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Inhibition of gastric cancer cell proliferation by resveratrol: role of nitric oxide

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Holian, Oksana, Shahid Wahid, Mary Jo Atten, and Bashar M. Attar. Inhibition of gastric cancer cell proliferation by resveratrol: role of nitric oxide. *Am J Physiol Gastrointest Liver Physiol* 282: G809–G816, 2002. First published January 30, 2002; 10.1152/ajpgi.00193.2001.—Resveratrol is a dietary phytochemical that has been shown to inhibit proliferation of a number of cell lines, and it behaves as a chemopreventive agent in assays that measure the three stages of carcinogenesis. We tested for its chemopreventive potential against gastric cancer by determining its interaction with signaling mechanisms that contribute to the proliferation of transformed cells. Low levels of exogenous reactive oxygen (H_2O_2) stimulated [^{3}H]thymidine uptake in human gastric adenocarcinoma SNU-1 cells, whereas resveratrol suppressed both synthesis of DNA and generation of endogenous O_2^- but stimulated nitric oxide (NO) synthase (NOS) activity. To address the role of NO in the antioxidant action of resveratrol, we measured the effect of sodium nitroprusside (SNP), an NO donor, on O_2^- generation and on [^{3}H]thymidine incorporation. SNP inhibited DNA synthesis and suppressed ionomycin-stimulated O_2^- generation in a concentration-dependent manner. Our results revealed that the antioxidant action of resveratrol toward gastric adenocarcinoma SNU-1 cells may reside in its ability to stimulate NOS to produce low levels of NO, which, in turn, exert antioxidant action. Resveratrol-induced inhibition of SNU-1 proliferation may be partly dependent on NO formation, and we hypothesize that resveratrol exerts its antiproliferative action by interfering with the action of endogenously produced reactive oxygen. These data are supportive of the action of NO against reactive oxygen and suggest that a resveratrol-rich diet may be chemopreventive against gastric cancer.

nitric oxide synthase; reactive oxygen species; gastric adenocarcinoma cells

A VARIETY OF NONPHAGOCYTIC cells generate low levels of reactive oxygen species in response to cytokines and peptide growth factors (10, 35), and the reactive oxygen generated by this miniburst interacts with guanine nucleotide binding proteins and with the Ras pathway to act as an intracellular messenger (17, 18). In transformed cells, the interaction between reactive oxygen and cellular signaling pathways results in transcription factor activation and modulation of gene expression, culminating in enhanced proliferation (5). On the basis of these observations, reactive oxygen species have been considered as procarcinogenic, whereas inhibition of reactive oxygen generation is now considered a plausible approach to suppress cancer development and progression. Resveratrol is a naturally occurring phytoalexin that was shown to inhibit the induction, promotion, and progression of experimentally induced cancer (19). Moreover, resveratrol inhibits transcription and activity of cyclooxygenase-2 (38, 39), an enzyme found to be upregulated in a number of transformed cells and various forms of cancer. Resveratrol also possesses antioxidant and anti-inflammatory activities, as evidenced by its ability to inhibit superoxide generation in stimulated neutrophils (33) and macrophages (19) and by its ability to protect low-density lipoproteins against oxidative damage (41, 46). Increased cellular levels of antioxidants function by *1*) directly scavenging reactive oxygen radicals, *2*) preventing the formation of cellular reactive oxygen, and/or *3*) increasing cellular detoxification mechanisms. The mechanism whereby resveratrol suppresses generation of reactive oxygen species in transformed cells has not yet been addressed.

Although the incidence of gastric cancer is on the decline, this disease remains a major health problem and a common cause of cancer mortality worldwide, because the disease is usually detected at an advanced stage, and the currently available chemotherapeutic agents are not highly effective. Development of gastric cancer is believed to be a slow process, with primary etiological determinants for gastric cancer being exposure to chemical carcinogens and/or infection with *Helicobacter pylori* (12, 21, 30). Several key events that follow a chemical insult or infection with *H. pylori* are *1*) an inflammatory response in the host gastric mucosa with release of numerous cytokines and reactive oxygen species, *2*) glandular atrophy, and eventually *3*) cellular proliferative changes such as metaplasia and dysplasia. Therefore, agents like resveratrol that can suppress neutrophil reactivity and the inflammatory response (33) and simultaneously inhibit proliferation of transformed gastric epithelial cells while remaining relatively nontoxic to the host (1) may

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constitute a new and effective defense against gastric carcinogenesis.

Prevention, suppression, or reversal of cancer induction through long-term use of naturally occurring compounds available in the diet is designated as chemoprevention. Epidemiological evidence indicates a protective effect of fruits and vegetables against gastric cancer, and this protection has been ascribed to their rich source of antioxidant vitamins and polyphenols. Among the polyphenolic compounds tested and proven somewhat effective against gastric cancer is curcumin (28, 36), shown to inhibit carcinogen-induced formation of gastric tumors by interacting with cellular signal transduction pathways to suppress cellular proliferation and induce apoptosis in the targeted cell. Some of the intermediate molecular events associated with the action of curcumin have been addressed (31).

We (1) have shown that resveratrol inhibits proliferation of gastric adenocarcinoma cells and reverses the stimulatory action of carcinogenic nitrosamines through a protein kinase C-mediated mechanism. Here we report on the antiproliferative action of resveratrol toward gastric adenocarcinoma SNU-1 cells through its efficacy as a modulator of reactive nitrogen and oxygen generation in these cells.

MATERIALS AND METHODS

Cells. The human gastric adenocarcinoma cell line SNU-1 (ATTC: CRL-5971) was routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 10 U/ml of streptomycin, and 0.25 μg/ml of amphotericin B at 37°C in a humidified incubator containing 5% CO_2. Freshly plated cells were always allowed to equilibrate 2–3 h before the addition of resveratrol (a kind gift from Pharmascience, Montreal, PQ, Canada), phorbol 12-myristate 13-acetate (PMA), sodium nitroprusside (SNP), or hydrogen peroxide (H_2O_2). PMA, SNP, and H_2O_2 were obtained from Sigma (St. Louis, MO). SNP and H_2O_2 were added as aqueous solutions, whereas resveratrol was dissolved in 95% ethanol, and the concentration of ethanol to which control and treated cells were exposed was always maintained at 0.1%. Because resveratrol is described as a light-sensitive and somewhat labile compound, its exposure to light was minimized, and fresh solutions were prepared on a weekly basis. PMA was dissolved in DMSO, and the concentration of DMSO to which cells were exposed was always maintained at 0.01%.

DNA synthesis. Synthesis of DNA was used as an index of cellular proliferation and was determined by the incorporation of [^{3}H]thymidine into cellular DNA. Cells at a density of 0.5×10^6 cells/5 ml of medium were plated in triplicate in six-well plates. Cells were allowed to equilibrate for 2 h at 37°C before the addition of 1 μCi of methyl-[^{3}H]thymidine ([^{3}H]thymidine; Amersham/Pharmacia Biotech, Piscataway, NJ) and the appropriate concentrations of resveratrol or SNP and were then incubated at 37°C for an additional 24 h. To determine the effects of H_2O_2 on DNA synthesis, SNU-1 cells were transferred to 0.1% FBS-supplemented medium for 48 h before the addition of H_2O_2 and [^{3}H]thymidine and were then incubated in 0.1% FBS-containing media with H_2O_2 in the absence and presence of resveratrol for an additional 24 h. At the end of treatment, cells were harvested in 5 ml PBS, centrifuged at 250 *g* for 4 min at 4°C, the PBS removed, and the resulting cell pellet suspended in 5 ml of 10% trichloroacetic acid (TCA) at 4°C for 30 min to precipitate protein-bound DNA. Formed precipitate was centrifuged at 1,500 *g* for 5 min at 4°C, washed once with 5 ml of ice-cold 10% TCA and solubilized in 0.25 ml of 0.1 N NaOH at 60°C, and added to 10 ml of scintillant. Amounts of incorporated [^{3}H]thymidine were quantified by liquid scintillation counting and are expressed as a percentage of their respective control.

Measurement of NO synthase activity. To determine the action of resveratrol on NO synthase (NOS) activity in SNU-1 cells, cells were incubated at 37°C for 16 h in CO_2-air with and without the specified concentrations of agonists in 25-cm^2 flasks at a density of 2.5×10^6 cells/10 ml media. At the end of treatment, cells were harvested, washed with 5 ml of PBS, resuspended in ice-cold 0.5 ml of 25 mM Tris·HCl (pH 7.4) containing 10 mM EDTA and 10 mM EGTA, and quick frozen in liquid nitrogen where they were stored for further use. Immediately before assay cells were lysed by two freeze-thaw cycles, NOS activity in cell lysates was determined by measuring the conversion of L-[2,3-^{3}H]-arginine to L-[^{3}H]citrulline by employing the NOS assay kit from Calbiochem (La Jolla, CA). Briefly, to 10 μl of cell lysate were added 40 μl of buffer to yield final concentrations of reagents as follows: 25 mM Tris·HCl, pH 7.4, 3 μM tetrahydrobiopterin, 1 μM FAD, 1 μM flavin mononucleotide, 1 mM NADPH (freshly prepared in 10 mM Tris·HCl, pH 7.4), 0.6 mM $CaCl_2$, and 1 μCi of L-[2,3-^{3}H]-arginine (New England Nuclear Life Science Products, Boston, MA). After incubation at 37°C for 60 min, the reaction was terminated by the addition of 400 μl of 50 mM HEPES, pH 5.5, containing 5 mM EDTA, followed by the addition of 100 μl of Dowex AG-50 WX-8 resin suspension in the above HEPES buffer. Samples were mixed, transferred to spin cups, and centrifuged at full speed for 20 s in an Eppendorf microfuge. Eluate, containing the formed L-[^{3}H]citrulline, was transferred to scintillation vials, and the amounts of generated L-[^{3}H]citrulline were quantified in a liquid scintillation counter. All individual treatment groups were performed in triplicate, and all findings were confirmed in at least three independent experiments. The amount of L-[^{3}H]citrulline generated was calculated per milligram lysate protein [protein content was determined by the method of Lowry (25)] and expressed as relative values, with NOS activity in PMA-treated cells assigned an arbitrary value of 1.0.

Measurement of superoxide release. The action of resveratrol on superoxide (O_2^-) generation by SNU-1 cells was determined using a modification of the LumiMax superoxide anion detection kit from Stratagene (La Jolla, CA). Immediately before measurement of O_2^- generation, proliferating SNU-1 cells were harvested, washed with 10 ml of sterile PBS supplemented with 0.1% FBS, and resuspended in this buffer at a density of 10×10^6 cells/ml and maintained at 37°C. Briefly, into a microcentrifuge tube containing 100 μl of cells (1×10^6 cells) were added 50 μl of 0.4 mM luminol and 50 μl of 0.4 mM lucigenin. Final dilutions of luminol and lucigenin, originally dissolved in DMSO, were made in the above-described HEPES buffer with the final content of DMSO always maintained at 0.1%. Generation of reactive oxygen by SNU-1 cells was initiated by the addition of 5 μl of 40 μM ionomycin (Sigma), and the emitted luminescence was recorded immediately thereafter for the duration of 5 min in an FB-12 luminometer (Zylux, Maryville, TN). Effects of resveratrol were determined by adding the appropriate concentration of the agent immediately after adding ionomycin. To determine the action of SNP on O_2^- generation, cells were incubated for 20 min at 37°C with appropriate concentrations of SNP in PBS containing 0.1% FBS before the addition of luminol, lucigenin, and ionomycin. Background luminescence, ob-

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tained in the absence of ionomycin, was negligible, and the resulting emitted light is expressed as relative light units generated by 1×10^6 cells when stimulated by 1 μM ionomycin.

Measurement of apoptosis. The percentage of apoptotic SNU-1 cells was determined using a photometric ELISA assay from Boehringer-Mannheim (Cell Death Detection ELISA^{PLUS}) that measures cytoplasmic histone-associated DNA fragments. SNU-1 cells plated in 96-well plates at a density of 1×10^4 cells/100 μl media were treated with resveratrol (10 and 100 μM) for 24 h or with 50 μM camptothecin for 48 h. Optimal apoptotic response (100% apoptotic cells) was observed after cell treatment with 50 μM camptothecin for 48 h, and this value was used to calculate the percentage of apoptotic SNU-1 cells after resveratrol treatment.

Measurement of cellular NADPH levels. The cellular levels of NADPH in resveratrol and ionomycin-treated cells were determined by utilizing bioreduction of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) tetrazolium compound (Owen's reagent from Promega, Madison, WI) to its colored formazan product. The quantity of formazan, directly dependent on the amount of NADPH in live cells, was measured by absorbance at 490 nm. To 10^4 cells in 100 μl of media were added the specified concentrations of resveratrol and ionomycin, and the cells were incubated in 96-well plates at 37°C in 5% CO_2-humidified air for 2 h. At the end of the 2-h incubation period were added 20 μl of the MTS reagent, and the plates were incubated for an additional 3 h to develop the colored formazan product, after which time absorbance at 490 nm was recorded using a 96-well plate reader from Molecular Devices (Sunnyvale, CA). Media alone were used as the blank and were subtracted from all other values. Results, expressed as absorbance (optical density at 490 nm), were derived from at least five individual experiments with each experimental value obtained from quadruplicate measurements.

RESULTS

Resveratrol inhibits DNA synthesis in gastric adenocarcinoma SNU-1 cells. Gastric adenocarcinoma SNU-1 cells proliferate in culture at a fairly rapid rate with a doubling time of ~24 h. Resveratrol treatment of these cells resulted in marked suppression of [^{3}H]thymidine incorporation into cellular DNA (Fig. 1) that was concentration-dependent with a calculated IC_{50} value of 25 μM. Treatment with 100 μM resveratrol, the highest concentration of resveratrol used in this study, resulted in 97% inhibition of [^{3}H]thymidine incorporation into SNU-1 cells.

Resveratrol stimulates NOS activity in SNU-1 cells. Under basal conditions, SNU-1 cells did not express measurable NOS activity. However, treatment of these cells for 16 h with PMA and/or resveratrol resulted in stimulation of NOS activity. NOS activity obtained after treatment with 0.1 μM PMA was designated as baseline activity and was assigned a value of 1.0 within each experiment to account for interexperimental variations. Cell treatment with resveratrol resulted in concentration-dependent stimulation of NOS activity with over threefold activation obtained after treatment with 100 μM resveratrol (Fig. 2). The stimulatory action of 0.1 μM PMA was found to be additive in the presence of 10 μM resveratrol, but there was no further response

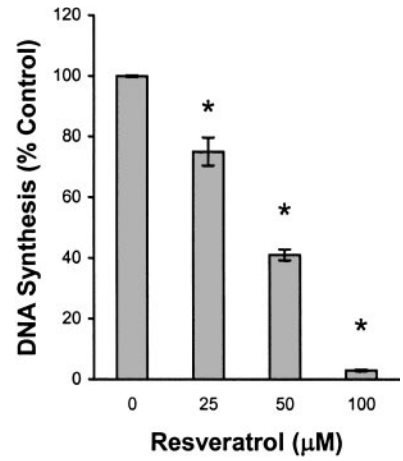

Fig. 1. Action of resveratrol on incorporation of methyl-[^{3}H]thymidine ([^{3}H]thymidine) into cellular DNA. SNU-1 cells (0.5×10^6 cells/well) were cultured for 24 h with 25, 50, or 100 μM resveratrol or with vehicle alone. After the addition of [^{3}H]thymidine (1.0 μCi/well) and resveratrol, cells were maintained for 24 h at 37°C in an air-CO_2 environment, after which they were harvested and treated with 10% trichloroacetic acid (TCA). TCA-precipitable radioactivity was used as a measure of [^{3}H]thymidine incorporation into cellular DNA. Data are presented as relative values and each point is the mean ± SE of 4 individual experiments with each experimental measurement derived from triplicate determinations. Resveratrol-induced inhibition of [^{3}H]thymidine uptake into DNA was statistically significant at all 3 concentrations of resveratrol (*$P < 0.001$ by ANOVA).

to 0.1 μM PMA when cells were treated with 100 μM resveratrol.

Action of SNP on DNA synthesis in SNU-1 cells. Because resveratrol stimulated NOS activity in SNU-1 cells, we explored the role of its product, NO, on DNA synthesis using SNP as a NO donor. As shown in Fig. 3, treatment of SNU-1 cells with low concentrations of SNP (0.05 and 0.1 mM) had no effect on [^{3}H]thymidine incorporation, whereas cell treatment with higher SNP concentrations (0.5–5 mM) resulted in significant ($P <$ 0.001) and dose-related suppression of [^{3}H]thymidine uptake into cellular DNA, with total inhibition of [^{3}H]thymidine uptake at 5.0 mM SNP.

Resveratrol inhibits H_2O_2-stimulated proliferation of SNU-1 cells. To determine the action of reactive oxygen on cellular DNA synthesis, serum-deprived SNU-1 cells were cultured in the absence and presence of H_2O_2. Incubation of SNU-1 cells with varying concentrations of H_2O_2 resulted in a concentration-dependent stimulation of [^{3}H]thymidine incorporation that peaked with a 2.5-fold increase at 10^{-8} M H_2O_2, remained elevated over the corresponding controls at 10^{-7} and 10^{-6} M H_2O_2, but declined to control levels at 10^{-5} M H_2O_2 (Fig. 4). Cell treatment with 100 μM resveratrol inhibited incorporation of [^{3}H]thymidine into SNU-1 cell DNA regardless of the presence of stimulating concentrations of H_2O_2. Resveratrol, therefore, effectively inhibited both basal (in the absence of exogenous H_2O_2) and H_2O_2-stimulated DNA synthesis.

SNP suppresses superoxide generation by SNU-1 cells. Quiescent human gastric adenocarcinoma SNU-1 cells do not release measurable amounts of endogenous

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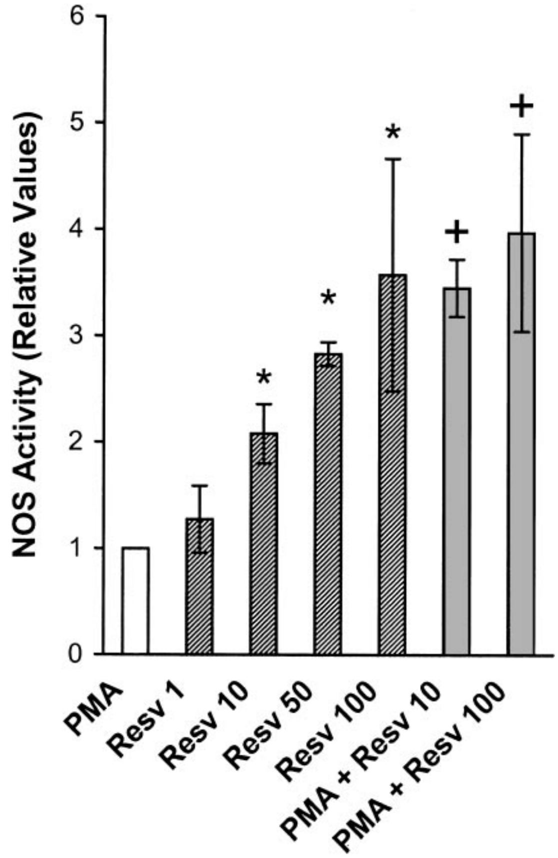

Fig. 2. Nitric oxide (NO) synthase (NOS) activity in SNU-1 cells after treatment with resveratrol (RESV) and/or phorbol 12-myristate 13-acetate (PMA). Cells were incubated with the agonists for 16 h, after which NOS activity in cell lysates was determined as described in MATERIALS AND METHODS. NOS activity obtained after cell treatment with 0.1 μM PMA was assigned a value of 1.0. Compared with treatment with PMA alone, significant stimulation of NOS activity was observed after cell treatment with 10, 50, and 100 μM resveratrol (*$P < 0.01$ by ANOVA) as well as after treatment of cells with 10 or 100 μM resveratrol plus 0.1 μM PMA (+$P < 0.01$ by Student's *t*-test).

reactive oxygen species. However, similar to several other cell types, they responded to ionomycin treatment with a small but measurable oxidative burst detectable only when the oxidation of luminol was enhanced by the addition of lucigenin. Subsequently, generation of reactive oxygen species by SNU-1 cells was routinely stimulated by treatment of the cells with 1 μM ionomycin and was measured in the presence of luminol and lucigenin as described in MATERIALS AND METHODS. To determine the action of NO on O_2^- generation, SNU-1 cells were treated with SNP for 20 min and then were stimulated with ionomycin, and generation of O_2^- was measured as described. Results, presented in Fig. 5, show that pretreatment of SNU-1 cells with SNP exerts a suppressing action on ionomycin-stimulated generation of O_2^- and suggests that cell treatment with the NO donor elicits an antioxidant-like response in these cells.

Resveratrol inhibits superoxide generation by SNU-1 cells. Ionomycin-stimulated oxidative burst in SNU-1 cells was also inhibited in a concentration-dependent manner by resveratrol (Fig. 6). The oxidative burst in response to 1 μM ionomycin decayed very rapidly, and resveratrol enhanced this decay with nearly total suppression of reactive oxygen generation observed within 3 min after the addition of 100 μM resveratrol.

Resveratrol and ionomycin deplete cellular NADPH. Generation of O_2^- by NADPH oxidase and production of NO by NOS utilize NADPH as the electron donor. We measured cellular NADPH levels after ionomycin treatment and after treatment with resveratrol (Table 1) and found decreased NADPH levels after cell exposure to either 1 μM ionomycin or to all concentrations of resveratrol. A significant drop in NADPH was already observed at 1 μM resveratrol, followed by further decrease in NADPH levels at higher concentrations of resveratrol. Loss of cellular NADPH seen after resveratrol treatment is believed to reflect its utilization during NOS activation, but it is also possible that resveratrol interacts with other, as yet undetermined, NADPH-utilizing reactions because significant NOS activity changes were observed only at 10 μM and higher resveratrol, whereas significant depletion of cellular NADPH was already evident after cell treatment with 1 μM resveratrol.

High-resveratrol concentration induces apoptosis in SNU-1 cells. Inhibition of cellular proliferation by exogenous agents often culminates in an apoptotic response. Therefore, we determined the apoptotic response of SNU-1 cells to treatment with resveratrol for the duration of time in which we observed inhibition of [^{3}H]thymidine incorporation and NOS activation. The effect of resveratrol on SNU-1 cell apoptosis is pre-

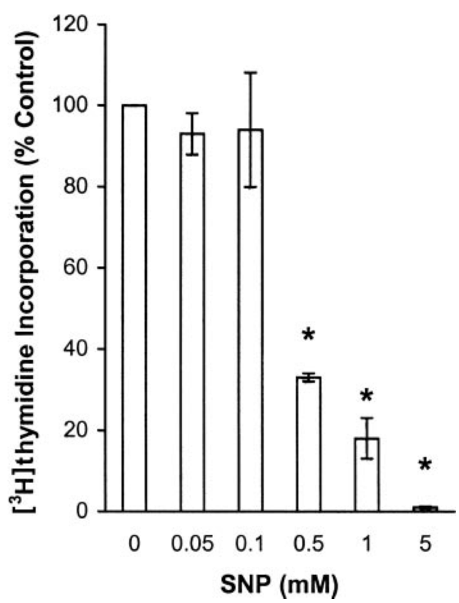

Fig. 3. Response of SNU-1 cells to treatment with sodium nitroprusside (SNP). Cells were incubated for 24 h at 37°C in humidified air-5% CO_2 with 1 μCi of [^{3}H]thymidine and with increasing concentrations of SNP. Incorporation of [^{3}H]thymidine into TCA-precipitable fraction was used as a measure of DNA synthesis and results, expressed as relative values, represent means ± SE of 3 individual experiments. Statistically significant inhibition of DNA synthesis occurred at 0.5 mM and higher SNP (*$P < 0.001$ by ANOVA).

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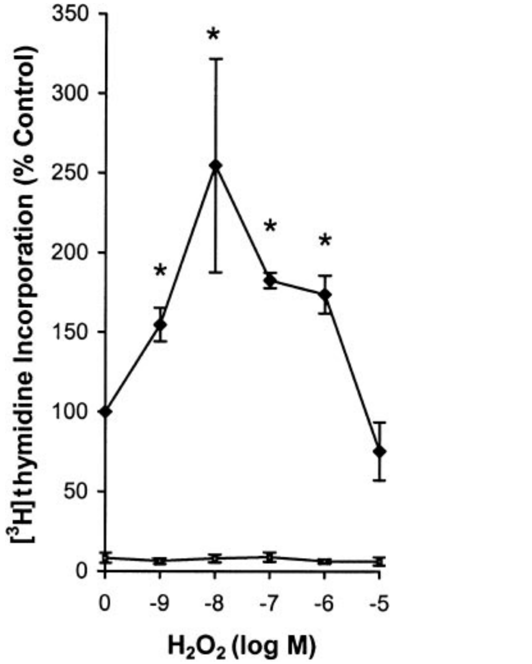

Fig. 4. Effect of H_2O_2 on [^{3}H]thymidine uptake in the absence and presence of resveratrol. SNU-1 cells (0.5×10^6) were cultured for 24 h with 1 μCi of [^{3}H]thymidine and with increasing concentrations of H_2O_2 in the absence (◆) and presence of 100 μM resveratrol (●). TCA-precipitable radioactivity was used as a measure of [^{3}H]thymidine uptake into DNA. Results, expressed as relative values with 100% incorporation designated in the absence of H_2O_2 and resveratrol, depict the means ± SE of 3 individual experiments with each experimental value derived from duplicate determinations. ANOVA analysis indicates a significant increase in [^{3}H]thymidine incorporation between 10^{-9} and 10^{-6} M H_2O_2 (*$P < 0.01$). Inhibition of DNA synthesis by resveratrol was statistically significant in the absence and presence of all H_2O_2 concentrations ($P < 0.001$, Student's *t*-test).

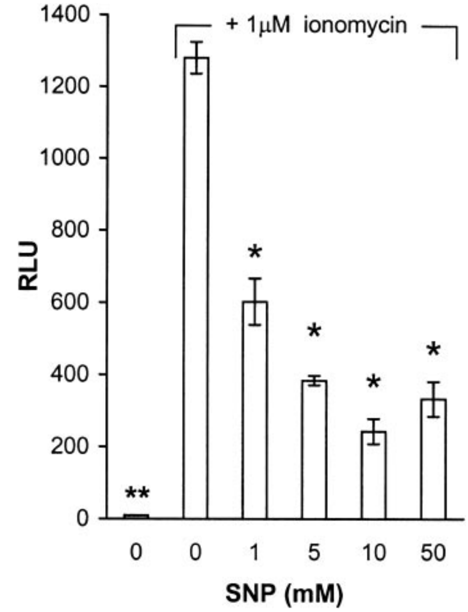

Fig. 5. Effect of SNP on ionomycin-stimulated O_2^- generation in SNU-1 cells. Cells were preincubated with increasing concentrations of SNP or with vehicle (PBS), O_2^- generation was stimulated by the addition of 1 μM ionomycin, and the generated O_2^- was quantitated by lucigenin-enhanced oxidation of luminol. Results are expressed as relative light units (RLU) emitted by 1×10^6 cells in the absence and presence of ionomycin, and represent means ± SE of 3 individual experiments. Ionomycin induced a significant increase in O_2^- levels (**$P < 0.001$) diminished by treatment of cells with SNP (*$P < 0.001$ by ANOVA).

sented in Fig. 7. Normally proliferating SNU-1 cells contained <5% apoptotic cells, and cell treatment with 10 μM resveratrol for 24 h, conditions that result in significant stimulation of NOS activity, did not increase the percentage of apoptotic cells. However, when cells were treated for 24 h with 100 μM resveratrol, there was a significant increase in apoptotic cells with nearly half of the cells exhibiting DNA fragmentation.

DISCUSSION

Chemoprevention, defined as the use of nontoxic substances to inhibit or reverse the process of carcinogenesis, is now considered an essential approach to cancer prevention and/or treatment. Because gastric cancer is known to have epigenetic origins, such as infection with *H. pylori* and/or exposure to carcinogenic nitrosamines (12, 30), it is thought to be preventable through appropriate intervention. However, at present, there are limited experimental data regarding specific agents that prevent or retard gastric carcinogenesis. Several polyphenolic compounds, among them curcumin, have demonstrated anticarcinogenic activities in experimental animal cancer models, and their potential as chemopreventive agents against gastric cancer has been discussed (28, 31, 36, 45). Recent evidence indicates that dietary intervention through supplementation with antioxidants like ascorbic acid and β-carotene results in regression of *H. pylori*-induced gastric dysplasia, a precursor event in gastric carcino-

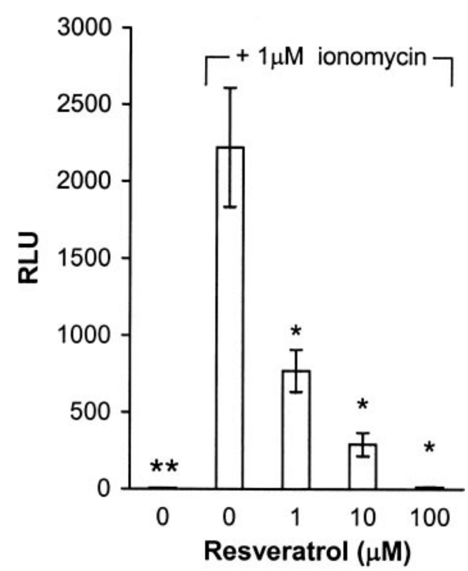

Fig. 6. Action of resveratrol on O_2^- generation by SNU-1 cells. Cell treatment with ionomycin resulted in statistically significant generation of O_2^- (**$P < 0.001$) suppressed by resveratrol in a concentration-dependent manner. Results are expressed as RLU emitted by 1×10^6 cells and values depict the means ± SE of 4 individual experiments. Inhibition of O_2^- generation was found to be significant at all concentrations of resveratrol (*$P < 0.001$ by ANOVA).

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Table 1. *NADPH-dependent reduction of MTS to formazan by SNU-1 cells*

Treatment	Formazan
None	0.607 ± 0.033
Ionomycin, 1 μM	0.254 ± 0.007*
Resveratrol, 1 μM	0.343 ± 0.020*
Resveratrol, 5 μM	0.283 ± 0.010*
Resveratrol, 10 μM	0.269 ± 0.009*
Resveratrol, 100 μM	0.262 ± 0.007*

Values shown represent the means ± SE of 4 individual experiments with each experimental point derived from quadruplicate determinations. Formazan optical density was at 490 nm. *Values significantly different from untreated controls ($P < 0.01$ by Student's *t*-test). MTS, 3-(4,5-dimethylthiazol-z-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

genesis (7). These studies indicate that polyphenolic compounds and antioxidants exert a protective action against gastric cancer. However, there is a paucity of experimental data regarding the cellular mechanisms engaged by these compounds in their chemopreventive action against gastric cancer.

We addressed the chemopreventive potential of resveratrol against gastric cancer by determining the action of resveratrol on the proliferation of gastric adenocarcinoma SNU-1 cells and on some of the molecular events associated with cellular proliferation and survival. There are sufficient data demonstrating that low levels of reactive oxygen act as intracellular messengers and promote the growth of transformed cells (5, 10, 17, 18, 35). In addition to reactive oxygen species, reactive nitrogen in the form of NO has also been implicated in regulation of cellular proliferation, but its role as a proliferative signal is not well defined, because it appears to depend on the cell type responsible for its release and the NOS isoforms within that cell, as well as on the concentration of released NO and on the composition of the intracellular milieu (22, 34, 44). The neuronal and endothelial isoforms are thought to be responsible for production of low levels of NO (37) and both isoforms have been identified in gastric mucosa (3, 32). NO is a lipophilic radical that, when produced in small amounts, can exert beneficial effects by reacting with O_2^- and, in this manner, behaves as an antioxidant. However, excess production of NO results in the accumulation of peroxynitrite, a reactive species that exerts deleterious effects on a variety of cells. Moreover, NO is rapidly oxidized to NO_2^- and NO_3^-, and the NO_2^--generating pathway was shown to exert an inhibitory effect on ribonucleotide reductase and on proliferation of murine adenocarcinoma cells (23). Activated macrophages produce high levels of cytostatic factors, NO being one, which inactivate tumor cells, and there is data indicating that NO, contributed by SNP or endogenously produced by endotoxins, is antiproliferative toward pancreatic tumor cells (13) and induces apoptosis in colonic epithelial cells (34). On the other hand, suppression of NO generation by cell treatment with the NOS inhibitor nitro-L-arginine methyl ester also resulted in antiproliferative effects on vascular smooth muscle cells (9).

Because resveratrol behaves as an antioxidant and can affect cellular NO production, we questioned whether its chemopreventive potential might result, in part, from its action on the generation of these two reactive species. We tested the antioxidant action of resveratrol by directly measuring its effect on ionomycin-stimulated reactive oxygen generation and by its action against H_2O_2-stimulated proliferation. Ionomycin induces generation of reactive oxygen in several cell types, and there is data indicating that ionomycin-stimulated generation of reactive oxygen is dependent on its action as a calcium ionophore (8). Normally, proliferating SNU-1 cells did not generate measurable levels of reactive oxygen, but treatment with ionomycin resulted in generation of low levels of reactive oxygen, and this was suppressed by resveratrol. SNU-1 cells responded to low concentrations of H_2O_2 with increased DNA synthesis, findings in line with reports showing that transformed cells respond to low levels of reactive oxygen species with increased proliferation. Resveratrol reversed the proliferative effect of H_2O_2, and at high concentration (100 μM) totally suppressed [^{3}H]thymidine uptake, suggesting that, in addition to its antioxidant action, resveratrol may also use other pathways to exert effective antiproliferative control. The response of SNU-1 cells to ionomycin treatment resulted in depletion of cellular NADPH, suggesting that in these cells, ionomycin activates an NADPH oxidase-like complex. Although resveratrol inhibited ionomycin-triggered generation of O_2^-, it did not reverse ionomycin-induced depletion of NADPH and, in fact, was, by itself, responsible for further NADPH depletion. Therefore, we hypothesize that resveratrol-induced depletion of NADPH may reflect utilization of NADPH for production of NO. Although resveratrol inhibits NO production (27, 42) and release of nitrite in lipopolysaccharide-activated murine macrophage RAW 264.7 cells (43), and suppresses lipopolysaccharide-

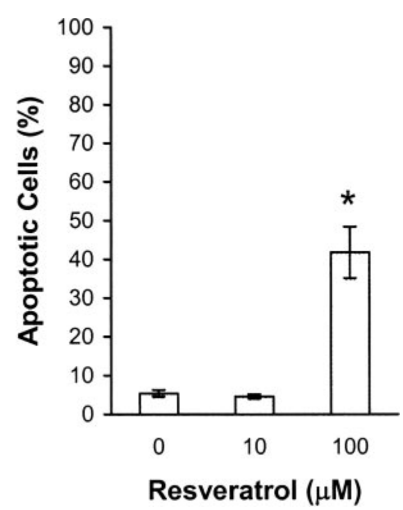

Fig. 7. Action of resveratrol on apoptosis. Percentage of apoptotic cells was determined as described in MATERIALS AND METHODS in untreated SNU-1 cells and after 24 h of cell treatment with 10 and 100 μM resveratrol. Significant increase in apoptotic cells was detected after treatment with 100 μM resveratrol (*$P < 0.01$).

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and interferon-γ-stimulated NOS in macrophages (26), resveratrol stimulates NOS activity and inhibits proliferation of pulmonary artery endothelial cells (15), suggesting a different mode of action in nonphagocytic cells. SNU-1 cells also responded to resveratrol treatment with increased NOS activity. PMA, which is known to stimulate all three NOS isoforms in a variety of cell types (2, 24, 29) also stimulated NOS in SNU-1 cells, and its action was additive with that elicited by low concentration of resveratrol. However, PMA had no further effect on NOS activity when cells were treated with high resveratrol concentration, indicating that 100 μM resveratrol elicits a maximal response with respect to both NOS activation and suppression of DNA synthesis.

To test the action of NO on cellular proliferation, we measured [^{3}H]thymidine incorporation over a range of SNP concentrations and observed that low concentrations of SNP had no effect on DNA synthesis in SNU-1 cells, whereas higher concentrations exerted an antiproliferative effect. Although we have no direct measurements of NO levels after cell treatment with SNP, our data indicate that production of low levels of NO is not detrimental to SNU-1 cells, but higher levels suppress cellular proliferation. This observation corroborates findings showing that exposure of SNU-1 cells to high resveratrol concentration (100 μM), which results in maximal NOS activation, also results in significant apoptotic response, whereas cell treatment for the same duration of time with lower concentrations of resveratrol (10 μM) does not induce apoptosis. Apoptosis at high resveratrol concentration may result from accumulation of peroxynitrite arising from production of significant levels of NO that react with endogenously generated O_2^-. Direct inhibition by SNP of ionomycin-stimulated generation of O_2^- in SNU-1 cells indicates that NO interferes with O_2^- generation and suggests that the inhibitory action of resveratrol on O_2^- generation may partly result from resveratrol-induced activation of NOS. Although suppression of O_2^- generation was observed at 1 mM and higher SNP, such concentrations of SNP may be needed to maintain a given NO level to counteract its rapid metabolic breakdown, and the actual level of NO that suppresses reactive oxygen generation may be much lower than that arising from SNP at any given time. Suppression of ionomycin-stimulated generation of O_2^- with concentrations of SNP that also inhibit [^{3}H]thymidine incorporation strongly indicates that NO directly inhibits O_2^- generation while also inhibiting SNU-1 proliferation. NO shares with resveratrol the ability to induce apoptosis (14) and inhibit ribonucleotide reductase, a rate-limiting step in DNA synthesis (4, 11), and inhibition of this enzyme may further contribute to the antiproliferative action of resveratrol.

We have shown that resveratrol treatment inhibits protein kinase C activity, induces cell cycle arrest, and suppresses nitrosamine-stimulated proliferation of gastric adenocarcinoma cells (1). The current data indicate that the antioxidant action of resveratrol resides, in part, in its ability to stimulate NOS and enhance production of NO that would interact with endogenously produced reactive oxygen to inhibit SNU-1 proliferation and eventually induce cell death by apoptosis. These observations lend further credence to the intermediary action of NO in resveratrol-elicited cellular responses (6, 16, 40), support existing evidence that the chemopreventive potential of resveratrol results from its interaction with cell signaling mechanisms that control cellular proliferation and apoptotic death, and argue that consumption of a resveratrol-rich diet may be protective against gastric cancer.

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