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Resveratrol-induced inactivation of human gastric adenocarcinoma cells through a protein kinase C-mediated mechanism

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Abstract

Resveratrol, a polyphenolic phytochemical present in berries, grapes, and wine, has emerged as a promising chemopreventive candidate. Because there is scant information regarding natural agents that prevent, suppress, or reverse gastric carcinogenesis, the aim of the present study was to determine the chemopreventive potential of resveratrol against gastric cancer by investigating cellular and molecular events associated with resveratrol treatment of human gastric adenocarcinoma cells. We determined the action of resveratrol on cellular function and cellular integrity by measuring DNA synthesis, cellular proliferation, cell cycle distribution, cytolysis, apoptosis, and phosphotransferase activities of two key signaling enzymes, protein kinase C (PKC) and mitogen-activated protein kinases (ERK1/ERK2), in human gastric adenocarcinoma KATO-III and RF-1 cells. Resveratrol inhibited [^{3}H]thymidine incorporation into cellular DNA of normally proliferating KATO-III cells and of RF-1 cells whose proliferation was stimulated with carcinogenic nitrosamines. Treatment with resveratrol arrested KATO-III cells in the G_0/G_1 phase of the cell cycle and eventually induced apoptotic cell death, but had a minimal effect on cell lysis. Resveratrol treatment had no effect on ERK1/ERK2 activity but significantly inhibited PKC activity of KATO-III cells and of human recombinant PKCα. Results indicate that resveratrol has potential as a chemopreventive agent against gastric cancer because it exerts an overall deactivating effect on human gastric adenocarcinoma cells. Resveratrol-induced inhibition of PKC activity and of PKCα, without any change in ERK1/ERK2 activity, suggests that resveratrol utilizes a PKC-mediated mechanism to deactivate gastric adenocarcinoma cells.

Keywords: Resveratrol; Cancer chemoprevention; Protein kinase C; Cell cycle; DNA synthesis; Apoptosis

1. Introduction

Gastric cancer is a common cause of cancer mortality worldwide, partly because most Western cancers are diagnosed at an advanced stage and partly because the currently available chemotherapeutic agents are ineffective against this type of cancer [1]. It is now well accepted that the primary etiological determinants for gastric carcinogenesis are exposure to chemical carcinogens and/or infection with *Helicobacter pylori* [2]. Based on these observations, intervention through chemopreventive measures to either arrest or reverse malignant transformation is a very attractive approach in combating gastric cancer.

Resveratrol, a polyphenolic phytoalexin present in various edible plants and extracted into foods produced from these plants, has emerged as a candidate chemopreventive agent because it exhibits antiproliferative and anti-inflammatory properties [3]. Resveratrol successfully suppresses the proliferation of a number of transformed [4–9] as well as normal cells [9–11] in culture, and has been shown to induce apoptosis [4,5,12], inhibit DNA polymerase [13], and inhibit ribonucleotide reductase [14]. In addition to its antiproliferative action, resveratrol may also act as a prophylactic against aryl hydrocarbon-induced carcinogenesis because it suppresses transcription and activity of cytochrome P450 1A1 in HepG2 and MCF-7 cells [15,16] and exhibits antagonist activity toward the aryl hydrocarbon receptor [17]. Resveratrol also behaves as an efficient antioxidant [18], an inhibitor of both COX-1 and COX-2 activities and expression [3,19], and modulates arachidonic

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Abbreviations: COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; PKC, protein kinase C; ERK1/ERK2, mitogen-activated protein kinases; NA, nitrosamine(s); TCA, trichloroacetic acid; and PMSF, phenylmethylsulfonyl fluoride.

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acid release and prostaglandin synthesis [20], properties thought to contribute to its chemopreventive potential. The anti-inflammatory property of resveratrol [21,22] and its growth inhibitory action against *H. pylori* [23] suggest that it may exert protection against *H. pylori*-induced gastric inflammation, further indicating its potential as a chemopreventive agent against gastric cancer.

Antiproliferative agents are known to exert an inhibitory effect on PKC and/or ERK1 and ERK2, key enzymes of cellular signaling cascades. PKC, comprised of a family of isozymes, is a pivotal constituent of the cellular signaling cascade regulating signal transduction for tumor promotion, differentiation, growth control, and apoptotic cell death [24, 25], whereas ERK1 and ERK2 are regulated by growth factors controlling normal cellular proliferation. PKC has become a target for the study of antineoplastic drugs because its function and activity become altered in certain malignancies, among them gastrointestinal cancers [26,27]. In turn, several chemotherapeutic agents and a number of naturally occurring flavonoids that exert protective action in experimentally induced carcinogenesis are among the compounds that inhibit PKC activity [28]. PKC has also been implicated in the regulation of apoptosis, since depletion of PKC isoforms and loss of PKC activity resulted in the apoptotic death of U937 promonocytic leukemia cells [29], and treatment with a PKC inhibitor potentiated mitomycin C-induced apoptosis of gastric cancer cells [30].

The present study was designed to elucidate cellular and molecular events that occur upon exposure of gastric cancer cells to resveratrol. Here, we report on the efficacy of resveratrol to inhibit DNA synthesis, arrest cells in the G_0/G_1 phase of the cell cycle, suppress proliferation, and induce apoptosis in human gastric adenocarcinoma cells, and on the relationship between these events and the enzymatic activities of PKC and ERK1/ERK2.

2. Materials and methods

2.1. Materials

Resveratrol was a gift from Pharmascience. Recombinant PKCα was purchased from Upstate Biotechnology, while NA, etoposide, and PMSF were purchased from the Sigma Chemical Co. [γ-^{32}P]ATP was obtained from New England Nuclear, and propidium iodide was a component of the Coulter DNA-Prep Reagents Kit used in flow cytometry. Gibco–Life Technologies supplied the PKC substrate (Ser-$^{25)}$PKC$_{19-31}$.

2.2. Cells

Human gastric adenocarcinoma KATO-III cells (ATCC: HTB-103) were cultured in Iscove's modified Dulbecco's medium, supplemented with 20% fetal bovine serum, 0.1 mM non-essential amino acids, 10 U/mL of penicillin, 10 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B in a CO_2–air mixture. Human gastric adenocarcinoma RF-1 cells (ATCC: CRL 1864) were cultured in Leibovitz's medium, supplemented with 10% fetal bovine serum, nonessential amino acids, and antibiotics–antimycotics as described for the KATO-III cells. RF-1 cells were cultured at 37^o in humidified air, because a mixture of CO_2 and air is detrimental to these cells.

2.3. Cell treatment

Freshly plated cells were allowed to equilibrate for 3 hr prior to the addition of the agonists. Resveratrol was dissolved in DMSO, and its exposure to light was minimized. The concentration of DMSO to which the cells were exposed was always 0.1%. A mixture of *N*-nitrosamines (*N*-nitrosodimethylamine, *N*-nitrosomethylethylamine, *N*-nitrosodiethylamine, *N*-nitrosopyrrolidine, *N*-nitrosopiperidine, *N*-nitroso-*n*-butylamine, and *N*-nitrosodiphenylamine) was dissolved in 95% ethanol, and the concentration of ethanol during exposure of RF-1 cells to *N*-nitrosamines was 0.1%. Control cells were always treated with the appropriate vehicle at identical concentrations after determining that DMSO or ethanol, at the indicated concentrations, were without effect on cellular integrity, cytotoxicity, proliferation, PKC activity, or apoptosis.

2.4. Cellular proliferation

KATO-III cells, plated in triplicates at 0.5×10^6 cells/3 mL in 6-well plates, were treated with increasing concentrations of resveratrol and incubated for the specified time, after which the medium was removed, cells were harvested in PBS, and the number of viable cells was determined using a hemocytometer (cells incorporating Trypan blue were subtracted from the total cell number).

2.5. DNA synthesis

Measurement of DNA synthesis was assessed by the incorporation of [^{3}H]thymidine into the 10% TCA precipitable fraction. For routine measurements, cells were plated in triplicate wells in 6-well plates at 0.5×10^6 cells/3.0 mL of medium and allowed to equilibrate for 3 hr, after which time 2 μCi/well of [^{3}H]thymidine and the specified concentration of resveratrol were added. Cells were then incubated for an additional 24 hr at 37^o under conditions appropriate for each cell line. At the end of the incubation time, the cells were harvested, centrifuged, washed once with 5 mL of PBS, and suspended in 2 mL of ice-cold 10% TCA with shaking for 30 min. This suspension was centrifuged at 1500 *g* for 5 min at 4°, the supernatant was discarded, and the remaining pellet was washed once with cold 10% TCA, and solubilized in 0.5 mL of 100 mM hyamine hydroxide in methanol; the resulting solution was added to 10 mL of

scintillant and counted in a Packard Liquid Scintillation counter.

2.6. Apoptotic index

KATO-III cells were incubated with 100 μM resveratrol or 50 μM etoposide or without agonists, and the percent of apoptotic cells was determined after 24 and 48 hr of treatment. DNA fragmentation was quantitated using a photometric ELISA assay from Boehringer-Mannheim (Cell Death Detection ELISA PLUS) that determines cytoplasmic histone-associated DNA fragments.

2.7. Preparation of cell fractions for enzyme assays

Untreated KATO-III cells were harvested, washed with 5 mL of ice-cold PBS, and centrifuged at 200 *g* for 4 min at 4°. PBS was aspirated, and cells were resuspended at 1×10^6 cells/0.1 mL of 20 mM Tris, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 10 mM mercaptoethanol, 1 mM PMSF, 50 μg/mL of leupeptin, and 100 μg/mL of trypsin inhibitor. The cell suspension was quick-frozen in liquid nitrogen and stored under liquid nitrogen until further use. Immediately before enzyme assay, cells were lysed by freeze–thaw in liquid nitrogen, and centrifuged at 20,000 *g* for 20 min at 4°; the resulting supernatant was used as the enzyme fraction. The protein content of each fraction was determined by the method of Lowry *et al.* [31].

2.8. PKC activity

For the measurement of PKC activity, 20 μL of KATO-III cell fraction or 20 ng of pure recombinant PKCα was added to 50 μL of a reaction mixture containing 20 mM Tris, pH 7.4, 0.5 mM $CaCl_2$, 6 mM $MgCl_2$, 33 μM [γ-^{32}P]ATP (200–400 cpm/pmol), 2.5 μg phosphatidylserine, 0.05 μg 1,2-dioleyl-rac-glycerol, and 5 μg of the PKC-preferred substrate molecule (Ser-^{25})PKC_{19-31}, and the indicated concentration of resveratrol. Nonspecific incorporation of radioactivity was determined in the absence of the substrate, since previous work has shown that incorporation of ^{32}P from [γ-^{32}P]ATP in the absence of the substrate is identical to incorporation of ^{32}P in the absence of calcium and the lipid cofactors [32]. Reaction was initiated by the addition of the enzyme fraction to the reaction components, was incubated at 30° for 5 min with shaking, and was terminated by the addition of 2 mL of 75 mM H_3PO_4. Reaction components were separated by passage through phosphocellulose filters (Whatman P-81), the filters were washed with 10 mL of 75 mM H_3PO_4, and the amount of radioactive phosphorylated peptide substrate adsorbed onto the phosphocellulose filters was quantified in a liquid scintillation counter. Nonspecific incorporation of ^{32}P in the absence of substrate was subtracted from that incorporated in the presence of substrate, and PKC activity was expressed as picomoles of ^{32}P incorporated per milligram of enzyme protein per minute.

2.9. ERK1/ERK2 activity

Activity of ERK1/ERK2 was determined by measuring the phosphorylation of myelin basic protein under conditions optimal for ERKl/ERK2. To 50 μL of reaction mixture containing 20 mM Tris buffer, pH 7.2, 10 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (300–500 cpm/pmol), 0.5 mM cAMP-dependent protein kinase inhibitor peptide, 0.5 mM sodium vanadate, and 1 mg/mL myelin basic protein was added 20 μL of the cell fraction. Reaction components were incubated at 30° for 5 min, and the reaction was terminated by the addition of 2 mL of 10% TCA followed by the addition of 25 μL of bovine serum albumin (5 mg/mL) as a protein co-precipitant. Reaction contents were filtered through glass fiber filters to remove unincorporated [γ-^{32}P]ATP, and were washed with 10 mL of 10% TCA; incorporation of ^{32}P into myelin basic protein was quantified in a liquid scintillation counter. Nonspecific incorporation of ^{32}P in the absence of myelin basic protein was subtracted from that in its presence, and ERK1/ERK2 activity was calculated and expressed as picomoles of ^{32}P incorporated per milligram of enzyme protein per minute. Activity of the treated cell fractions was always compared with that of untreated controls from the same experiment.

2.10. Cell cycle

The distribution of KATO-III cells in different phases of the cell cycle was measured by flow cytometric analysis after reaction of cellular DNA with propidium iodide. KATO-III cells (1×10^6) were seeded in culture flasks at a concentration of 0.25×10^6 cells/mL of medium with and without resveratrol (10 or 100 μM), and maintained in a 5% CO_2 atmosphere for 4 and 24 hr. At the end of the treatment, cells were harvested, washed once with cold PBS, and reacted with propidium iodide to label the DNA for flow cytometric analysis.

2.11. Cytotoxicity/cytolysis

The cytotoxic/cytolytic effect of resveratrol on KATO-III cells was determined using the Cyto Tox 96 Non-Radioactive cytotoxicity assay from the Promega Corp. Cells were suspended at 0.25×10^6/mL of medium, and 200 μL of cells was treated with increasing concentrations of resveratrol for 24 hr in 96-well plates in triplicate. Untreated cells were used as controls, and medium was used as the blank. At the end of the incubation, the plates were centrifuged, and the supernatant fraction was used to measure the amount of lactate dehydrogenase, a stable cytosolic enzyme released from lysed cells into the culture supernatants. This assay is based on the conversion of a tetrazolium salt (INT) into its red formazan product by

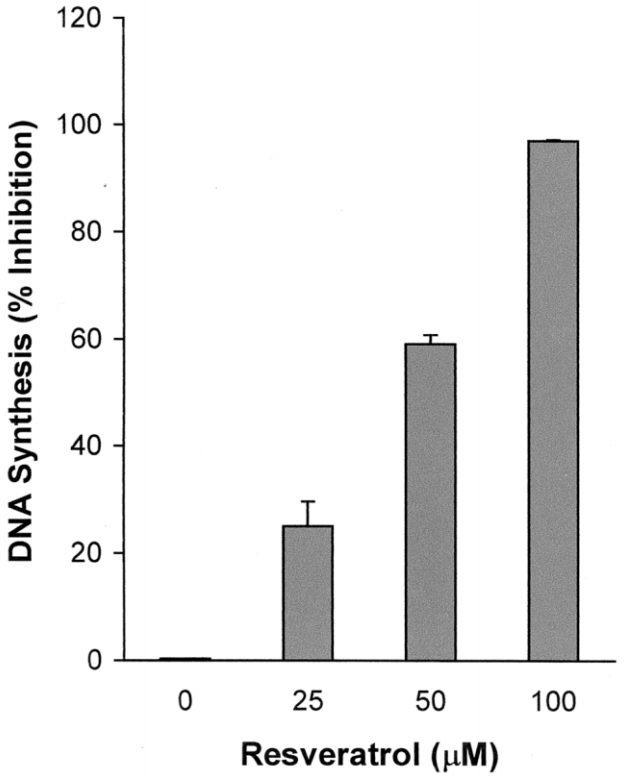

Fig. 1. Inhibition of DNA synthesis by resveratrol in human gastric adenocarcinoma cells. KATO-III cells were treated for 24 hr with increasing concentrations of resveratrol, and DNA synthesis was assessed by incorporation of [^{3}H]thymidine into the TCA precipitable fraction of cell lysates. Results are expressed as percent of inhibition and represent the means ± SEM of five separate experiments with each experimental value derived from triplicate measurements.

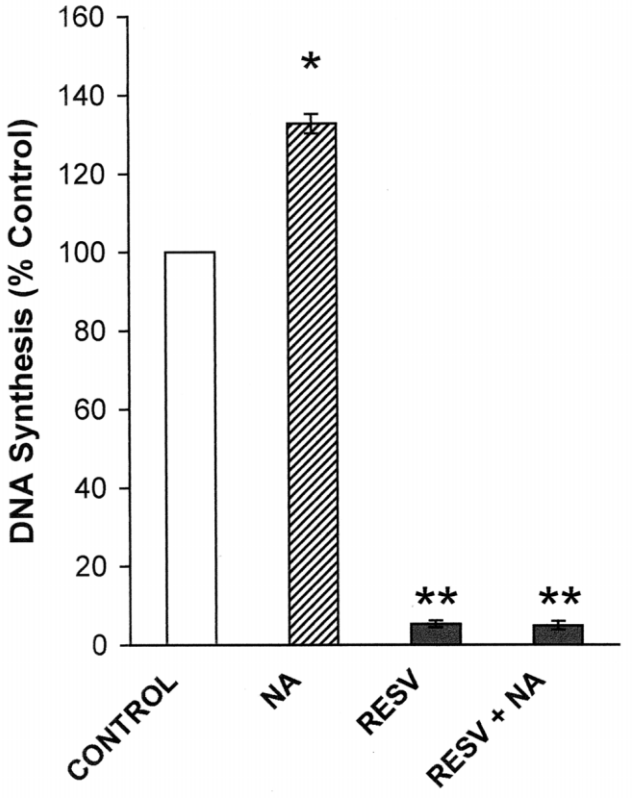

Fig. 2. Effect of resveratrol on NA-stimulated DNA synthesis in human gastric adenocarcinoma cells. RF-1 cells were treated with 50 μM nitrosamines (NA), 100 μM resveratrol (RESV), or 50 μM nitrosamines plus 100 μM resveratrol (RESV + NA). Results, expressed as relative values, are derived from four individual experiments with each experimental value determined in triplicate, and are expressed as the means ± SEM. Key: (*) $P < 0.02$; and (**) $P < 0.001$ as analyzed by Student's *t*-test.

lactic dehydrogenase-generated NADPH, and the amount of color generated reflects the degree of cell lysis.

3. Results

3.1. Effect of resveratrol on DNA synthesis

Incorporation of [^{3}H]thymidine into cellular DNA was used as an index of DNA synthesis. Exposure of KATO-III cells to resveratrol for 24 hr suppressed cellular DNA synthesis in a concentration-dependent manner with an IC_{50} value of approximately 35 μM, and with total inhibition of [^{3}H]thymidine incorporation after treatment with 100 μM resveratrol (Fig. 1). Inhibition of [^{3}H]thymidine incorporation into cellular DNA was also seen in resveratrol-treated RF-1 cells (Fig. 2). We have already shown that exposure of these cells to NA results in increased cellular proliferation and increased [^{3}H]thymidine uptake [33]. In the present study, a 24-hr exposure of RF-1 cells to 50 μM NA resulted in a 30% increase in [^{3}H]thymidine uptake, and treatment with 100 μM resveratrol totally abolished this increase. Resveratrol-induced inhibition of DNA synthesis in KATO-III cells was found to be partially reversible (Fig. 3). We compared the uptake of [^{3}H]thymidine into KATO-III cells that were exposed to resveratrol for 24 hr, after which time resveratrol was washed out and the cells were resuspended in fresh medium, with uptake into cells treated with resveratrol for the duration of the experiment. Inhibition of [^{3}H]thymidine incorporation was reversible, but the degree of reversibility was dependent upon the resveratrol concentration, with total recovery observed when cells were treated with 10 μM resveratrol but only partial recovery when cells were exposed to higher concentrations of resveratrol. As shown in Fig. 3, constant exposure of KATO-III cells to 100 μM resveratrol inhibited [^{3}H]thymidine incorporation by 92%, whereas after resveratrol washout [^{3}H]thymidine incorporation was inhibited by 56%.

3.2. Effect of resveratrol on cellular proliferation

Proliferation of KATO-III cells was determined by cell count after 24–96 hr of treatment with increasing concentrations of resveratrol (0–100 μM). Results show a concentration-dependent inhibition of cellular proliferation, with total suppression of proliferation at 50 μM and higher resveratrol (Fig. 4).

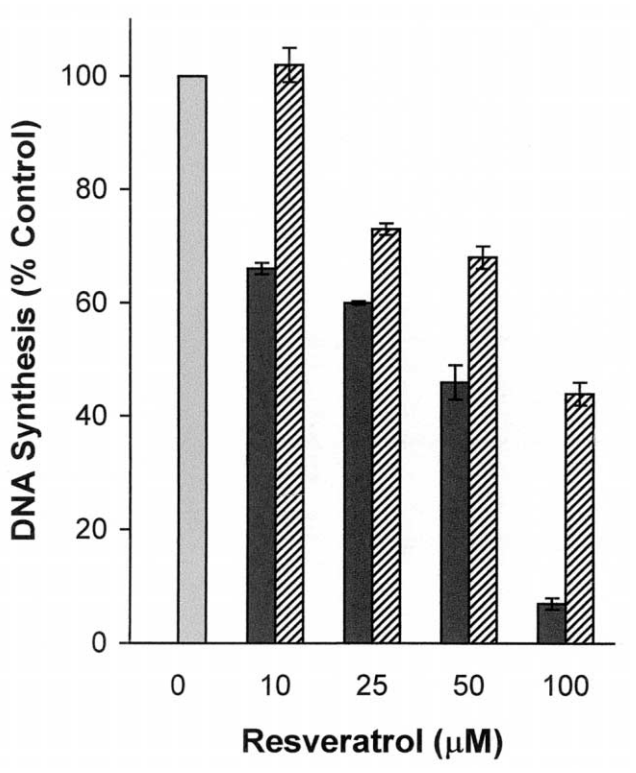

Fig. 3. Reversible effect of resveratrol on inhibition of DNA synthesis. KATO-III cells were incubated with the indicated concentrations of resveratrol and 2 μCi of [^{3}H]thymidine for 24 hr (black bars) or were preincubated with resveratrol for 24 hr, washed, and resuspended in fresh medium, and the incorporation of [^{3}H]thymidine was initiated (hatched bars). Results are expressed as means ± SEM of relative values derived from three individual experiments, with each experimental value obtained from triplicate measurements.

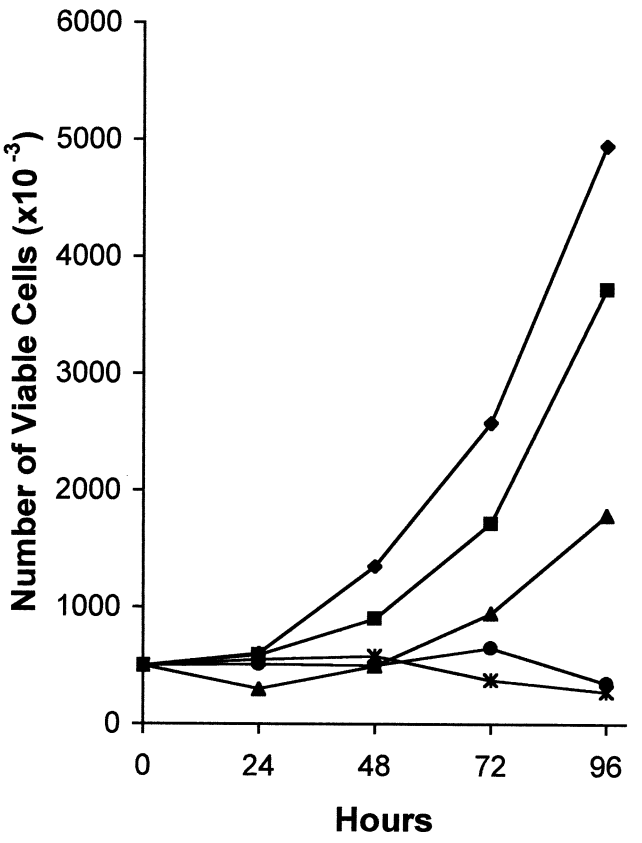

Fig. 4. Effect of resveratrol on the growth of KATO-III cells. KATO-III cells were treated with or without resveratrol [(♦) 0, (■) 10 μM, (▲) 30 μM, (●) 50 μM, or (*) 100 μM] for 24–96 hr, and viable cells were determined using a hemocytometer. Values represent an average of three individual experiments with each experimental value derived from duplicate measurements.

3.3. Effect of resveratrol on cytolysis

The action of resveratrol on the lysis of KATO-III cells was determined by measuring cellular release of lactate dehydrogenase. Results show that some cell lysis did occur after resveratrol treatment, but even at the highest tested concentration of resveratrol (100 μM) only 13% of cells lost membrane integrity (Fig. 5).

3.4. Effect of resveratrol on the cell cycle

Flow cytometric analysis revealed that resveratrol treatment induced changes in cell cycle distribution, with increased accumulation of KATO-III cells in the G_0/G_1 phase and compensation for this change by a decrease of cells in the G_2/M phase. These changes were already apparent after 4 hr of cell treatment with 100 μM resveratrol and became intensified after 24 hr of cell exposure to resveratrol (Fig. 6).

3.5. Effect of resveratrol on apoptosis

To determine whether the growth inhibitory and antiproliferative effects of resveratrol were ultimately associated with cell death by apoptosis, we measured DNA fragmentation in resveratrol-treated KATO-III cells (Fig. 7). After 24 hr with 100 μM resveratrol, only 8.3% of cells had fragmented DNA, but after 48 hr of treatment nearly 53% of cells displayed DNA fragmentation. Induction of apoptosis by resveratrol was compared with apoptosis stimulated by etoposide, a chemotherapeutic agent known to induce apoptosis in gastric adenocarcinoma cells. Cell treatment with 50 μM etoposide for 24 hr induced DNA fragmentation in 52.6% of the treated cells, whereas after 48 hr of treatment with 50 μM etoposide nearly all cells (91.5%) had fragmented DNA. These findings reveal that resveratrol is a slow inducer of apoptosis when compared with etoposide, and suggest that loss of cells after prolonged treatment with high concentrations of resveratrol may result from apoptotic cell death.

3.6. Effect of resveratrol on PKC activity

The activity of cell free PKC was determined by transfer of the terminal phosphate from [γ-^{32}P]ATP to a PKC-preferred substrate peptide. Results depicted in Fig. 8 show concentration-dependent inhibition of PKC phosphotrans-

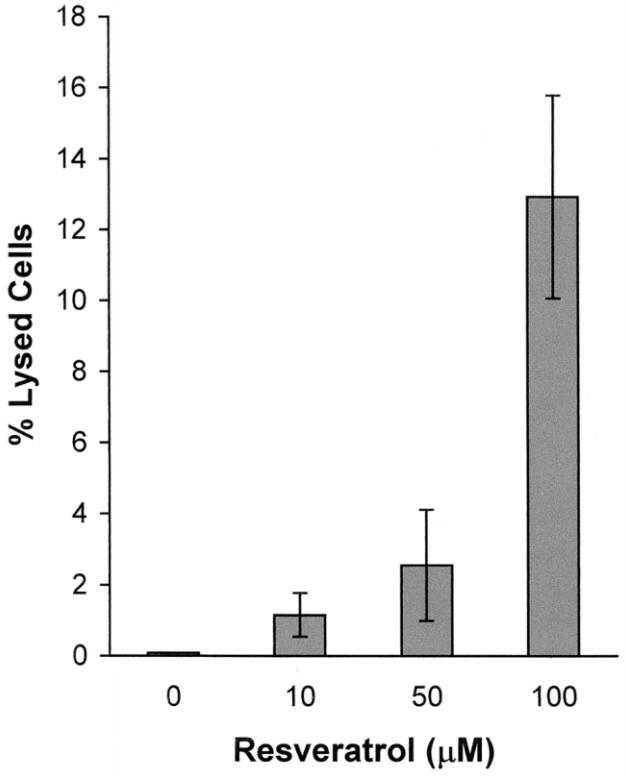

Fig. 5. Action of resveratrol on cell lysis. KATO-III cells (5×10^4) were treated with resveratrol, and the percentage of lysed cells was determined as described in "Materials and methods." Values represent means ± SEM and are derived from quadruplicate determinations of three individual experiments.

ferase activity by resveratrol. Resveratrol also displayed inhibitory activity toward pure recombinant PKCα, although the degree of inhibition was not of the same magnitude as toward PKC in KATO-III cytosol. However, resveratrol had no effect on either PKCϵ or PKCζ activity (data not shown), indicating that only the classical PKCα isoform was affected, whereas the novel (ϵ) and the atypical (ζ) isoforms did not respond to resveratrol treatment.

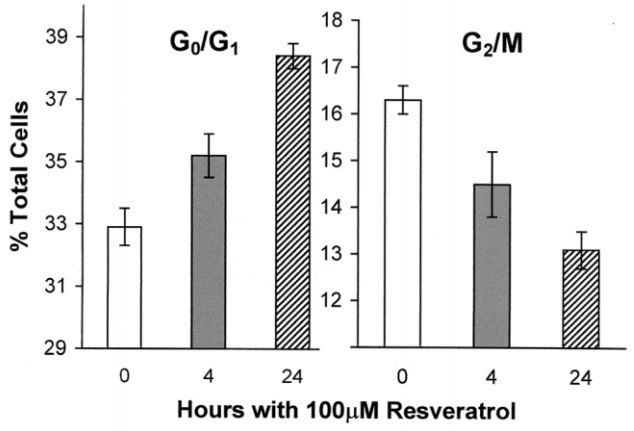

Fig. 6. Effect of resveratrol on the distribution of KATO-III cells between G_0/G_1 and G_2/M phases of the cell cycle. Cells (1×10^6) were cultured without or with 100 μM resveratrol for 4 and 24 hr, and cell distribution was determined by flow cytometric analysis of DNA contents. Results, expressed as the percent of total cells, represent means ± SEM of four individual experiments.

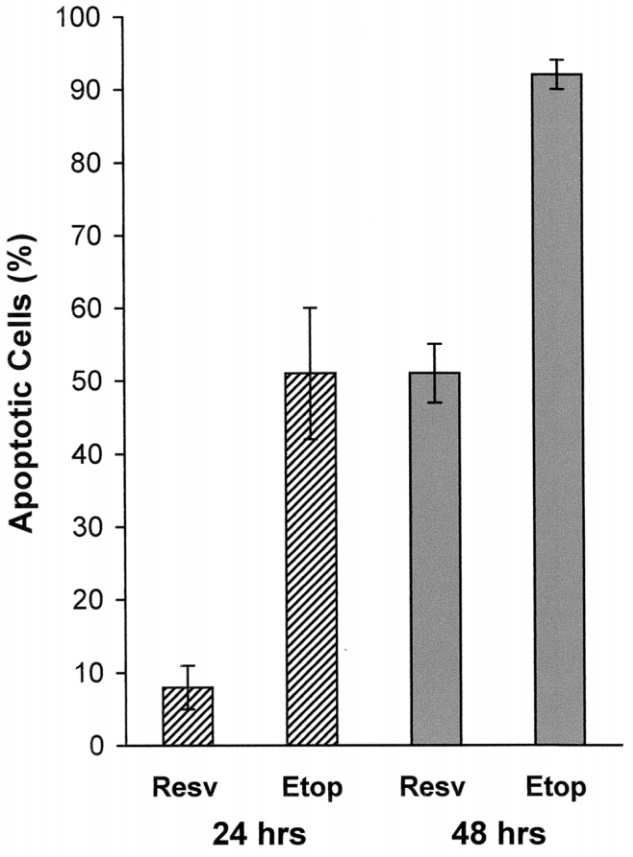

Fig. 7. Effects of resveratrol and etoposide on cellular apoptosis. KATO-III cells (starting number of cells was 10,000 in a total volume of 200 μL) were incubated for 24 or 48 hr without or with either 100 μM resveratrol or 50 μM etoposide, and the percent of apoptotic cells was determined as described in "Materials and methods." Values represent the means ± SEM of four individual experiments with each experimental value derived from quadruplicate measurements.

3.7. Effect of resveratrol on ERK1/ERK2 activity

Transfer of terminal phosphate from [γ-^{32}P]ATP to a mitogen-activated protein kinase-preferred substrate was used to measure the direct action of resveratrol on ERK1/ERK2 activity of KATO-III cells. Resveratrol had no direct effect on ERK1/ERK2 activity, suggesting that its antiproliferative action against KATO-III cells is exerted either downstream of ERK1/ERK2 or through a mechanism that bypasses the ERK1/ERK2 kinases (Table 1).

4. Discussion

The role of dietary factors in the chemoprevention of colon cancer is well accepted due to ample documenta-

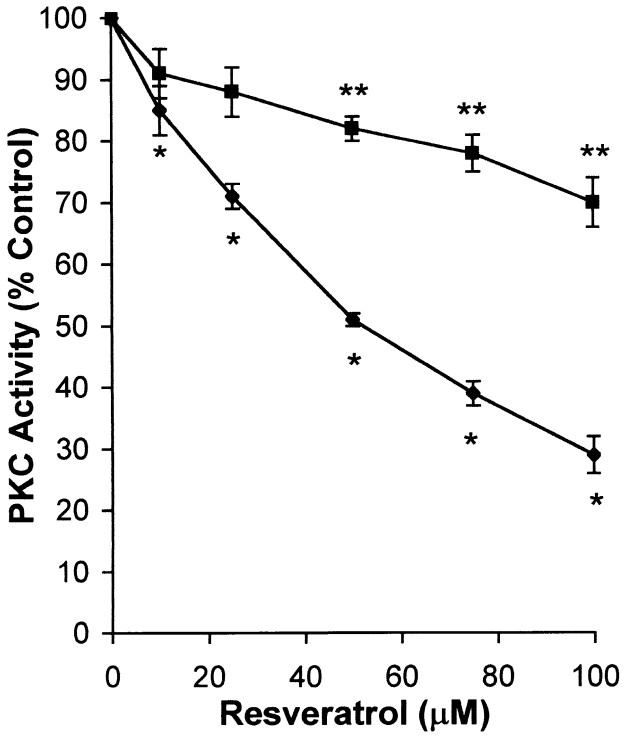

Fig. 8. Inhibition of PKC activity by resveratrol. The phosphotransferase activity of PKC in cell cytosol of KATO-III cells (♦) and the activity of pure recombinant PKCα (■) were measured in the absence and presence of resveratrol as described under "Materials and methods." In the absence of resveratrol, PKC activity in KATO-III cell cytosol was 127 ± 3 pmol of ^{32}P transferred/mg protein/min, and the activity of recombinant PKCα was 1410 ± 67 nmol of ^{32}P transferred/mg protein/min. Results are expressed as relative values and represent the means ± SEM of duplicate determinations from three separate experiments. Inhibition of PKCα by a resveratrol concentration of 50 μM and higher, and the inhibition of KATO-III cell PKC by a resveratrol concentration of 10 μM and higher were found to be significant (**P = 0.02, *P = 0.0001 by ANOVA).

tion that a diet rich in fruits and vegetables is protective against colon cancer, and this protection is ascribed to the high concentrations of folic acid, calcium, and antioxidant vitamins such as A, C, and E [34]. In addition to dietary components, nonsteroidal anti-inflammatory agents with the ability to inhibit COX-2 activity suppress the growth of colorectal cancer cells and colorectal polyps [35], and inhibit colon cancer development [36]. Although at the present time there is scant information regarding the role of dietary chemopreventive candidates in conferring protection against gastric cancer, a growing body of literature indicates that flavonoids and polyphenols exert protection against a number of cancers [28]. Resveratrol is a polyphenol with anti-inflammatory and antiproliferative properties whose potential as a candidate cancer chemopreventive agent has been demonstrated in assays that measure the three major stages of carcinogenesis, showing that resveratrol interferes in cellular events associated with tumor initiation, promotion, and progression [3]. Resveratrol is also an antioxidant [18] and inhibits COX-2 activity as well as its expression [19], and it is thought that these actions of resveratrol are responsible, in part, for its chemopreventive potential. Whether inhibition of COX-2 activity in gastric mucosa contributes to protection against the genesis of gastric cancer has not been elucidated, although there is some indication that overexpression of COX-2 protein may contribute to an early event in gastric cancer development [37].

Table 1
Effect of resveratrol on ERK1/ERK2 phosphotransferase activity in KATO-III cells

	ERK1/ERK2 phosphotransferase activity (pmole/mg protein/min)
Control	253 ± 14
RESV, 10 μM	220 ± 9
RESV, 100 μM	225 ± 11

ERK1/ERK2 phosphotransferase activity was determined as described in "Materials and methods." Values represent means ± SEM of two individual experiments and each experimental point was derived from duplicate determinations.

We show that resveratrol inactivates human gastric adenocarcinoma cells by (a) interfering with cell cycle progression, (b) inhibiting DNA synthesis, (c) suppressing cellular proliferation, and (d) eventually inducing apoptosis. Exposure of KATO-III and RF-1 cells to 100 μM resveratrol resulted in total suppression of [^{3}H]thymidine incorporation. Moreover, resveratrol suppressed NA-stimulated DNA synthesis in RF-1 cells, showing that, in addition to suppressing normal cellular proliferation, resveratrol was able to reverse carcinogen-stimulated proliferation.

Resveratrol-induced inhibition of [^{3}H]thymidine incorporation was reversible, but the degree of recovery was concentration dependent, with total recovery noted when cells were treated with low concentrations of resveratrol but partial reversal at higher concentrations. Reversibility of resveratrol-induced inhibition of DNA synthesis indicates that cellular inactivation by resveratrol is a revocable event and implies that, in order to achieve effective suppression of proliferation, cells require continuous exposure to resveratrol. As only a small percentage of KATO-III cells succumbed to apoptosis and cytolysis within 24 hr of treatment, it is probable that removal of resveratrol allows resumption of DNA synthesis, whereas prolonged exposure to resveratrol results in significant DNA fragmentation and apoptotic cell death.

Some data suggest that overexpression of PKC is associated with carcinogenesis, whereas inactivation of PKC is associated with tumor suppression, cell cycle arrest, decreased proliferation, and apoptosis. Inhibitors of PKC have been studied as potential anticancer agents precisely because they are effective in inducing apoptosis [38]. Resveratrol inhibited PKC activity in KATO-III cells and in-

hibited the activity of pure recombinant PKCα, suggesting that the antiproliferative signal from resveratrol may be partially transmitted through PKCα, since neither novel nor atypical isoforms of PKC responded to resveratrol, and of the classical isoforms, only PKCα was present in KATO-III cells (Holian O, unpublished observations). The target of PKC-mediated suppression of proliferation in gastric cancer cells is not known. Resveratrol has been shown to inhibit COX-2 expression [3] and suppress COX-2 promoter activity stimulated by phorbol 12-myristate 13-acetate (PMA) and by transforming growth factor α (TGFα) [39], and it is noted that the suppression of COX-2 expression in human gastric cancer MKN-74 cells was preceded by down-regulation of PKCα and was associated with apoptosis [40]. Moreover, our data demonstrating that induction of apoptosis is preceded by arrest of cells in the G_0/G_1 phase of the cell cycle suggest that resveratrol interacts with components of the cell cycle engine, among which are cyclin-dependent kinases and phosphorylation events regulated by other kinases. In HL-60 cells resveratrol up-regulated cyclin A and cyclin E expression and induced cell arrest at S/G_2 phase transition [4]. Such findings infer that the actions of resveratrol on cell cycle progression may be cell-type dependent.

Another likely candidate for PKC-mediated inhibition of cellular proliferation is NF-κB, the nuclear transcription factor responsible for regulating expression of genes involved in the inflammatory process and in promotion of cell survival [41,42]. NF-κB is activated by the addition of PKC and by PMA, whereas PKC inhibitors decrease basal NF-κB activity [43]. Resveratrol down-regulates NF-κB activation [44,45] by inhibiting IκB kinase activity [46], whereas *H. pylori* infection, considered an initiating event in gastric carcinogenesis, is associated with the opposite effects, namely increased NF-κB expression and activation and IκB degradation [47,48]. It has been demonstrated that *H. pylori* produce *cis*-9,10-methyleneoctadecanoic acid which, in turn, stimulates PKC activity and DNA synthesis in gastric cells [49]. Resveratrol, on the other hand, incorporates into phosphatidylserine-containing multilamellar vesicles and inhibits PKCα activity [50]. Based on these observations, we hypothesize that activation of PKC and NF-κB is associated with pro-carcinogenic events, whereas PKC inhibition by compounds like resveratrol leads to suppression of NF-κB activity and decreased DNA synthesis, and can be considered anti-carcinogenic. Our findings support this hypothesis and suggest that PKCα may play a critical role in resveratrol-controlled regulation of KATO-III proliferation.

ERK1/ERK2 activity of KATO-III cells remained unaffected by resveratrol treatment, suggesting that either PKC is the primary antiproliferative target of resveratrol in these cells, or that resveratrol interferes with signaling events downstream of ERK1/ERK2. Nonetheless, others have reported that resveratrol inhibits ERK1/ERK2 activity in coronary smooth muscle strips [51], while treatment of neuroblastoma cells with low concentrations of resveratrol stimulates ERK1/ERK2 activity, but this effect is reversed at higher resveratrol concentrations [52]. Such differences in results may arise from different experimental conditions, as we measured phosphotransferase activity whereas the above-cited studies determined the phosphorylation state of ERK1 and ERK2, or they may be cell-type dependent.

The ability of transformed cells to multiply without constraints is attributed, in part, to their unchecked proliferation and their failure to undergo apoptosis. Thus, a candidate chemopreventive agent should exhibit strong antiproliferative action and induce apoptosis, while remaining relatively non-cytotoxic. The findings that resveratrol delays tumor onset but is well tolerated in significant doses by mice [53], has no adverse effect on the health of rats [54], and inhibits the growth of *H. pylori* [22], along with our observations that it inactivated gastric adenocarcinoma cells, suggest resveratrol as a valuable chemopreventive candidate against gastric cancer.

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