NS398). We note that NS398 decreased the synthesis of PGs to less than 10% of the control level (Fig. 2*B*). This means that more than 90% of basal COX activity in mammary epithelial cells was because of the COX-2 isoform; the presence of COX-2 under basal conditions is consistent with its transformed phenotype (6–8, 28). As resveratrol (30 μM) caused approximately a 70% decrease in basal production of PGE_2 (Fig. 2*A*), our data show that the predominant inhibitory effect of resveratrol on synthesis of PGs was via inhibition of the COX-2 isoform of cyclooxygenase.

A second experiment was conducted to confirm that resveratrol directly inhibited COX-2 enzyme activity (Fig. 3). Phorbol esters are potent inducers of COX-2 (25), so we also examined the effect of resveratrol on the synthesis of PGs by cells in which COX-2 was induced by PMA. In this experiment, mammary epithelial cells were treated with vehicle or PMA for 4.5 h. Fresh medium containing either resveratrol or NS398 was then added. 30 min later, synthesis of PGE_2 was measured. Resveratrol caused concentration-dependent suppression of PMA-stimulated synthesis of PGE_2 with complete inhibition at 30 μM resveratrol (Fig. 3*A*). As shown in Fig. 3*B*, 0.1 μM NS398 was required to completely suppress PMA-mediated induction of PG synthesis. Immunoblot analysis demonstrated that levels of COX-2 protein did not decrease during treatment with resveratrol or NS398 (data not shown). These results strongly suggest that resveratrol inhibited COX-2 activity.

To further evaluate the effects of resveratrol on COX-2 activity, we utilized baculovirus-expressed human recombinant COX-2 in a cell-free assay. Resveratrol caused dose-dependent inhibition of PGE_2 synthesis (Fig. 4). As expected from the findings in cell culture (Figs. 2 and 3), resveratrol (IC_{50} value, 32.2 μM) was a less potent inhibitor of COX-2 activity than the synthetic inhibitors NS398 (IC_{50} value, 3.2 μM) and indomethacin (IC_{50} value, 1.9 μM).

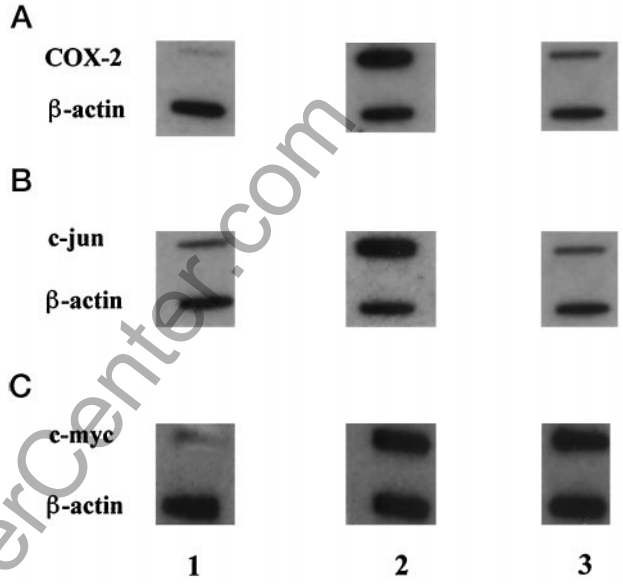

FIG. 8. **Resveratrol inhibits PMA-mediated induction of COX-2 transcription.** 184B5/HER cells were treated with vehicle (*lane 1*), PMA (50 ng/ml, *lane 2*), or PMA (50 ng/ml) and resveratrol (15 μM, *lane 3*) for 30 min. Nuclear run-offs were performed as described under "Experimental Procedures." The *COX-2* (*A*), c-*jun* (*B*), c-*myc* (*C*), and *β-actin* cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled nascent RNA transcripts.

Resveratrol Inhibits the Induction of COX-2 by Phorbol Esters—We also investigated the possibility that resveratrol inhibited PMA-mediated induction of PG synthesis by suppressing the induction of COX-2. In these experiments, cells were cotreated for 4.5 h with PMA and the indicated concentrations of resveratrol. The medium then was replaced, and the synthesis of PGs was measured in the absence of resveratrol over the next 30 min. PMA in this setting caused about a 2-fold increase in synthesis of PGE_2. This effect was suppressed by resveratrol in a dose-dependent manner (Fig. 5). To confirm that these effects of resveratrol were not unique to mammary epithelial cells, we also determined whether resveratrol inhibited PMA-mediated induction of PG synthesis in a premalignant, oral leukoplakia cell line. Treatment of these cells with PMA led to a 2-fold increase in PG synthesis. This effect was inhibited completely by 20 μM resveratrol (data not shown).

To determine whether the above effects on production of PGE_2 could be related to differences in levels of COX, Western blotting of cell lysate protein was carried out. Fig. 6*A* shows that PMA induced COX-2 in human mammary epithelial cells. Cotreatment with resveratrol caused a dose-dependent decrease in PMA-mediated induction of COX-2; the maximal drug effect was observed at 15–20 μM. Neither PMA nor resveratrol altered amounts of COX-1 (data not shown). It is noteworthy that the effects of resveratrol on PGE_2 synthesis mediated by PMA (Fig. 5) were greater than the degree of suppression of amounts of COX-2 protein induced by PMA (Fig. 6*A*). For

example, 5 μM resveratrol decreased PG synthesis to levels detected in uninduced cells while only partially blocking PMA-mediated induction of COX-2 protein. This finding is consistent with the idea that resveratrol inhibited PMA-mediated induction of PG synthesis both by suppressing levels of COX-2 protein and by direct inhibition of COX-2 activity. The down-regulation of COX-2 expression by resveratrol was not limited to mammary cells but was also demonstrable in oral epithelial cells (Fig. 6*B*).

To further elucidate the mechanism responsible for the changes in amounts of COX-2 protein, we determined steady-state levels of COX-2 mRNA by Northern blotting. Treatment with PMA resulted in a marked increase in levels of COX-2 mRNA, an effect that was suppressed by resveratrol in a concentration-dependent manner (Fig. 7).

Resveratrol Inhibits Phorbol Ester-mediated Increases in the Transcription of COX-2—Differences in levels of mRNA could reflect altered rates of transcription or changes in mRNA stability. Nuclear run-offs were performed to distinguish between these possibilities. As shown in Fig. 8, we detected higher rates of synthesis of nascent COX-2 mRNA after treatment with PMA, consistent with the differences observed by Northern blotting. This effect was suppressed by resveratrol. We also investigated whether resveratrol suppressed PMA-mediated induction of c-*jun* (Fig. 8*B*) and c-*myc* (Fig. 8*C*) transcription. Resveratrol caused a marked decrease in the activation of c-*jun* expression. In contrast, resveratrol did not significantly inhibit PMA-mediated induction of c-*myc* transcription.

To further investigate the importance of PMA and resveratrol in modulating the expression of *COX-2*, transient transfections were performed using a human *COX-2* promoter-luciferase construct. Treatment with PMA increased *COX-2* promoter activity about 6-fold. Resveratrol caused dose-dependent inhibition of PMA-mediated induction of *COX-2* promoter activity (Fig. 9). We next attempted to define the region of the *COX-2* promoter that responded to PMA and resveratrol. This was accomplished using a series of human *COX-2* promoter deletion constructs. As shown in Fig. 10*A*, both PMA-mediated increases in *COX-2* promoter activity and inhibition of promoter activity by resveratrol were detected with all *COX-2* promoter constructs except the −52/+59 construct. A CRE is present between nucleotides −59 and −53, suggesting that this element may be responsible for mediating the effects of PMA and resveratrol. To test this notion, transient transfections were performed utilizing *COX-2* promoter constructs in which specific enhancer elements including the CRE were mutagenized. As shown in Fig. 10*B*, mutagenizing the CRE site

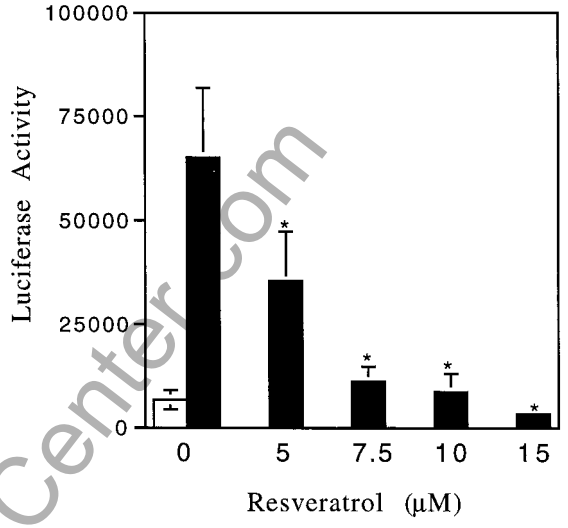

FIG. 9. **Resveratrol suppresses PMA-mediated induction of COX-2 promoter activity.** 184B5/HER cells were cotransfected with 1.8 μg of human *COX-2* promoter construct ligated to luciferase (−1432/+59) and 0.2 μg of pSVβgal. After transfection, cells were treated with vehicle (*open column*), PMA (50 ng/ml, *black column*), or PMA (50 ng/ml) and resveratrol (5–15 μM). Reporter activities were measured in cellular extract 6 h later. Luciferase activity represents data that have been normalized with β-galactosidase activity. Six wells were used for each of the conditions. *Columns*, means; *bars*, S.D. *, $p < 0.001$ compared with PMA treatment.

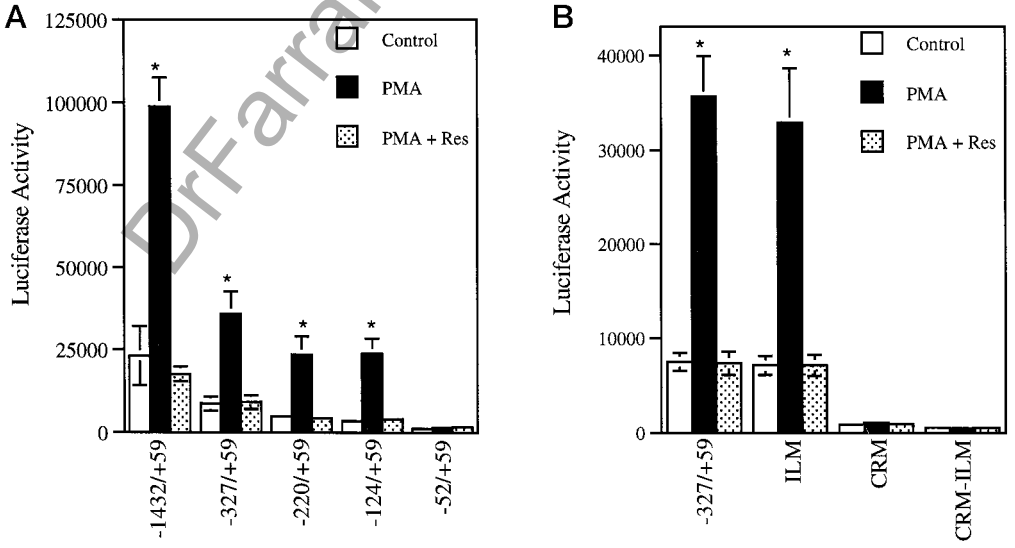

FIG. 10. **Localization of region of COX-2 promoter that mediates the effects of phorbol ester and resveratrol.** *A*, 184B5/HER cells were transfected with 1.8 μg of a series of human *COX-2* promoter deletion constructs ligated to luciferase (−1432/+59, −327/+59, −220/+59, −124/+59, −52/+59), and 0.2 μg of pSVβgal. *B*, 184B5/HER cells were transfected with 1.8 μg of a series of human *COX-2* promoter-luciferase constructs (−327/+59; ILM, CRM, CRM-ILM) and 0.2 μg of pSVβgal. ILM represents the −327/+59 *COX-2* promoter construct in which the NF-IL6 site was mutagenized; CRM refers to the −327/+59 *COX-2* promoter construct in which the CRE was mutagenized; CRM-ILM represents the −327/+59 *COX-2* promoter construct in which both the NF-IL6 and CRE elements were mutagenized. After transfection, cells were treated with vehicle (*open columns*), PMA (50 ng/ml, *black columns*), or PMA (50 ng/ml) and resveratrol (15 μM, *stippled columns*). Reporter activities were measured in cellular extract 6 h later. Luciferase activity represents data that have been normalized with β-galactosidase. Six wells were used for each of the conditions. *Columns*, means; *bars*, S.D. *, $p < 0.001$ compared with control.

had several effects, including a decrease in basal promoter activity and a loss of responsiveness to both PMA and resveratrol. By contrast, mutagenizing the NF-IL6 site had little effect on *COX-2* promoter function.

Defining the Mechanism by Which Resveratrol Inhibits PMA-mediated Induction of COX-2—One of the ways that PMA regulates gene expression is by activating the PKC signal transduction pathway (37). A key feature of this mechanism is the redistribution of PKC activity from cytosol to membrane. We therefore investigated the possibility that resveratrol inhibited the redistribution of PKC activity that was mediated by PMA. As shown in Fig. 11, resveratrol completely inhibited the translocation of PKC activity from cytosol to membrane. To further investigate the effects of resveratrol on the PKC signal transduction pathway, a series of transient transfections were performed. As shown in Fig. 12, overexpressing PKC-α or ERK1 caused 4.7- and 5.1-fold increases in *COX-2* promoter activity, respectively. These effects were suppressed by resveratrol. We also determined the effects of resveratrol on c-Jun-mediated induction of *COX-2* promoter activity. As shown in Fig. 13*A*, c-Jun caused an approximately 4-fold increase in *COX-2* promoter activity. This effect was also blocked by resveratrol. Resveratrol also suppressed PMA-mediated activation of an AP-1 reporter plasmid (2 $\times$ TRE-luciferase) (Fig. 13*B*).

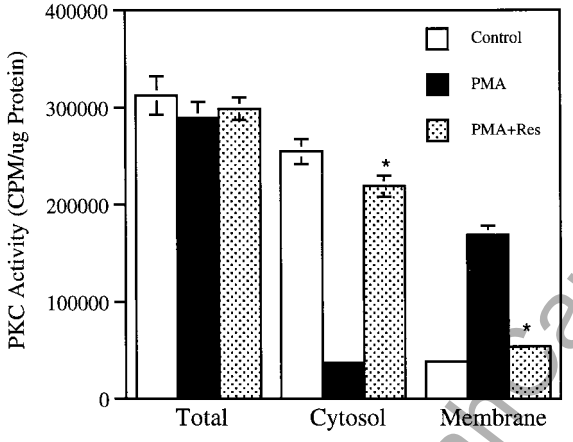

FIG. 11. **Resveratrol inhibits the redistribution of PKC activity induced by PMA.** 184B5/HER cells were treated with vehicle (*open columns*), PMA (50 ng/ml, *black columns*), or PMA (50 ng/ml) plus resveratrol (15 μM) (*stippled columns*) for 30 min. Total PKC activity, cytosolic PKC activity, and membrane PKC activity were measured. *Columns*, means; *bars*, S.D. $n = 6$, *, $p < 0.01$ *versus* PMA.

DISCUSSION

An expanding body of evidence indicates that inhibitors of COX-2 are useful for treating inflammation and preventing cancer (13, 15, 38). Drugs that interfere with the signaling mechanisms that up-regulate COX-2 should also be useful in this regard because they too decrease total COX-2 activity (25, 26). We have shown in the present experiments that resveratrol suppressed PMA-mediated induction of PG synthesis by inhibiting *COX-2* gene expression and the enzyme activity of COX-2. This is the first report of a compound that inhibits COX-2 by both mechanisms. Because effects on gene transcription and enzyme activity were observed over a similar range of concentrations of resveratrol, it will be of interest to determine whether the anti-inflammatory effects of resveratrol relate to one or both of these mechanisms.

In regard to the mechanism by which resveratrol modulates gene expression, it suppressed PMA-mediated activation of *COX-2* transcription in human mammary epithelial cells by inhibiting the PKC signal transduction pathway at multiple levels. It blocked both PMA-induced translocation of PKC activity from cytosol to membrane (Fig. 11) and the 4.7-fold increase in *COX-2* promoter activity mediated by PKC-α (Fig. 12*A*). Resveratrol also blocked the induction of *COX-2* promoter activity by ERK1 (Fig. 12*B*) and c-Jun (Fig. 13*A*); PMA-mediated induction of c-*jun* (Fig. 8*B*) and AP-1 activity (Fig. 13*B*) were suppressed by resveratrol. These inhibitory effects could be explained, in part, by the antioxidant properties of resveratrol as other phenolic antioxidants inhibit both phorbol ester-mediated activation of PKC (39) and AP-1 (40). These results are significant because PKC activity is up-regulated in some cancers (41, 42) and is considered a potential target for anti-cancer therapy (43). Additionally, as AP-1 has been implicated in promoting carcinogenesis, these effects are likely to contribute to the anti-tumor activity of resveratrol.

The inductive effects of PMA and suppressive effects of resveratrol on COX-2 expression are mediated via the CRE (Fig. 10). Xie and Herschman (44) showed that c-Jun, a component of the AP-1 transcription factor complex, activated the murine *Cox-2* promoter via the CRE (44). Thus, it seems likely that resveratrol blocks PMA-mediated induction of COX-2 by suppressing AP-1-dependent transactivation via the CRE (45). The finding that resveratrol inhibited PMA-mediated induction of

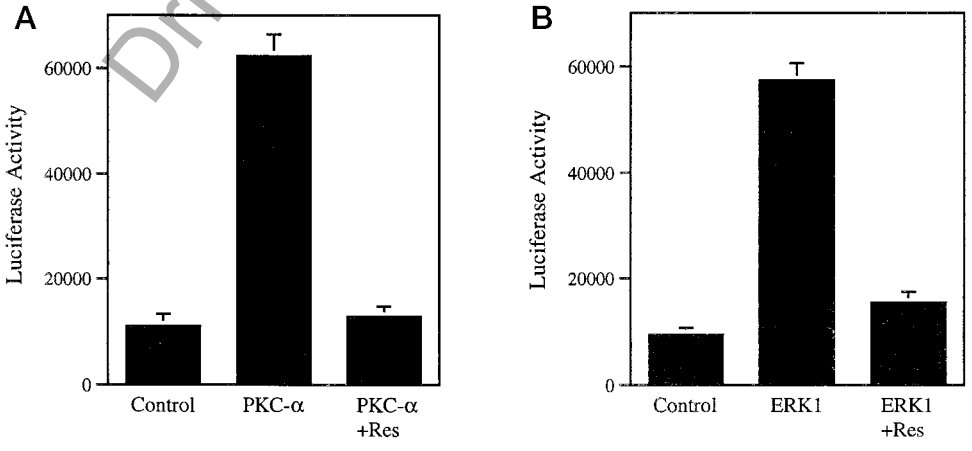

FIG. 12. **Resveratrol suppresses PKC-α and ERK1-mediated induction of COX-2 promoter activity.** *A*, cells were transfected with 0.9 μg of human *COX-2* promoter construct (−327/+59) (control) or 0.9 μg of *COX-2* promoter construct plus 0.9 μg of expression vector for PKC-α. *B*, cells received 0.9 μg of human *COX-2* promoter construct (−327/+59) (control) or 0.9 μg of *COX-2* promoter construct plus 0.9 μg of expression vector for ERK1. All cells received 0.2 μg of pSVβgal. The total amount of DNA in each reaction was kept constant at 2 μg by using empty vector. Immediately after transfection, cells were treated with vehicle (0.2% Me_2SO) or resveratrol (15 μM) for 24 h. Luciferase activity represents data that have been normalized with β-galactosidase. *Columns*, means; *bars*, S.D. $n = 6$.

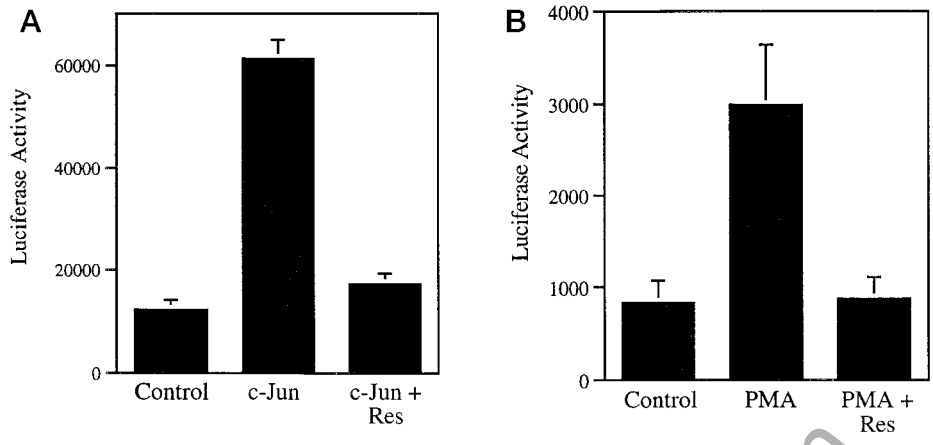

FIG. 13. **Resveratrol inhibits AP-1-mediated induction of COX-2 promoter activity.** *A*, cells were transfected with 0.9 μg of a human *COX-2* promoter construct ligated to luciferase (−327/+59) (control) or 0.9 μg of *COX-2* promoter construct and 0.9 μg of expression vector for c-*jun*. All cells received 0.2 μg of pSVβgal. The total amount of DNA in each reaction was kept constant at 2 μg by using empty vector. Immediately after transfection, cells were treated with vehicle or resveratrol (15 μM) for 24 h. *B*, cells were cotransfected with 1.8 μg of 2 × TRE-luciferase and 0.2 μg of pSVβgal. 24 h after transfection, cells were treated with vehicle, PMA (50 ng/ml), or PMA (50 ng/ml) and resveratrol (15 μM) for 6 h. Luciferase activity represents data that have been normalized with β-galactosidase activity. Six wells were used for each of the conditions. *Columns*, means; *bars*, S.D.

c-Jun expression (Fig. 8*B*) is consistent with this idea. Another possibility is that resveratrol will induce Fra expression like other phenolic antioxidants (46). Heterodimers of c-Jun and Fra do not activate AP-1-mediated gene expression as effectively as c-Jun homodimers or c-Jun/c-Fos heterodimers (47).

We reported previously that retinoids blocked PMA-mediated induction of COX-2 in oral epithelial cells (25). The same effect of retinoids was observed in the human mammary epithelial cells used in this study. However, whereas resveratrol and retinoids both block PMA-mediated induction of *COX-2* transcription, they seem to do so via different mechanisms. Thus, in contrast to resveratrol, retinoids did not block the PMA-induced redistribution of PKC activity from cytosol to membrane (data not shown). Additionally, resveratrol and retinoids antagonize AP-1 activity via different mechanisms. Retinoids antagonize AP-1 activity via a receptor-dependent mechanism (48), whereas our data suggest that resveratrol blocks PMA-mediated stimulation of AP-1-activity by inhibiting the PKC signaling cascade. This distinction between resveratrol and retinoids is important for the design of chemopreventive strategies utilizing combinations of drugs that act via different mechanisms.

Based on the results of an oxygen consumption assay, Jang *et al*. (27) reported that resveratrol did not inhibit the cyclooxygenase activity of COX-2. However, the results of the present study clearly show that resveratrol suppressed the synthesis of PGE_2 by inhibiting COX-2 enzyme activity (Figs. 2–4). One possible explanation for these apparently contradictory results is the difference in assays used to measure COX-2 activity.

Resveratrol was found recently to be a phytoestrogen that stimulates the growth of estrogen-dependent breast cancer cells (49). The mammary cell line used in our work was derived from normal human breast tissue and was insensitive to estrogen (28). Therefore, we do not know yet whether resveratrol will have similar effects on COX-2 in estrogen-dependent and -independent mammary cell lines. Another interesting but unanswered question is whether the same structural properties of resveratrol account for inhibition of COX-2 enzyme activity and *COX-2* transcription. Analogues of resveratrol are needed to determine the relationship between its structure and these different functions. Finally, based on the finding that resveratrol inhibited COX-2, further studies are warranted to determine how effective this compound or its analogues will be in preventing or treating inflammation and cancer.

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Resveratrol Inhibits Cyclooxygenase-2 Transcription and Activity in Phorbol Ester-treated Human Mammary Epithelial Cells

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