Apoptosis Induced by Capsaicin and Resveratrol in Colon Carcinoma Cells Requires Nitric Oxide Production and Caspase Activation

MIN YOUNG KIM[1], LAURA J. TRUDEL[1] and GERALD N. WOGAN[1,2]

Departments of [1]Biological Engineering and [2]Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

Abstract. *Although many studies have focused on anticarcinogenic properties of capsaicin and resveratrol, molecular mechanisms by which they selectively induce apoptosis are incompletely characterized. We examined the role of nitric oxide ($NO^{\bullet}$) and influence of p53 status during apoptosis induced by these agents in two isogenic HCT116 human colon carcinomas, wild-type p53 (p53-WT) and complete knockout of p53 (p53-null) cells. Capsaicin and resveratrol, alone or in combination, inhibited cell growth and promoted apoptosis by the elevation of $NO^{\bullet}$; combined treatment in p53-WT cells was most effective. Increased $NO^{\bullet}$ production after treatment uniformly stimulated p53 and Bax expression through Mdm2 down-regulation in p53-WT cells, whereas all were unaffected in p53-null cells. Both cell types underwent a reduction in the levels of anti-apoptotic Bcl-2 protein, cytochrome c loss from mitochondria and activation of caspase 9 together with caspase 3, independently of p53 status. Concomitantly, we observed DR4, Fas(CD95) and caspase 8 activation, suggesting that these compounds activate both the mitochondrial and death receptor pathways working together to induce apoptosis. These findings provide insight into the mechanism of apoptotic action of capsaicin and resveratrol based on p53 status and indicate manipulation of $NO^{\bullet}$ may offer exciting opportunities to improve the effectiveness of colon cancer treatment.*

Colon cancer is a leading cause of cancer mortality in western countries and is increasing in prevalence in Asia (1-3). Current evidence indicates that dysregulated production of $NO^{\bullet}$ is an important factor in the etiology of this disease (4, 5). Modalities for treating or preventing this disease are limited, and potential alternatives that have been suggested include naturally occurring compounds of various types (6-8). Capsaicin and resveratrol are prominent examples of phytochemicals that are candidates for potential use as cancer chemopreventive or chemotherapeutic agents.

Capsaicin (*N*-[(4-hydroxy-3-methoxy-phenyl)methyl]-8-methyl-non-6-enamide), a principal ingredient of hot peppers of the genus *Capsicum*, possesses substantial antimutagenic and anticarcinogenic activities (7, 9). It preferentially represses growth of immortalized and malignant cells (10) and induces apoptosis in transformed cells (11-13), although mechanisms involved are incompletely characterized. Resveratrol (*trans*-3,5,4'-trihydroxystilbene) a polyphenolic phytoalexin found in grapes, red wine and other plant foods is an effective chemopreventive agent in multiple murine models of human cancers, affecting tumor initiation, promotion and progression (14). Extensive studies in human cell cultures reveal that it exerts antiproliferative and proapoptotic actions on different cancer cell types through diverse mechanisms including antioxidant effects, growth factor and hormone receptor binding, and direct or indirect interactions with nucleic acids (14). However, these effects are cell type-specific and the underlying molecular mechanisms are not fully understood.

Capsaicin stimulates inducible NOS in rat pheochromocytoma, PC-12 (15), and C6 glioma (11) tumor cells. Certain *in vivo* responses to resveratrol are mediated by $NO^{\bullet}$, including protection of the heart, kidney and brain from ischemic-reperfusion injury through up-regulation of $NO^{\bullet}$ production (16, 17). *In vitro* studies also show that resveratrol can induce apoptosis through elevation of $NO^{\bullet}$ production in MCF-7 human breast cancer cells (18) and exert antiproliferative action on HepG2 hepatocellular carcinoma cells by NOS activation (19). Based on the collective evidence that capsaicin and resveratrol share the ability to stimulate $NO^{\bullet}$ production in certain cell types, we hypothesized that they may act synergistically in the induction of apoptosis *via* this pathway.

Correspondence to: Professor G.N. Wogan, Department of Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, U.S.A. Tel: +1 6172533188, Fax: +1 6172589733, e-mail: wogan@mit.edu

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To test this hypothesis, we examined mechanisms of death signaling induced by capsaicin and resveratrol with special emphasis on the role of $NO^{\bullet}$, employing HCT116 human colon carcinoma cells. In addition, wild-type p53 (p53-WT) and p53-null cells were used to evaluate the influence of p53 status, given its importance as a genomic sensor implicated in activation of the intrinsic apoptosis pathway.

Materials and Methods

Cell cultures and chemicals. Two isogenic HCT116 colon carcinoma cells (p53-WT and p53-null), kindly provided by Dr. C.C. Harris (National Cancer Institute, NIH), were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine at 37˚C with 5% CO_2 in a humidified atmosphere. Sources of reagents were as follows: cell culture reagents, Lonza (Walkersville, MD, USA); capsaicin, resveratrol, Ac-DEVD-CHO and GenElute™ mammalian genomic DNA miniprep kit, Sigma Chemical (St. Louis, MO, USA); *N*-methyl-L-arginine monoacetate (NMA), CalBiochem (Salt Lake City, UT, USA); total $NO^{\bullet}$ quantitative detection kit, R&D Systems (Minneapolis, MN, USA); annexin V-FITC apoptotic assay kit, Clontech Laboratories (Palo Alto, CA, USA); mitochndrial/cytosol fractionation kit, Biovision (Mountain View, CA, USA); ECL™ western blotting detection reagents, GE Healthcare Bio-Sciences (Piscataway, NJ, USA); ApoAlert caspase-3 colorimetric assay kit, Clontech Laboratories (Mountain View, CA, USA); RIPA lysis buffer, anti-NOS1 (neuronal NOS, nNOS) antibody, anti-NOS2 (iNOS) antibody, anti-NOS-3 (endothelial NOS, eNOS) antibody, anti-bcl-2 antibody, and secondary goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase, Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-Bax antibody, anti-caspase 8 antibody and anti-caspase 3 antibody, Cell Signaling Technology (Beverly, MA, USA); anti-DR4 antibody and anti-Fas antibody, StressGen Biotechnologies Corp (Victoria, BC, Canada); anti-caspase 9 antibody, anti-Mdm2 antibody and anti-cytochrome c antibody, BD PharMingen (San Diego, CA, USA); and anti-p53 antibody and anti-actin antibody, Oncogene (Cambridge, MA, USA).

Cytotoxicity assay. Cytotoxicity 24 h after treatment was determined by trypan blue exclusion to determine dose-response and time-dependent effects of capsaicin and resveratrol on colon carcinoma cells. Cells were treated with capsaicin or resveratrol (0-200 μM) for 24 h and were then incubated with concentration which showed around 35% relative survival of capsaicin or resveratrol, or to a mixture of the two for different periods of time (0-48 h).

Determination of $NO^{\bullet}$ production rate. After each period of exposure, total $NO^{\bullet}$ production was measured with kits quantifying nitrate (NO_3^-) plus nitrite (NO_2^-) concentrations in cell supernatants. Briefly, 50 μL of supernatant was allowed to react with 100 μL of Griess reagent and incubated at room temperature for 10-30 min. NADH and NO_3^- reductase were added prior to reaction with Griess reagent. Optical density was measured using a microplate reader at 540 nm, with fresh culture media serving as the blank. Total $NO^{\bullet}$ concentrations were calculated from standard curves derived from NO_3^- standard solution provided with the kit, and $NO^{\bullet}$ production rate was expressed in pmoles per 10^6 viable (trypan blue-excluding) cells per second.

Apoptosis analysis. Three million cells were incubated in a 60-mm tissue culture dish containing capsaicin and/or resveratrol. Cells were harvested by trypsinization and centrifugation, then analyzed in a Becton Dickinson FACScan (excitation at 488 nm) equipped with CellQuest software after staining with annexin V-FITC and propidium iodide according to a previously described (20) protocol. Apoptotic cells stained with annexin V (early apoptosis) or with both annexin V and propidium iodide (late apoptosis), necrotic cells stained with propidium iodide, and living cells did not contain either stain. Cells treated with DMSO and 50 μM etoposide were used as negative and positive controls, respectively.

DNA fragmentation. For analysis of DNA fragmentation by agarose gel electrophoresis, total DNA was isolated from cells treated with capsaicin and/or resveratrol, using a GenElute™ mammalian genomic DNA miniprep kit. Isolated DNA was suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 9.0) and quantified by absorbance at 260 nm. Fragmented DNA was loaded onto 1.8% agarose gel containing 1 × TBE buffer and separated by electrophoresis for 2 h at 50 V, then photographed after staining with 0.5 ng/mL ethidium bromide.

Whole cell extract and mitochondria-free cytosolic fraction preparation. Cells were harvested after treatment and lysed in 450 μL of ice-cold RIPA lysis buffer [1 × TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1 mM phenylmethylsufonyl fluoride (PMSF), sodium orthovanadate supplemented with 20 μg/mL protease inhibitor cocktail; Santa Cruz Biotechnology Inc.] for 30-60 min on ice, centrifuged at 10,000 × g for 10 min at 4˚C. Protein concentration in the resulting supernatant was measured by the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA) before Western blot analysis.

Cytosolic fractions from control and treated cells were prepared using a kit from BioVision. Briefly, cells (3×10^6) were treated, collected, pelleted by centrifugation and washed with ice-cold phosphate-buffered saline (PBS). They were then suspended in cytosol extraction buffer (as supplied by the manufacturer), incubated on ice for 10 min, then homogenized using an ice-cold Dounce tissue grinder. The homogenate was centrifuged at 10,000 × g for 30 min to separate cytosol and mitochondria, and the cytosolic supernatant fraction was collected and stored at –80˚C for cytochrome c analysis.

Western blot analysis. About 60 μg of protein from whole-cell lysate or cytosolic fractions were denatured, separated electrophoresis on 15% (or 7.5% for NOS isoforms) SDS-PAGE gels and blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked with 5-7% (w/v) nonfat dry milk in Tris-buffered saline/0.1% Tween-20 (TBS/T, pH 7.6) for 1-3 h at room temperature, then incubated at 4˚C overnight with antibodies, diluted as indicated, against the following : NOS1 (1:500); NOS2 (1:500); NOS3 (1:1000); p53 (1:1000); Mdm2 (1:1000); Bax (1:1000); bcl-2 (1:1000); DR4 (1:1000); Fas (CD95) (1:2000); caspase 8 (1:1000); caspase 9 (1:1000); cytochrome *c* (1:2000); caspase 3 (1:1000), or actin (1:8000). After two 10 min washes with TBS/T, blots were incubated with the corresponding peroxidase-conjugated secondary goat anti-rabbit or mouse IgG (diluted 1:8000) for 1 h at room temperature, followed by washing two times for 5 min and four times for 10 min. Enhanced chemiluminescence was detected by exposure to Hyperfilm ECL.

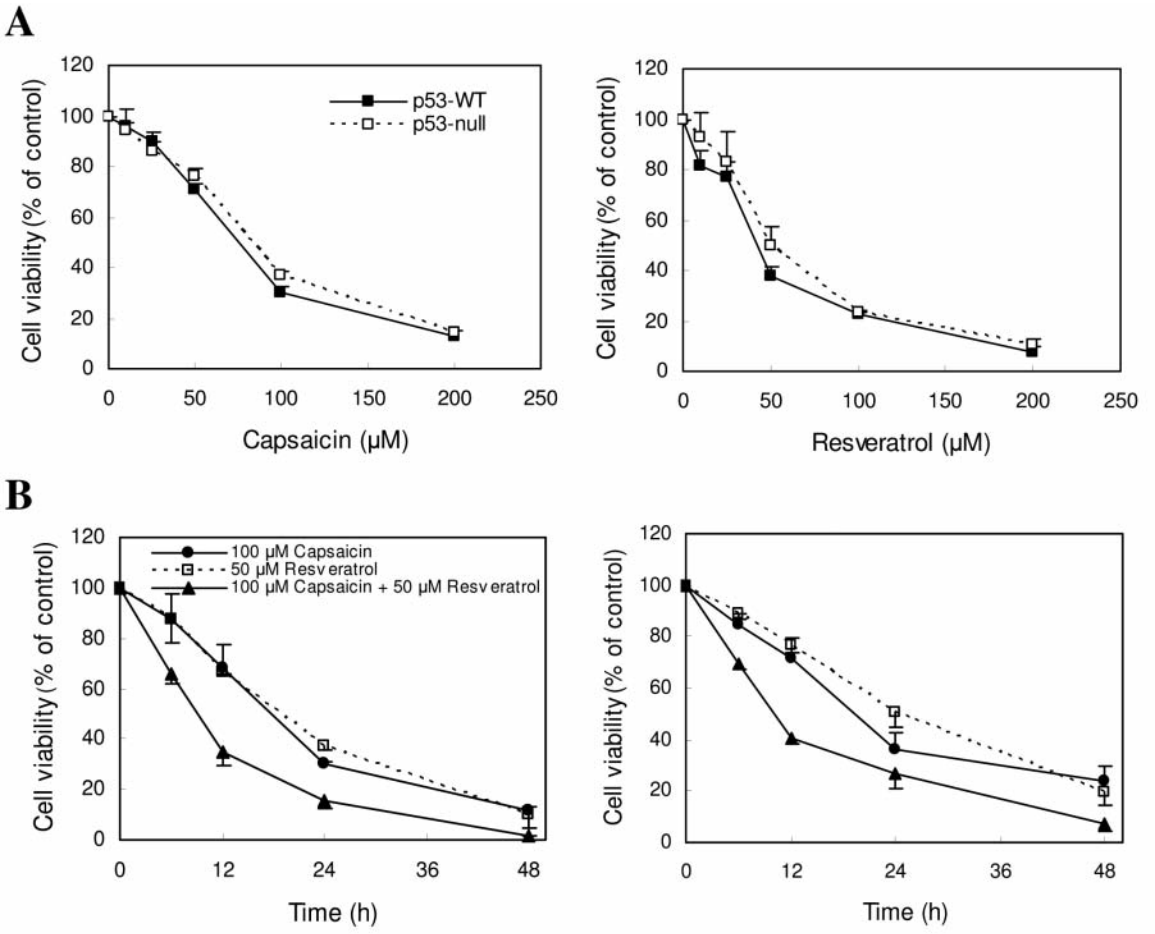

Figure 1. *The percentage of viable p53-WT and p53-null HCT116 cells after capsaicin and/or resveratrol treatment. The cells were treated with the indicated concentrations for 24 h (A), or 100 μM of capsaicin and/or 50 μM of resveratrol for 6, 12, 24 and 48 h (B). Each point is the mean±SD of three experiments.*

Caspase-3 activity assay. Caspase-3 activity of treated cells was determined using an ApoAlert caspase-3 colorimetric assay kit. Two million cells were centrifuged at 400 × g for 5 min, resuspended in 50 mL of chilled cell lysis buffer, and incubated on ice for 10 min. Cell lysates were centrifuged at 16,000 × g for 10 min at 4˚C to precipitate cellular debris. To each supernatant were added 50 μL of 2 × reaction buffer/DTT Mix and 50 μM of caspase 3 substrate DEVD-pNA, after which samples were incubated at 37˚C for 1-3 h in a water-bath, and read at 405 nm using a μQuant plate reader from Biotek Instruments Inc. (Winooski, VT, USA).

Statistical analysis. The two-tailed Student's *t*-test was used for statistical comparisons of responses in capsaicin- and/or resveratrol-treated and control groups.

Results

Loss of cell viability. Cells treated with 0, 10, 25, 50, 100 and 200 μM of capsaicin or resveratrol for 24 h responded similarly, with respect to viability, in that both treatments reduced the percentage of viable cells dose-dependently (Figure 1A). Exposure to 100 μM capsaicin for 24 h, reduced viability in both cell types to 30 and 37%, respectively, whereas comparable values after treatment with 50 μM resveratrol for 24 h, were 38 and 50% (Figure 1A).

We next assessed the time-course of responses to doses causing 35% of relative survival rate, *i.e*. 100 μM capsaicin, 50 μM resveratrol and a mixture of the two, in cells treated for 6, 12, 24 and 48 h (Figure 1B). As expected, viability decreased in a time-dependent manner, and the combination was more effective than either agent alone (Figure 1B).

Expression of NOS isoenzyme proteins and total $NO^{\bullet}$ production. Western blot analysis showed that all untreated cells contained nNOS, iNOS and eNOS proteins at different concentrations, and that both cell types responded differently to treatment with capsaicin and/or resveratrol (Figure 2A). In p53-WT cells, resveratrol, capsaicin and the mixture induced increases in each NOS isoform, with apparently equal potency; however, the response of eNOS to each agent

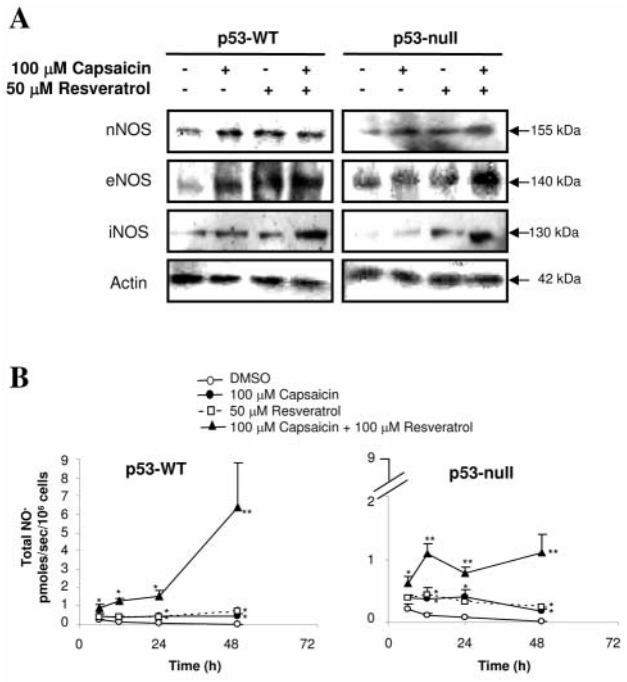

Figure 2. *Effect of capsaicin and/or resveratrol on the expression of NOS isoforms (A) and total NO• production (B) in p53-WT and p53-null HCT116 cells. The results are shown as the mean±SD (n=3). *p<0.05 and **p<0.01, as compared with DMSO control.*

was greater than that of the other isoforms. The p53-null cells showed prominent responses of nNOS and eNOS to all treatments, including more pronounced increases in eNOS and iNOS to the combination treatment.

Rates of total NO• production were calculated from concentrations of NO_3^- plus NO_2^- in culture media following treatment for 6 to 48 h. As summarized in Figure 2B, time-dependent increases in rates of total NO• production were observed in each cell type in response to all treatments. Cells treated with either capsaicin or resveratrol alone for 48 h showed 10- to 40-fold increases in NO• production rate, as compared with controls, and the increases were further enhanced by combination treatment. p53-WT cells produced NO• at higher rates than p53-null cells (Figure 2B); for example, the rate in p53-WT cells (1.55 pmoles/s/10^6 cells) was twice that of p53-null cells (0.78 pmoles/s/10^6 cells) after 24 h of combined treatment.

NMA reduces loss of viability induced by capsaicin and/or resveratrol. As shown in Figure 2, capsaicin and/or resveratrol were potent NO• inducers. To assess the contribution of NO• to the growth inhibition caused by these treatments (Figure 1), we incubated treated cells with 1 or 2 mM NMA, an NOS inhibitor. Increasing concentrations of NMA resulted in a dose-dependent attenuation of the loss of viability induced by capsaicin and/or resveratrol (Table I), confirming that NO• or its products were major contributors to the cytotoxicity of these agents.

Table I. *Effect of NMA on cell viability by capsaicin and/or resveratrol treated for 48 h.*

Group	p53-WT	p53-null
DMSO	100	100
+ 1 mM NMA	100.6±9.33	96.2±3.36
+ 2 mM NMA	99.9±4.98	98.3±3.04
100 μM Capsaicin	34.9±6.46**	31.7±6.73**
+ 1 mM NMA	67.6±12.08*	54.2±1.84**
+ 2 mM NMA	68.1±15.40*	71.7±7.45
50 μM Resveratrol	32.0±3.65**	38.6±8.35**
+ 1 mM NMA	61.6±7.62	45.8±1.84*
+ 2 mM NMA	77.8±9.70*	88.0±4.07*
100 μM Capsaicin + 50 μM Resveratrol	10.1±2.44**	12.0±4.07**
+ 1 mM NMA	14.8±1.94**	20.2±5.60**
+ 2 mM NMA	29.4±4.57**	40.0±11.20*

Results are presented as a percentage of control cells (mean±SD, n=3); *p<0.05 and **p<0.01, as compared with DMSO control.

Table II. *Induction of apoptosis (%) by capsaicin and/or resveratrol treated for 48 h.*

Group	p53-WT	p53-null
DMSO	6.7±0.31	10.6±0.96
100 μM Capsaicin	14.4±1.91*	12.8±2.23
50 μM Resveratrol	23.0±7.12*	24.1±1.53*
100 μM Capsaicin + 50 μM Resveratrol	49.1±7.05**	30.6±5.89*
50 μM Etoposide	15.9±2.17*	15.4±0.03*

Results are presented as a percentage of control cells (mean±SD, n=3); *p<0.05 and **p<0.01, as compared with DMSO control.

Apoptosis identified by flow cytometric analysis. Table II shows that capsaicin and/or resveratrol induced apoptosis in each of the cell types. Approximately 14% of p53-WT cells were apoptotic after capsaicin treatment and 23% after resveratrol treatment (2.1- and 3.4-fold, respectively, over control level), whereas p53-null cells were less responsive (1.2- and 2.3-fold increases) (Table II). A stronger apoptotic response was induced by combination treatment than by capsaicin or resveratrol alone, inducing maximum frequencies of 31-49% in both cell lines (1.6-3.4-fold elevation over controls) (Table II).

Internucleosomal DNA fragmentation induced. Since a major biochemical feature of apoptosis is fragmentation of the genomic DNA, we analyzed genomic DNA after treating the

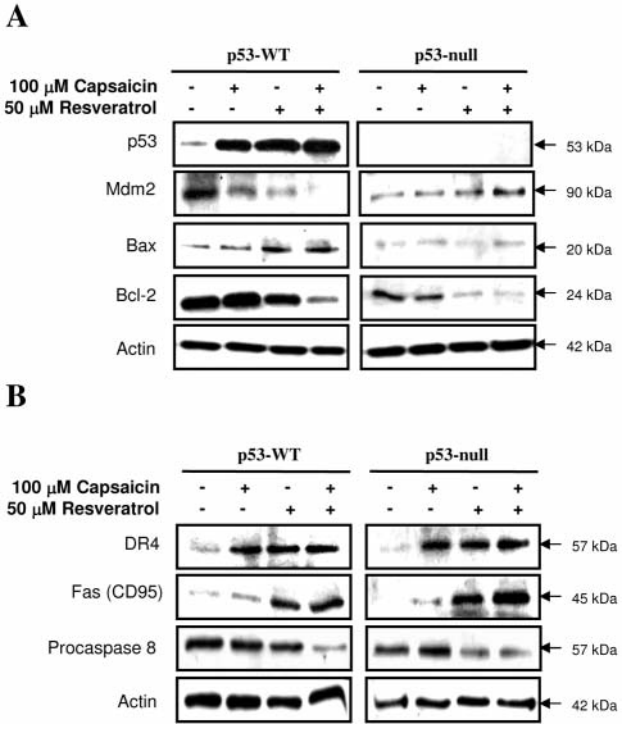

Figure 3. *Representative Western blot showing changes in the levels of p53, Mdm2, Bax and Bcl-2 (A), and DR4, Fas(CD95) and caspase-8 (B) in p53-WT and p53-null HCT116 cells. Cell were treated with capsaicin and/or resveratrol for 48 h and cell lysates were prepared and subjected to Western blot analysis using specific antibodies as described in Materials and methods. Equal protein loading was confirmed by reaction with actin antibody. The results are representative of two independent experiments.*

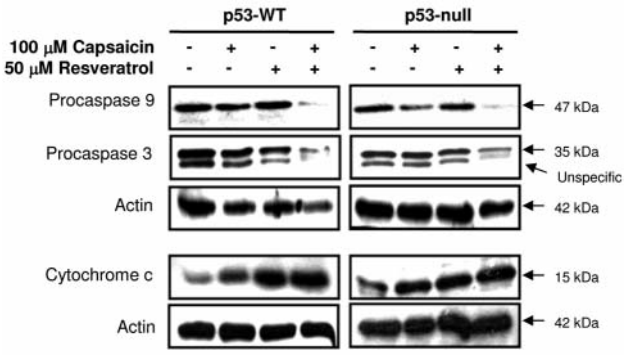

Figure 4. *Participation of caspases in apoptosis in p53-WT and p53-null HCT116 cells. Effect of capsaicin and/or resveratrol on degradation of procaspases-9 and -3, and the release of cytochrome c from mitochondria into cytosol. Equal protein loading was confirmed by reaction with actin antibody. The results are representative of two independent experiments.*

cells for 48 h. All treatments induced DNA ladder formation, and combined treatment was more effective than either agent alone, confirming that apoptosis was a major contributor to cell death induced by these compounds (data not shown).

Altered expression of proteins regulating intrinsic and extrinsic apoptotic pathways. We further investigated mechanisms underlying cell death induced by capsaicin and resveratrol, with a focus upon proteins regulating the apoptotic process. We first examined expression of p53, Mdm2, Bax, and Bcl-2 proteins. Treatment of the cells for 48 h led to a substantial elevation of p53, accompanied by a reciprocal decrease of Mdm2 in p53-WT cells (Figure 3A). The highest p53 and lowest Mdm2 levels were caused by the combination treatment. The *Bax* gene, a proapoptotic member of the Bcl-2 family, is an important target for p53. Our results showed a slight elevation of Bax protein and a pronounced down-regulation of the anti-apoptotic Bcl-2 protein in p53-WT cells following capsaicin and/or resveratrol treatment (Figure 3A).

Conversely, treatment of p53-null cells caused no changes in Mdm2 or Bax expression, but a decrease in Bcl-2 expression (Figure 3A), indicating that a Bax- and a p53-independent down-regulation of Bcl-2 played a role in apoptosis induced under these conditions.

To evaluate the role of the extrinsic apoptotic pathway in treatment-induced cell death, we analyzed the activation of DR4, Fas(CD95) and caspase 8. Western blot analyses summarized in Fig. 3B show increases in DR4 and Fas(CD95), and a decrease in procaspase 8 proteins after treatment. In general, maximal and minimal levels of these proteins following combined treatment were similar to those of the intrinsic pathway proteins. Increased protein degradation of the cleaved form of caspase 8 in both cell types could explain the lack of this active form (43 and 18 kDa) in the Western blot analysis. These findings suggest that both intrinsic and extrinsic apoptotic pathways contribute to cell death induced by capsaicin and/or resveratrol treatment.

Activation of the caspase-dependent cascade (caspase-9, cytochrome c and caspase-3). Because many stress stimuli lead to caspase activation, we evaluated the role of this process in apoptotic death induced by the treatment regimen. Both cell types responded with a decrease in the level of the precursor form of caspase-9 (Figure 4), and mitochondrial release of cytochrome *c*. Western blot analyses showed that degradation of procaspase-3 was accompanied by a significant increase of caspase-3 activity in a colorimetric assay based on the cleavage of the synthetic peptide Ac-DEVD-pNA (Table III). Caspase-3 activity was highest in p53-WT cells following the combination treatment. To investigate the significance of caspase 3 activity in treatment-induced growth inhibition,

we pretreated cells with the caspase 3 inhibitor, Ac-DEVD-CHO. This agent substantially attenuated growth inhibition, as shown in Table IV, supporting the involvement of caspase 3 in this response.

Discussion

Previous studies of the induction of apoptosis by capsaicin and resveratrol have produced contradictory evidence regarding signaling events involved. For instance, it has been variously reported that cell death was caspase activation dependent (18, 21) or independent (22); cytochrome *c* release, p53 and Bax dependent (23) or independent (24); and death receptor dependent (25). Whereas decreased Bcl-2 levels are in many cases associated with cytochrome *c* release from mitochondria and caspase-mediated apoptosis, in MCF-7 cells, resveratrol failed to cause cytochrome *c* leakage into the cytosolic compartment even at high doses (18). These observations suggest that the cytotoxicity of capsaicin and resveratrol is influenced by multiple factors, including concentration, duration of exposure, availability and efficiency of antioxidant capacity, and cell type.

The aim of this study work was to dissect biochemical and molecular events associated with cell death induced by these agents in HCT116 colon carcinoma cells. Our results clearly demonstrate that they inhibited growth and induced cell death in p53-WT and p53-null cells, alone or in combination (Figure 1). Interestingly, they also show that they caused up-regulation of endogenous NOS activity and, in turn, an increase in $NO^{\bullet}$ production leading to cell death (Figures 1 and 2). Combination treatment induced the highest level of NOS expression and 6- to 15-fold higher $NO^{\bullet}$ production rates over 48 h, compared with treatment with either agent alone (Figure 2). Moreover, cells exposed to the NOS inhibitor NMA grew at rates similar to those of untreated controls (Table I), confirming that the induced increase in $NO^{\bullet}$ production was a critical determinant of cell viability. DNA fragmentation as well as flow cytometric analysis showed that apoptosis was a major contributor to cell death induced in both cell types (Table II); nearly half of p53-WT and 31% of p53-null cells stained with PI and annexin V 48 h after combination treatment (Table II).

Conflicting evidence exists regarding the ability of capsaicin and resveratrol to modulate $NO^{\bullet}$ production. While some investigators found that they enhance $NO^{\bullet}$ production, others observed suppression. For example, capsaicin was reported to promote iNOS expression and $NO^{\bullet}$ production, leading to inhibited proliferation of PC-12 (15) and C6 glioma (11) cells. Resveratrol was shown to increase $NO^{\bullet}$ production and inhibit cell proliferation of MCF-7 human breast cancer cells (18), HepG2 hepatocellular carcinoma cells (19) and gastric adenocarcinoma (26) cells. On the other hand, it was reported that capsaicin (27-29) and resveratrol (30) inhibited IFN-γ and LPS-mediated $NO^{\bullet}$ production and iNOS protein expression in RAW264.7 macrophages, and resveratrol reduced $NO^{\bullet}$ production in leukemic cells (31). Our data indicate a direct relationship between increased $NO^{\bullet}$ production and loss of cell viability (Figures 1 and 2, and Table II) caused by both resveratrol and capsaicin.

Because mechanisms through which these compounds induce cell death are incompletely understood, we undertook examination of their effects on apoptotic signaling pathways. The intrinsic and extrinsic apoptosis pathways, shared by all mammalian cells, both converge on activation of initiator and effector caspases (32-34). The intrinsic pathway is dependent on release of mitochondrial cytochrome *c* and other proapoptotic molecules into the cytoplasm. Cytochrome *c* associates with Apaf1 to form the apoptosome, which activates caspase-9 and this in turn activates downstream caspases (32-34). In the extrinsic pathway, binding of specific death ligands to their respective cell surface receptors, *e.g.* Fas (to CD95), TNF (to TNFR) and TRAIL (to DR4 and DR5), actives downstream pathways by recruiting initiator caspase-8 which then cleaves effector procaspases (32, 33). Wild-type p53 is

Table III. *Caspase 3 activity by capsaicin and/or resveratrol treated for 48 h.*

	p53-WT	p53-null
DMSO	100	100
100 μM Capsaicin	206.8±76.68*	166.9±8.67*
50 μM Resveratrol	259.7±62.88*	228.8±40.02*
100 μM Capsaicin + 50 μM Resveratrol	520.2±81.59**	413.9±5.55**

Results are presented as a percentage of control cells (mean±SD, n=3); *$p<0.05$ and **$p<0.01$, as compared with DMSO control.

Table IV. *Total number of cells by capsaicin and/or resveratrol treated for 48 h in the absence or presence of caspase 3 inhibitor, Ac-DEVD-CHO, pre-incubated for 1 h.*

	p53-WT	p53-null
DMSO	100	100
100 μM Capsaicin	42.8±7.70**	53.1±0.58**
+ Ac-DEVD-CHO	69.6±14.16*	79.0±3.08*
50 μM Resveratrol	36.2±5.50**	47.1±4.35**
+ Ac-DEVD-CHO	64.6±7.10*	68.0±2.09*
100 μM Capsaicin + 50 μM Resveratrol	7.5±3.50**	11.0±3.19**
+ Ac-DEVD-CHO	23.4±2.27**	30.6±3.86**

Results are presented as a percentage of control cells (mean±SD, n=3); *$p<0.05$ and **$p<0.01$, as compared with DMSO control.

responsible for sensing DNA damage and increases cell passage into to apoptosis, whereas mutated p53 or p53 deletion renders cells less sensitive (32). In part, p53 exerts this influence on apoptosis by regulating expression of Bcl-2 family proteins that modulate the mitochondrial pathway (32-34). Accordingly, our results show that p53-null cells treated with capsaicin and/or resveratrol were more resistant to apoptosis than p53-WT cells (Table II), reflecting the importance of p53 in this process.

In order to understand more fully p53-related mechanisms involved in the apoptotic response to the compounds being studied, we examined expression of p53, Mdm2 and members of the Bcl-2 family (Figure 3A). Treatment uniformly stimulated p53 and Bax expression, whereas protein expression of the the p53-regulator gene *Mdm2* was distinctly down-regulated in p53-WT cells. In contrast, Mdm2 and Bax proteins were unaffected by treatment in p53-null cells. Interestingly, down-regulation of Bcl-2, an anti-apoptotic p53-downstream target, was also detected in both cell types irrespective of p53 status (Figure 3A). These results agree with previous findings that decreased Bcl-2 expression similarly enhanced apoptosis induced by these agents in other tumor cell types (11, 35, 36).

Caspases-8 and -9 are initiators that transduce apoptotic signals by directly activating the downstream executioner, caspase-3 (32-34). Our experiments showed that treatment with capsaicin and/or resveratrol activates caspase-9 and caspase-3, causes cytochrome *c* loss from mitochondria, activates caspase-8 and up-regulates DR4 and Fas(CD95) (Figures 3B and 4). The caspase-3 inhibitor, Ac-DEVD-CHO, restored normal growth in treated cells (Table IV). Thus, they induce apoptosis through both the intrinsic and extrinsic pathways (Figure 3). Compared to capsaicin or resveratrol alone, a combination of both compounds was more effective in several activities, including $NO^{\bullet}$ production, DNA fragmentation and induction of apoptosis. In addition, our experiments show that p53-WT cells were more sensitive to the induction of apoptosis than p53-null cells.

Taken together, our results indicate that apoptotic responses induced by capsaicin and resveratrol in HCT116 cells are mediated by stimulation of endogenous $NO^{\bullet}$ production by the tumor cells, and involves both extrinsic and intrinsic pathways. While our findings may suggest a potential use of these compounds for colon cancer chemoprevention, further research will be required to define more specifically the mechanisms through which they act. Recognition that $NO^{\bullet}$ is a critical intermediate will facilitate progress towards that goal.

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