JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Resveratrol Induces Apoptosis through ROS-Dependent Mitochondria Pathway in HT-29 Human Colorectal Carcinoma Cells

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trans-Resveratrol is a polyphenol found in blueberries, grapes, and wine with cancer chemopreventive properties. The low bioavailability of this compound enhances its concentration in the luminal content and becomes a potential chemopreventive agent against colon cancer. In the present study, the antiproliferative and pro-apoptotic effects on the human colorectal carcinoma HT-29 cells as well as the mechanisms underlying these effects were examined. Proliferation, cytotoxicity, and apoptosis were measured by fluorescence-based techniques. Studies of dose-dependent effects of *trans*-resveratrol showed antiproliferative activity with an EC_{50} value of 78.9 ± 5.4 μM. Caspase-3 was activated in a dose-dependent manner after incubation for 24 h giving an EC_{50} value of 276.1 ± 1.7 μM. Apoptosis was also confirmed with microscopic observation of changes in membrane permeability and detection of DNA fragmentation. The activity of *trans*-resveratrol on the mitochondria apoptosis pathway was evidenced by the production of superoxide anions in the mitochondria of cells undergoing apoptosis. In conclusion, *trans*-resveratrol inhibits cell proliferation without cytotoxicity and induces apoptosis in HT-29. Results of the present study provide evidence demonstrating the antitumor effect of *trans*-resveratrol via a ROS-dependent apoptosis pathway in colorectal carcinoma.

KEYWORDS: Resveratrol; proliferation; apoptosis; caspase-3; ROS; colon cancer

INTRODUCTION

In recent years, the growing interest in the role of nutrition in the prevention of cancer has prompted the identification of specific diet constituents with chemotherapeutic properties (*1*). Among the dietary bioactive compounds stands out *trans*-resveratrol, which belongs to a class of defense molecules called phytoalexins. These are produced by several plants in response to stress, injury, UV radiation, and fungal infections. *trans*-Resveratrol is normally found in dietary products such as grapes (50−100 μg/g), red wine (0.1−14.3 mg/L), peanuts (0.02−1.92 μg/g), and various berries (0.07−5.8 μg/g) (*2*).

trans-Resveratrol is gaining acceptance as a potential antitumor agent because of its pleitropic effects described in different experimental models of carcinogenesis (*3, 4*). This bioactive compound was shown to inhibit the growth of tumor cell lines derived from various human cancers (*5–8*). This effect has been associated with the ability of resveratrol to arrest cell cycle progression (*5, 9*) and to induce programmed cell death (*6, 10, 11*). These properties accompanied with the lack of harmful effects (*12*) makes *trans*-resveratrol an attractive chemotherapy and chemopreventive drug for cancer treatment (*3*).

Despite the numerous studies describing the intracellular changes leading to cell cycle arrest or apoptosis in response to *trans*-resveratrol treatment, the effects are often cell type specific, and the underlying mechanism of action remains to be fully identified (*4*). Several studies have indicated that reactive oxygen species (ROS) production in mitochondria may mediate apoptosis induction (*13, 14*). The roles of ROS and mitochondria in *trans*-resveratrol induced apoptosis are still undefined. Consequently, the present study examines the antiproliferative and pro-apoptotic effects on the human colorectal carcinoma HT-29 of *trans*-resveratrol and elucidates the possible involvement of ROS in the antitumoral activity of this promising bioactive compound.

MATERIAL AND METHODS

Chemicals and Reagents. *trans*-Resveratrol (Sigma, Tres Cantos, Madrid, Spain) was chemically pure. Before use, its purity was assessed by HPLC coupled to a diode-array UV detector, and a chromatogram

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10.1021/jf800175a CCC: $40.75
Published on Web 06/04/2008

that showed a single peak at 306 nm, its maximum absorbance, was obtained. The use of a diode-array UV detector allowed the confirmation of the identity of the peak by its spectrum (data not shown). The preparations of the medium containing this compound as well as all cell culture treatments were performed in dim light to avoid photochemical isomerization of *trans*-resveratrol to the *cis* form. Media and supplements for cell culture were from Invitrogen (Karslruhe, Germany). Cell culture plates were from Renner (Dannstadt, Germany), and SYTOX-Green was obtained from Bioprobes (Leiden, Netherlands). The fluorogenic caspase-3 substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-amino-4-methyl-coumarine (Ac-DEVD-AMC) was obtained from Calbiochem (Bad Soden, Germany). All other reagents were commercially available, analytical grade chemicals.

Cell Culture. HT-29 cells (passage 106) were provided by American type Culture Collections and were used between passages 150 and 200. Cells were cultured and passaged in RPMI 1640, supplemented with 100 mL/L fetal calf serum and 2 mM glutamine. Antibiotics added to the media were 100,000 U/L penicillin and 100 mg/L streptomycin. The cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Fresh medium was given every second day and on the day before the experiments were done. Cells were passaged at preconfluent densities, using a solution containing 0.05% trypsin and 0.5 mM EDTA. *trans*-Resveratrol was dissolved in dimethyl sulfoxide (DMSO), and all cells received DMSO to a final concentration of 2% (v/v). Controls were always treated with the same amount of DMSO.

Necrosis. The potential nonspecific toxicity of *trans*-resveratrol in HT-29 cells was determined as described previously (*15*). Cells were seeded at a density of $5 \cdot 10^4$ cells/well onto 24-well cell culture plates and allowed to adhere for 4 h. Subsequently, the medium was replaced by a fresh one, and the cells were exposed to 1, 10, 25, 50, 75, 100, 150, 200, 250, 300, and 400 μM of this polyphenol for 3 h. Necrotic cell death was evaluated with SYTOX-Green, which becomes fluorescent after DNA binding. The percentage of dead cells was determined by comparing SYTOX-fluorescence prior to cell lysis with the fluorescence measured after the cells were solubilized with 1% (v/v) Triton X-100 in isotonic NaCl. Cell numbers were determined on the basis of a calibration curve. The calibration curve was measured using cell numbers between $1 \cdot 10^3$ and $1.5 \cdot 10^5$ cells, which had been adjusted after determining the cell numbers in a Neubauer chamber. Fluorescence of the corresponding cell numbers were measured at 538 nm, after excitation at 485 nm using a fluorescence multiwell plate reader (Fluoroskan Ascent, Thermo Electron, Dreieich, Germany).

Cell Proliferation. Cell proliferation was measurement following the method described previously (*15*). HT-29 cells were seeded at a density of $5 \cdot 10^3$ cells/well onto 24-well cell culture plates and allowed to adhere for 24 h. After that, the medium was substituted by a fresh one containing 1, 10, 25, 50, 75, 100, 150, 200, 250, 300, and 400 μM of *trans*-resveratrol, and cells were allowed to grow for another 72 h. Total cell counts were determined by SYTOX-Green that becomes fluorescent after DNA binding. Therefore, cells were then lysed with 1% Triton X-100 in isotonic NaCl, and DNA was stained with SYTOX-Green. Cell numbers were measured using the fluorescence multiwell plate reader.

Caspase-3-Like Activity. Caspase-3-like activity was measured according to the method described previously (*16*). This activity was used as an early apoptosis marker. Briefly, cells were seeded at a density of $5 \cdot 10^5$ per well onto 6-well plates and allowed to adhere for 24 h. The time course of caspase-3 activation was evaluated in an initial set of experiments. Cells were exposed to 150 and 250 μM of *trans*-resveratrol for 3, 8, 12, 24, 36, and 48 h. Thereafter, cells were trypsinized once the incubation had finished. Cell numbers were then determined, and the cells were centrifuged at 2500*g* for 10 min. Cytosolic extracts were prepared by adding 750 μL of a buffer containing 2 mM EDTA, 1.63 μM 3-((cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS), 5 mM dithiotreitol (DTT), 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 10 mg/L pepstatin A, 20 mg/L leupeptin, 10 mg/L aprotinin, and 10 mM HEPES/KOH (pH 7.4) to each pellet and homogenizing with 10 strokes. The homogenate was centrifuged at 100,000*g* at 4 °C for 30 min, and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-amino-4-methylcoumarin at a final concentration of 20 μM. The caspase-3 substrate was cleaved. The emission at 460 nm was then determined, after excitation at 390 nm using the fluorescence plate reader.

The study of the time-dependent activation of caspase-3 indicated that 24 h was an adequate incubation time. Consequently, the dose-dependent activation of this caspase was evaluated after HT-29 cells were exposed to 10, 25, 50, 100, 150, 200, and 250 μM of *trans*-resveratrol for 24 h.

Membrane Permeability. Early changes in membrane permeability were detected subsequent to incubating $3 \cdot 10^4$ HT-29 cells/well on glass slides placed in Quadriperm wells following the method described previously (*17*). Cells were allowed to adhere for 24 h. Cells were incubated with 150 μM of *trans*-resveratrol for 8, 16, 20, and 24 h. At the end of the incubation, cells were stained with 1 mg/L Hoechst 33342, and the rate of accumulation of the dye in early apoptotic cells was detected using an inverted fluorescence microscope (Leica DMIRBE, Bensheim, Germany), equipped with a band-pass excitation filter of 340−380 nm and a long-pass emission filter of 425 nm. Photographs were taken of at least three independent cell batches, and images were evaluated on a blindly coded basis. Apoptotic cells were determined by the number of cells showing elevated fluorescence versus the total cell counts.

Nuclear Fragmentation. Nuclear fragmentation as a late marker of apoptosis was determined by staining DNA with Hoechst 33258 (*17*). HT-29 cells ($3 \cdot 10^4$ cells/well) were then incubated with 150 μM of *trans*-resveratrol for 8, 16, 20, and 24 h. Cells were washed with PBS, allowed to air-dry for 30 min, and fixed with 2% paraformaldehyde before staining with 1 mg/L Hoechst 33258. Images were evaluated on a blindly coded basis. Apoptotic cells were determined by the number of cells displaying chromatin condensation and nuclear fragmentation versus total cell counts.

Detection of Superoxide Radicals. The production of superoxide radicals in the mitochondria of HT-29 cells was visualized using a confocal laser scanning microscope (Leica TCS SP2, Bensheim, Germany) as described previously (*17*). Cells were seeded at a density of $3 \cdot 10^4$ per well on glass slides placed in Quadriperm wells. Cells were grown for 24 h to allow adhesion to the slides. Subsequently, the medium was substituted with a fresh one containing 150 μM of *trans*-resveratrol. Cells were incubated for 4 h, and 50 μM proxyl fluorescamine was loaded into the cells for the last 2 h of incubation. The aim was to determine the production of superoxide anions in the mitochondria. Mitochondria were stained with 500 nM MitoTracker Red CMXRos. This was loaded into the cells for the last 30 min of incubation. Superoxide radicals were detected after excitation with the UV-laser at emissions of 440−480 nm. Mitochondria were visualized after excitation at 543 nm, at emissions of 590−650 nm.

Statistical Analysis. Data were given as the mean ± SE. We applied a nonlinear approximation model, using the least-squares method, to derive the EC_{50} values for growth inhibition. This model was based on a competition curve using one component (Graph Pad Prism). Data were evaluated by one-way ANOVA and posthoc Tukey's Multiple Comparison tests (Graph Pad Prism). Statistical differences between time and dose were tested by two-way ANOVA and Bonferroni's post-test. At least three independent experiments were carried out for each variable. A $P < 0.05$ level was taken as significant.

RESULTS

***trans*-Resveratrol Does Not Exert Nonspecific Cytotoxicity.** HT-29 cells were exposed to increasing concentrations of *trans*-resveratrol for 3 h. Cell viability was unaffected (95.1 ± 0.2%) even for concentrations as high as 400 μM.

***trans*-Resveratrol Inhibits Cell Proliferation of HT-29 Human Colon Cancer Cells.** *trans*-Resveratrol induced growth inhibition in a dose-dependent manner (**Figure 1**). The concentration that induced a 50% inhibition of cell proliferation as compared to controls was 78.2 ± 4.7 μM.

***trans*-Resveratrol Is an Effective Apoptosis Inducer in HT-29 Cells.** The time course of activation of caspase-3 was

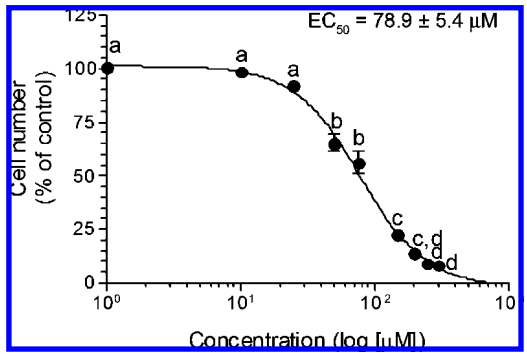

Figure 1. Effects of *trans*-resveratrol on proliferation in HT-29 cells. Proliferation was measured over 72 h in the absence (control) or presence of *trans*-resveratrol at 1, 10, 25, 50, 75, 100, 150, 200, 250, 300, and 400 μM. Values are means ± SE. Statistical differences between concentrations: a > b > c > d, where means without a common letter differ, $P < 0.05$.

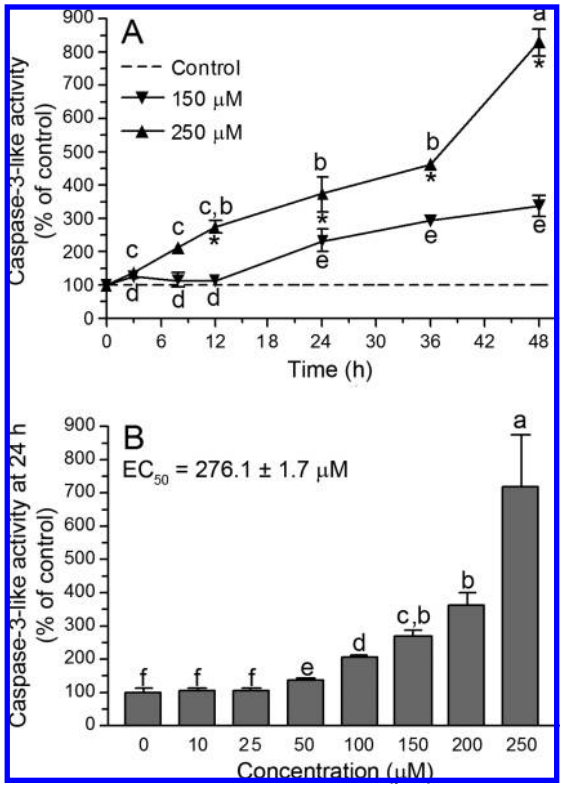

Figure 2. Effects of *trans*-resveratrol on caspase-3 activity in HT-29 cells. (**A**) Caspase-3-like activity was determined at 3, 8, 12, 24, 36, and 48 h of incubation with 150 and 250 μM of *trans*-resveratrol. Values are the means ± SE. Statistical differences between time: 150 μM, a > b > c; and 250 μM, e > d. Means without a common letter differ. *Statistical differences between concentrations, $P < 0.05$. (**B**) Caspase-3 activity determined after the incubation with 10, 25, 50, 100, 150, 200, and 250 μM of *trans*-resveratrol for 24 h. Values are means + SE; $n = 3$. Statistical differences between concentrations: a > b > c > d > e > f; $P < 0.05$.

assessed by exposing HT-29 cells to 150 or 250 μM of *trans*-resveratrol from 3 to 48 h. Caspase-3-like activity of control cells was set at 100% (*18*). Cells exposed to 150 μM of *trans*-resveratrol for 24, 36, and 48 h showed an increase in the activity of this protease of 250%, 300%, and 340% that of control cells ($P < 0.001$), respectively (**Figure 2A**). When HT-29 cells were incubated with 250 μM of *trans*-resveratrol, the increase in caspase-3-like activity started at 12 h, with a value of 275% above that in control cells. Treatment of HT-29 cells with this

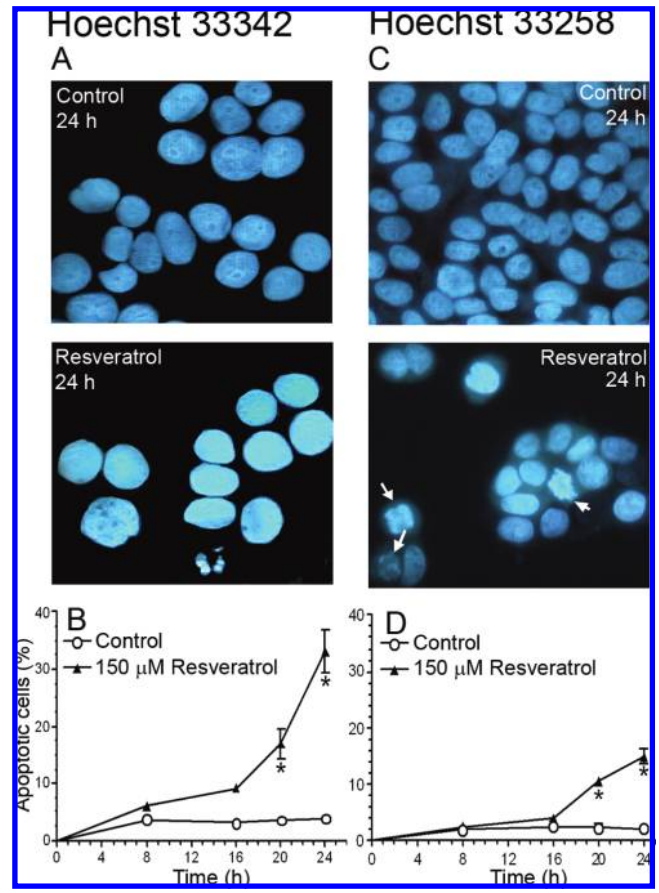

Figure 3. Determination of early (**A** and **B**) and late apoptosis events (**C** and **D**) in human HT-29 cells grown under control or 150 μM of *trans*-resveratrol. Cells that accumulated Hoechst 33342 dye because of membrane disintegration (**A**) were counted and expressed as the percentage of apoptotic cells under control conditions or after 150 *trans*-resveratrol treatment (**B**) at different time points. Cells that displayed nuclear fragmentation (**C**) were counted and expressed as the percentage of apoptotic cells (**D**). Values are means ± SE. *Different from control, $P < 0.001$. Arrows indicate apoptotic cells due to membrane disintegration (early apoptosis) or nuclear fragmentation (late apoptosis).

polyphenol for 24, 36, and 48 h induced an increment of 375%, 450%, and 800% with respect to the control cells ($P < 0.001$), respectively. Taking into account that at 150 μM of *trans*-resveratrol the significant increase was observed at 24 h, this time was selected for the study of the effect of different concentrations of this compound on caspase-3- like activity.

The effect of the incubation of HT-29 cells with concentrations ranging from 10 to 250 μM of *trans*-resveratrol for 24 h on caspase-3-like activity is shown in **Figure 2B**. HT-29 cells incubated with 100 μM of *trans*-resveratrol increased this protease activity by 200% ($P < 0.001$), and higher concentrations induced an enhancement in the activation, in increments of 270, 360, and 750% above control cells ($P < 0.001$) at 150, 200, and 250 μM, respectively. Caspase-3 activation by *trans*-resveratrol after having incubated HT-29 cells with different concentrations gave an EC_{50} value of 276.1 ± 1.7 μM.

The activation of caspase-3 was mirrored by an augmented accumulation of the Hoechst dye 33342 in cells exposed to 150 μM of *trans*-resveratrol that serves as an indicator of early phases of apoptosis characterized by impaired membrane permeability. When cells were incubated with this polyphenol for 20 and 24 h, 17.0 ± 2.7% and 33.0 ± 3.7% ($P < 0.001$) of cells became strongly fluorescent (**Figure 3A** and **B**). Staining

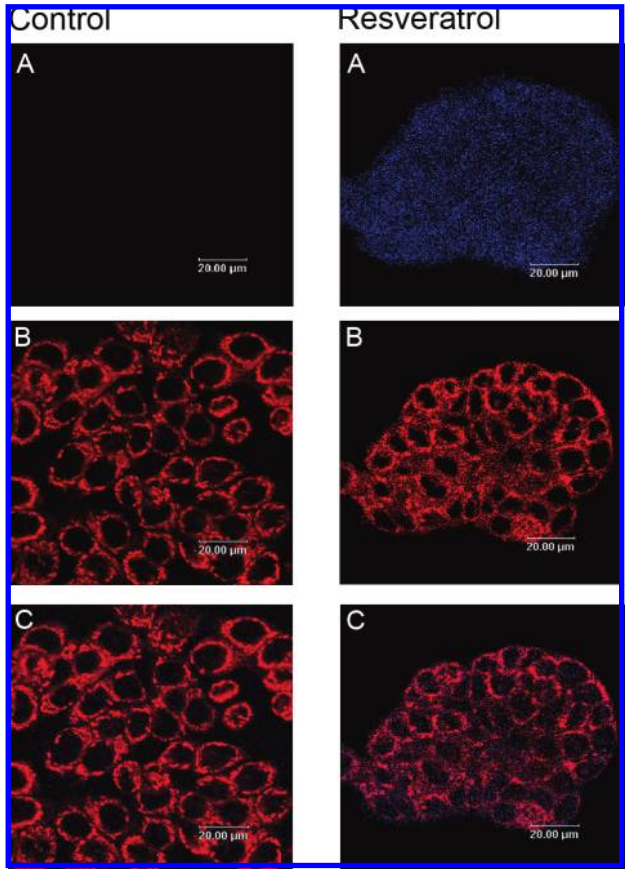

Figure 4. Detection of superoxide radicals in the mitochondria of HT-29 cells incubated with medium alone (control) and with 150 μM of *trans*-resveratrol for 4 h. Cells were loaded with proxylfluorescamine (**A**) in combination with MitoTracker (**B**). The overlay of **A** and **B** is displayed in **C**. Blue fluorescence (emission at 440−480 nm) in **A** indicates the presence of superoxide anions, whereas red fluorescence (emission at 590−650 nm) in **B** allowed the visualization of the mitochondria.

of cells with Hoechst 33258, which served as an indicator of the late phases of apoptosis, revealed 14.9 ± 1.4% ($P < 0.001$) of cells with intense nuclear fragmentation after 24 h of exposure to 150 μM of *trans*-resveratrol (**Figure**s **3C** and **D**).

ROS production is implicated in apoptosis and has been described as an early event. Therefore, cells were treated with 150 μM of *trans*-resveratrol for only 4 h before confocal microscopic analysis with the fluorescent reporter for superoxide anions. As shown in **Figure 4**, cells exposed to this polyphenol displayed a pronounced blue fluorescence, which indicated the production of $O_2^{-\bullet}$ in the mitochondria of HT-29 cells. Thus, proving that *trans*-resveratrol induces an early increase in mitochondrial ROS production upstream of caspase activation.

DISCUSSION

trans-Resveratrol, a natural plant polyphenol, has gained interest as a nontoxic (*12*) chemopreventive agent capable of inducing tumor cell death in a variety of cancer types (*3*). However, the early molecular mechanisms of *trans*-resveratrol-induced apoptosis are not well defined. Here, we demonstrate that exposure of HT-29 cells to 10−300 μM of *trans*-resveratrol reduced cell proliferation rates, with half-maximal effects for growth inhibition of around 80 μM. Of note, this polyphenol did not induce necrosis even at concentrations that caused full inhibition of cell growth. The antiproliferative activity of *trans*-resveratrol may be attributed to cell cycle arrest at G2 phase as has been previously indicated for HT-29 cells (*9*) and Caco-2 cells (*7, 19*).

Our results in HT-29 cells indicate that *trans*-resveratrol increased the activity of caspase-3 in a time- and a dose-dependent manner, with the highest activation of 7-fold above that in control cells at a concentration of 250 μM. The lowest concentration that induced the activation of caspase-3 corresponded to 100 μM that incremented the activity of this protease in 200% that of control cells. Previous studies have documented that resveratrol-induced apoptosis involved caspase activation, both in vitro (*7, 20*) and in vivo (*8, 10*), which was further confirmed in the cell line tested in our research. Once activated, caspases cleave a battery of cellular substrates, leading finally to morphological changes as a hallmark of apoptosis (*21*). The increase in caspase-3-like activity caused by *trans*-resveratrol was followed by full execution of apoptosis with a disintegration of the plasma membrane and finally a pronounced nuclear fragmentation.

In cancer cells, there is a crucial role of ROS in cell growth and apoptosis. However, this role might be that of a double-edge sword. ROS can initiate cell transformation by causing alterations leading to mutations during DNA replication (*22*), whereas in already transformed cells, ROS play an important role in the initiation and execution of apoptosis (*23, 24*). The balance of ROS and antioxidant levels therefore critically determines apoptosis in cancer cells, and overcoming the antioxidative defense systems by accelerating ROS production could promote apoptosis (*24*).

In the present study, we show, for the first time, the generation of mitochondrial ROS in HT-29 cells exposed to *trans*-resveratrol for 4 h. This time was chosen since ROS production in mitochondria has been characterized as an early pro-apoptotic event that takes place before the commitment of the cells to undergo apoptosis (*23*). Our results are in agreement with previous studies that pointed out superoxide anions as secondary messengers in apoptosis provoked by anticancer agents, such as paclitaxel and cisplatin (*13, 14*). Our findings are also consistent with a study that indicated that treatment of prostate cancer cells with *trans*-resveratrol resulted in the generation of reactive oxygen species, prior to the release of mitochondrial proteins to the cytosol, activation of effector caspase-3, and caspase-9, and induction of apoptosis (*11*). Recently, it has been published that HT-29 cells pretreated with 100 μM of *trans*-resveratrol for 30 min and then exposed to 100 μM of etoposide for 24 h, exhibited cellular ROS and cell apoptosis measured by Hoechst 33342 dye (*25*).

trans-Resveratrol, because of its polyphenolic structure, exerts antioxidant effects, including the scavenging of reactive oxygen species (*26*). Recent results have provided interesting insight into the effect of resveratrol on intracellular redox state; these results seem to support both anti- and pro-oxidant activities of this compound, depending on the concentration of *trans*-resveratrol and the cell type (*27, 28*). Here, we indicate that in human colon cancer HT-29 cells, 150 μM *trans*-resveratrol increases mitochondrial superoxide anions, as a mechanism of apoptotic induction, given that ROS play an important role as a signal transduction leading to apoptotic cell death (*22*). This mechanism of action has been stated in HT-29 cells for other compounds such as the antioxidant α-lipoic acid (*24*), flavone (*17*), and an olive fruit extract composed by maslinic and oleanolic acid (*18*).

trans-Resveratrol stands out as a potent chemopreventive agent that has been shown to elicit a broad range of effects that interfere with signaling pathways that control cell proliferation

and/or cell death. Two principal pathways of apoptosis have been described, such as mitochondria-mediated intrinsic pathway and death receptor-induced extrinsic pathway (*21*). However, recent data suggest that lysosomes and the endoplasmic reticulum also play important roles in the process (*29*). *trans*-Resveratrol triggers cell death in HT-29 cells through lysosomes and demonstrates a hierarchy of the proteolytic pathways involved in its cytotoxic mechanism in which lysosomal cathepsin D acts upstream of caspase activation (*30*). Moreover, this polyphenol has been reported to promote apoptosis through the endosplasmic reticulum (ER) (*31*) and the induction of CHOP/GADD153 gene expression, which has been acknowledged as a proapoptosis gene (*32*).

The amounts of *trans*-resveratrol found in peanuts and berries are very low; therefore, red wine constitutes the most important dietary source of this compound (*2*). Assuming that the average concentration of *trans*-resveratrol in wine is 7 mg/L and that the moderate daily consumption of wine is 375 mL, or about two glasses of wine, the mean daily intake of *trans*-resveratrol in these conditions is approximately 12 μmols. Numerous studies in animals and humans have shown that the bioavailability of *trans*-resveratrol is very low (*33*). On the basis of the assumption that an estimated 70% of this compound is not absorbed in the small intestine and reaches the colon unaltered in a distribution phase of around 200 mL, the concentration of *trans*-resveratrol would be around 40 μM. This concentration is not sufficient to attain cellular concentrations that displayed the cancer chemopreventive activity shown here. Only the consumption of wine containing 14 mg/kg could provide a luminal concentration of around 80 μM of *trans*-resveratrol, the concentration that induced 50% inhibition of cell proliferation.

In conclusion, we demonstrate that the naturally occurring *trans*-resveratrol inhibits cellular proliferation at nontoxic concentrations and more importantly restores apoptosis sensitivity in human colon adenocarcinoma cells. The linkage between ROS generation and resveratrol shown in this study provides a new molecular mechanism in HT-29 cells that may contribute to the antitumorigenic activities of *trans*-resveratrol.

ACKNOWLEDGMENT

This work was performed at the Molecular Nutrition Unit, Technical University of Munich, Freising, Germany.

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Received for review January 17, 2008. Revised manuscript received April 11, 2008. Accepted April 14, 2008. This work was supported by grant AGL2005-05728 from Ministerio de Ciencia y Tecnología and grant 2005-SGR-00632 from Generalitat de Catalunya, Spain.

JF800175A