Endocytosis of Resveratrol via Lipid Rafts and Activation of Downstream Signaling Pathways in Cancer Cells

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Abstract

trans-Resveratrol has been proposed to prevent tumor growth and to sensitize cancer cells to anticancer agents. Polyphenol entry into the cells has remained poorly understood. Here, we show that [3H]-resveratrol enters colon cancer cells (SW480, SW620, HT29) and leukemia U937 cells through a monensin (5–20 μmol/L) -sensitive process that suggests clathrin-independent endocytosis. Uptake of the molecule can be prevented by methyl-β-cyclodextrin (2–12 mg/mL), nystatin (12 ng/mL), and filipin (1 μg/mL), which all disrupt plasma membrane lipid rafts. Accordingly, radiolabeled resveratrol accumulates in sphingomyelin- and cholesterol-enriched cell fractions. Interestingly, extracellular signal–regulated kinases (ERK), c-Jun NH2-terminal kinases (JNK), and Akt also accumulate in lipid rafts on resveratrol exposure (IC50 at 48 h ≈ 30 μmol/L in SW480 and U937 cells). In these rafts also, resveratrol promotes the recruitment, by the integrin αvβ3 (revealed by coimmunoprecipitation with an anti-integrin αvβ3 antibody), of signaling molecules that include the FAK (focal adhesion kinase), Fyn, Grb2, Ras, and SOS proteins. Resveratrol-induced activation of downstream signaling pathways and caspase-dependent apoptosis is prevented by endocytosis inhibitors, lipid raft–disrupting molecules, and the integrin antagonist peptide arginine-glycine-aspartate (500 nmol/L). Altogether, these data show the role played by lipid rafts in resveratrol endocytosis and activation of downstream pathways leading to cell death. Cancer Prev Res; 4(7); 1095–106. ©2011 AACR.

Introduction

Epidemiologic and experimental studies have suggested favorable effects of dietary polyphenols through their antioxidative and anticarcinogenic properties. Resveratrol (trans-3,4′,5-trihydroxystilbene), a wine grape microcomponent, may be one of the most efficient of these polyphenols (1) in that it could prevent the occurrence of vascular diseases, neurodegenerative processes, and some malignant tumors (see for review ref. 2). These chemopreventive properties are supported by observations at the cellular and molecular levels (3–5) and reinforce the interest in grape products and dietary supplements for cancer therapy.

The potential ability of trans-resveratrol to prevent the occurrence of colon cancer and other carcinomas is related to its effects on the tumor cell cycle (5–7) through activation of signaling pathways that involve c-Jun NH2-terminal kinases (JNK) or extracellular signal–regulated kinases (ERK; refs. 8, 9) and/or its ability to trigger tumor cell death by apoptosis (4, 10–12). There is also compelling evidence that resveratrol can sensitize tumor cells to various anticancer drugs (13, 14) and cytokines such as TRAIL (11), possibly because of the clustering of death receptors in detergent-resistant membranes (DRM) known as lipid rafts (4, 11, 15).

An unsolved question remains the mechanisms of resveratrol entry into cells and how resveratrol initiates its biological effects in tumor cells. This study shows that resveratrol-induced apoptosis involves dependent mitogen-activated protein kinase (MAPK) activation requiring accumulation in lipid rafts and monensin-sensitive active endocytosis. These 2 events are essential for the downstream activation of kinase-dependent signaling pathways and caspase activation leading to cell death. Interestingly, resveratrol also promotes the redistribution of MAPKs and the integrin β3 protein into lipid rafts, which seem as key platforms for anticancer effects of the anticancer effects of this polyphenol. These findings contribute new information on...
resveratrol uptake–associated mechanisms and on activation of downstream signaling pathways in tumor cells.

Materials and Methods

Cell lines

SW480, SW620, HT29, and HCT116 human colon carcinoma cells, human leukemic monocyte lymphoma U937 cells, and rat nontransformed small intestinal IEC-18 cells were obtained from the American Type Culture Collection. U937 cells were maintained in RPMI 1640 and SW480, SW620, HT29, HCT116, and IEC18 cells were cultured in Eagle’s minimum essential medium. Both media were complemented with 10% (v/v) fetal calf serum (Sigma-Aldrich). Cells were also maintained at different pH values (7.4 and 6.0) before 24 hours and during resveratrol treatment. Human peripheral blood monocytes were obtained from healthy donors with informed consent according to recommendations of an independent scientific review board, in accordance with the Declaration of Helsinki. Cells were enriched by the use of a monocyte isolation kit with autoMACS Separator according to the manufacturer’s instructions (Miltenyi Biotec) and cultured as previously described (16).

Drugs, chemical reagents, and antibodies

[1H]-trans-Resveratrol (specific activity: 74 GBq/mmol) labeled in ortho and para positions of benzenic rings (Amer sham). All chemicals were obtained from Sigma-Aldrich unless specified. We used rabbit polyclonal antibodies (Abs) against human phospho-JNKs (p-JNK), JNKs, phospho-ERK1/2 (p-ERK1/2), ERK1/2, phospho-Akt (p-Akt), Akt, Fyn, FAK, Ras, Grb2, PARP, procaspase-3, caspase-3 active fragments, integrin β3 (Cell Signaling Technology), against caveolin-2 (Santa-Cruz Biotechnology), against SOS1 (Abcam), and mouse monoclonal Abs against human flotillin (BD Transduction Lab), integrin αvβ3 (Santa-Cruz Biotechnology), and β-actin (Sigma-Aldrich).

Fluorescence microscopy

Cells were grown on glass coverslips in 12-well plates, exposed to resveratrol for indicated times, quickly rinsed 3 times with cold PBS and mounted on slides in PBS. Conventional fluorescence microscopic analysis using a 4′,6-diamidino-2-phenylindole (DAPI) filter was immediately carried out with an Axioskop (Zeiss) after UV excitation to visualize resveratrol autofluorescence (17).

To detect acidic compartment, cells were grown on glass coverslips in 12-well plates and incubated with 1 μmol/L LysoSensor probe (Invitrogen/Molecular Probes) for 30 minutes with or without resveratrol during the last 6 minutes. Cells were then rinsed 3 times with cold PBS and quickly mounted on slides. A 488-nm wavelength was used to visualize LysoSensor green using a fluorescein isothiocyanate (FITC) filter.

Resveratrol uptake measurement

Resveratrol uptake was examined by incubating cells seeded in 6-well plates over different times with [1H]-resveratrol (30 μmol/L; 0.5 μCi/mL) at various concentrations in complete, serum-free, HBSS-enriched, bovine serum albumin (BSA)-enriched medium and at different pH values (7.4 and 6.0). We carried out cis-inhibition experiments by adding unlabeled resveratrol to incubation medium. Resveratrol uptake assays were carried out at 37°C and 4°C, with or without endocytosis inhibitors (10 μmol/L monensin, 10 μmol/L phenylarsine oxide, 50 μmol/L chlorpromazine, 50 μmol/L monodansylcavender, 50 μmol/L amiloride, 80 μmol/L dynasore). Rafts mediated resveratrol uptake was also detected by treating cells with [1H]-resveratrol after raft disruption by methyl-β-cycloexodrin (MβCD) pretreatment (30 minutes, 6 mg/mL). The integral involvement in resveratrol uptake was examined by incubating SW480 cells with an integrin peptide inhibitor [500 nmol/L RGD peptide (Arg-Gly-Asp), 24 hours] and with [1H]-resveratrol (30 μmol/L). At the end of the incubation period, the labeled media were removed, the cells were washed 3 times with cold PBS, and lysed in a lysis buffer (0.1 mol/L NaOH, 2% Na2CO3, 0.1% SDS). Cell lysates and media radioactivity were counted in a liquid scintillation analyzer.

Resveratrol binding studies

trans-Resveratrol (5 μmol/L) was incubated for different times at 37°C with standard cell-free culture medium. Unbound ligand was extracted by ethyl acetate and then quantified by high performance liquid chromatographic (HPLC) analysis. The analyses were carried out on a reversed phase. Nucleosil C18 columns were from Touzart and Matignon (Waters). A Waters 625 LC system, was used, together with a Waters 486 tunable absorbance detector and a SP4400 ChromJet integrator (Spectra-Physics). The UV detector was set at 306 nm and resveratrol was eluted from the column with a gradient containing water and acetonitrile. Resveratrol concentrations in culture media were calculated using a standard curve of resveratrol.

Cell fractionation

Colon carcinoma cells were seeded in 6-well plates and were treated with 30 μmol/L [1H]-resveratrol for 1, 3, 6, and 10 minutes. Cells were thoroughly washed with cold PBS and lysed on ice in a lysis buffer (10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol) in the presence of protease inhibitor cocktail (Roche Diagnostics). After 15 minutes, the extracts were homogenized with 0.6% NP40 during 15 seconds and centrifuged for 10 minutes at 1,200 × g. The nuclear fractions were dissolved in lysis buffer containing 1% NP40, and the cytoplasmic fractions were centrifuged twice more to remove nuclear contaminations. Fractions were estimated for proteins concentrations and submitted to radioactivity measurement in a liquid scintillation analyzer.

Flow cytometric analyses

Cancer cells were seeded in 6-well plates and were treated with 30 μmol/L resveratrol for 6 minutes. Cells were
trypsinized, washed with PBS, centrifuged for 5 minutes at 400 × g, and incubated for 30 minutes with the fluorescent fluid marker Sulforhodamine-101 (25 μg/mL) with or without monensin (5 μmol/L). Cells were washed in PBS, fixed in 1% paraformaldehyde, and analyzed with a FACScan flow cytometer (BD Biosciences).

Rafts isolation and biochemical characterization
This study was conducted as described (4). Briefly, cells were starved for 6 hours and treated with MβCD (30 μmol/L, 6 minutes) with or without MβCD, lysed in 2 mL (containing Tris, NaCl, and EDTA), and centrifuged at 20,000 rpm for 2 hours at 4°C. The lysates were diluted with 2 mL buffer containing 80% sucrose (w/v), placed at the bottom of a linear sucrose gradient and centrifuged at 39,000 rpm for 20 hours at 4°C before collecting eleven 1-mL fractions. Sixty micro liters of each fraction was subjected to SDS-PAGE and immunoblotted. Lipids were extracted and analyzed as described (4) in the indicated conditions. Flotillin and caveolin-2 described as markers for rafts were analyzed in each fraction by Western blotting.

Immunoprecipitation
SW480 cells were seeded into 80-cm² flasks (2 × 10⁶ cells) for 48 hours and starved for 6 hours before resveratrol treatment. After 15 minutes, cells were washed with cold PBS and proteins were cross-linked with disuccinimidyl suberate (DSS) according to the manufacturer’s instructions (Pierce, Thermo Fisher Scientific). Cells were then incubated in a lysis buffer (50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 10 mmol/L EDTA, 1% Triton X-100, pH 7.4 containing antiproteases) for 30 minutes at 4°C, and passed through an ice-cold cylinder homogenizer. The lysates were diluted with 2 mL buffer containing 80% sucrose (w/v), placed at the bottom of a linear sucrose gradient and centrifuged at 39,000 rpm for 20 hours at 4°C before collecting eleven 1-mL fractions. Sixty micro liters of each fraction was subjected to SDS-PAGE and immunoblotted. Lipids were extracted and analyzed as described (4) in the indicated conditions. Flotillin and caveolin-2 described as markers for rafts were analyzed in each fraction by Western blotting.

Western blotting
Standard Western blot analyses were carried out as described in Supplementary Materials and Methods.

Proliferation assays
Cells were seeded in quadruplicates in 24-well plates and treated with 30 μmol/L of resveratrol at different pH values (7.4 and 6.0). After indicated times, cells were washed with PBS, stained with crystal violet [0.5% (w/v) crystal violet in 25% (v/v) methanol] for 5 minutes, and rinsed with water 3 times. Absorbance was read at 540 nm after extraction of the dye with 0.1 mol/L sodium citrate in 50% ethanol.

Apoptosis identification
Apoptosis was identified by staining the nuclear chromatin of trypsinized cells with 1 μg/mL Hoechst 33342 (Sigma-Aldrich) for 15 minutes at 37°C. The percentage of apoptotic cells was determined by analyzing 300 cytospins. MAPK implication in resveratrol-induced apoptosis was assessed by pretreating cells during 2 hours by 10 μmol/L of U0126 [MAP/ERK kinase (MEK) inhibitor], LY294002 [phosphoinositide 3-kinase (PI3K)/Akt inhibitor], or SP600125 (JNK inhibitor) before resveratrol exposure.

Densitometry and statistical significance
The densitometry of blots was realized by the use of ImageJ software (NIH). Unless indicated in the legends of figures, the reported values represent the means ± SD of 3 representative experiment repeated 3 times. Statistical significance was determined using the Mann–Whitney test at P < 0.05, P < 0.01, or P < 0.001.

Results
Resveratrol rapidly accumulates in colon cancer cell lines
Resveratrol exhibits a spontaneous blue fluorescence on UV excitation (17), which allows the visualization of its time- and dose-dependent accumulation in the cells. Fluorescence was detected in SW480 colon cancer cells 1 minutes after the beginning of their treatment (Fig. 1A). Intracellular fluorescence was obvious at 10 minutes at resveratrol concentrations ranging from 0.5 to 50 μmol/L with an apparent dose-dependent increase. This rapid uptake of the molecule was also observed in HCT116, HT29, and SW620 colon cancer cell lines (not shown). To quantify this uptake, cancer cells were incubated with 30 μmol/L [3H]-resveratrol, and intracellular and extracellular radioactivity was monitored. This resveratrol concentration was chosen in accordance with our results showing that it induces a marked antiproliferative (6) and proapoptotic effect on colon cancer cells and leukemia cells without inducing toxicity and apoptosis in normal human monocytes and in normal rat intestinal IEC18 cells (Supplementary Fig. S1). After adding [3H]-trans-resveratrol at 37°C, it was readily taken up by cancer cell lines (Fig. 1B, diamonds) and accumulated with time to reach a maximum at 6 minutes after the beginning of cell treatment. Thereafter, the radioactivity decreased gradually to reach at 48 hours the level obtained after 1 minutes of treatment (Fig. 3E), suggesting a prevalence of efflux over influx. When these experiments were conducted at 4°C, resveratrol uptake at 6 minutes was lower than 50% that measured at 37°C (Fig. 1B, triangles). This transport in the tumor cells was...
specific to resveratrol, as we failed to detect any metabolite in the culture medium, up to 1 hour after the beginning of incubation with the polyphenol (Fig. 1C).

Serum removal from the culture medium increased resveratrol uptake at both 4°C and 37°C, indicating that the polyphenol was trapped by serum constituents (Fig. 1D). The molecule uptake decreased in a dose-dependent manner by the addition of BSA, suggesting that the major serum protein albumin plays a role in its trapping (Fig. 1D). The kinetics of resveratrol binding to serum proteins indicated a 50% decrease in unbound molecule after 2 hours of incubation in the presence of serum and a virtually complete trapping of resveratrol after 24 hours (Fig. 1D, insert).

**Resveratrol is internalized by endocytosis**

For many drugs, passive diffusion and carrier-mediated transport coexist, the latter being an active or a facilitated transport that has a limited capacity and thus can reach saturation. Concentration dependence of resveratrol
uptake reveals that intracellular radioactivity varied linearly with the incubated concentration (Fig. 2A). When the total uptake measured at 37°C was corrected from the passive uptake measured at 4°C, the curve obtained, which appears to be saturated, corresponds to a carrier-mediated transport (Fig. 2A). To confirm this mechanism, cis-inhibition experiments were undertaken. When unlabeled resveratrol was added to the radiolabeled molecule at 37°C, the intracellular radioactivity measured at 6 minutes decreased in SW480 and HT29 colon cancer cells (Fig. 2B), which was not observed at 4°C (Fig. 2B). This observation suggests that cancer cells could uptake resveratrol, at least in part, by an active process. Similar observations were made in SW620, HCT116, and U937 cells (not shown). To further explore this active process, the 5 cell lines studied were pretreated for 30 minutes in a medium containing 5 μmol/L monensin (18), an inhibitor of endocytosis that disrupts the membrane electropotential and the pH value. Monensin pretreatment provoked 50% decrease in resveratrol uptake at 3 and 6 minutes (see 3 of the cell lines in Fig. 2C). At this concentration, monensin also inhibited the accumulation of Sulfonamide-101, a fluorescent marker for clathrin-independent fluid-phase endocytosis, in these cells (Fig. 2D) and counteracted resveratrol-induced apoptosis (Supplementary Fig. S1). Altogether, these results suggest that resveratrol taken up in cancer cells was mediated by endocytosis.

**Resveratrol endocytosis depends on lipid rafts integrity**

To better characterize the endocytic process, we used specific inhibitors of clathrin-mediated endocytosis (phe- nyllarsine oxide, chlorpromazine, monodansylcadaverine; refs. 19–21), macropinocytosis (amiloride; ref. 22), dyna- min-dependent endocytosis (dynasore; ref. 23), and lipid raft–mediated endocytosis (MβCD). Under cotreatment with these inhibitors, only monensin and MβCD blocked [3H]-resveratrol uptake (Fig. 2E). Two polyene antibiotics (filipin and nystatin), which are other disruptors of lipid rafts, also decreased resveratrol internalization (Fig. 2F). At the concentrations used, MβCD, nystatin, and filipin did not cause any plasma membrane leakage, as determined by trypan blue exclusion assay (not shown). The ability of MβCD to decrease resveratrol internalization into SW480 cells was dose dependent and reached 70% at 12 mg/mL (Fig. 2G). Similar results were obtained in other cell lines studied (not shown). These combined results argue for a lipid raft–dependent endocytosis of resveratrol.

**Resveratrol accumulates in lipid rafts of colon cancer cells**

The lipophilic structure of resveratrol suggests that this compound could accumulate in plasma membrane lipid rafts before endocytosis. When SW480 cells were incubated with 30 μmol/L [3H]-resveratrol, most of the radioactivity accumulated within minutes in the membrane/particulate fraction (Fig. 3A). Lysates of SW480 cells exposed for 6 minutes to 30 μmol/L [3H]-resveratrol were fractionated on a sucrose gradient, and the lipid content of each fraction was determined by HPLC-coupled mass spectrometry to identify sphingomyelin- and cholesterol-enriched fractions corresponding to lipid rafts (Fig. 3B) and flotillin and caveolin-2 confirmed the DRMs enrichment in fractions 3 and 4 (Fig. 3B). Radioactivity analysis showed that resveratrol was found mainly in these 2 fractions (Fig. 3B). In the presence of MβCD (6 mg/mL), resveratrol was dispersed in the various fractions. Furthermore, we observed that there was no incorporation of resveratrol in membrane phospholipids in a covalent manner (not shown). Interestingly, endocytosis of resveratrol via lipid rafts was associated with an accumulation of LysoSensor-positive acidic vesicles within cytoplasm (Fig. 3C). The antiproliferative effect of resveratrol was not affected by an acidification of the medium (Fig. 3D). Moreover, resveratrol was taken up by SW480 cells indifferently of pH conditions (Fig. 3E) and the lipid composition remained unchanged (Supplementary Fig. S2).

**Disruption of resveratrol endocytosis prevents polyphenol-induced signaling pathways**

In agreement with the data on prostate cancer cells and melanoma cells (24, 25), resveratrol induced a rapid activation of JNK, Akt, and ERK1/2 in colon carcinoma cells and leukemia cells but not in human normal monocytes (Fig. 4A). The combination of resveratrol with an ERK1/2 inhibitor (U0126), PI3K/Akt inhibitor (LY294002), or INK inhibitor (SP600125) prevented resveratrol-induced apoptosis in these cells (Fig. 4B). Pretreatment of cancer cells with monensin decreased resveratrol-induced MAPK activation in a dose-dependent manner (Fig. 4C; Supplementary Fig. S3), without affecting their total levels in cancer cells. These results underline the importance of resveratrol endocytosis in kinase activation.

Interestingly, fractionation of cell lysates on a sucrose gradient showed that JNKs, Akt, ERK1/2, and their phosphorylated forms, which are not detected in the fractions enriched in cholesterol and sphingomyelin of untreated cells, accumulated in these fractions on resveratrol treatment (Fig. 5A and B). Moreover, resveratrol induced redistribution of integrin β3, which is a resveratrol receptor (25), and its associated proteins including FAK, Fyn, and Ras (Fig. 5B). It is noteworthy that MβCD suppressed the resveratrol-induced redistribution of these proteins in lipid rafts (Fig. 5B), the activation of JNKs, Akt, and ERK1/2 (Fig. 5C; Supplementary Fig. S4), and caspase-dependent apoptosis (Supplementary Fig. S5).

**Integrin αvβ3 is involved in resveratrol uptake**

Considering the most important role played by integrin in signal transduction, we then analyzed whether the specific redistribution of FAK, Fyn, and Ras, together with integrin β3, in the DRMs was associated with a resveratrol-induced formation of the integrin signaling complex. Coimmunoprecipitation experiments using anti-integrin αvβ3 Ab showed recruitment of FAK, Fyn, Grb2, Ras, and to a lesser extent SOS proteins to integrin αvβ3 in SW480 cells.
Figure 2. Resveratrol uptake involves a lipid raft–dependent endocytosis. A, concentration-dependent uptake of resveratrol. SW480 and HT29 cells were exposed to [3H]-resveratrol (0–300 μmol/L) at 37°C (●) and 4°C (○) for 6 minutes. The carrier-mediated transport obtained by subtracting the total 37°C uptake from the passive 4°C uptake is also plotted (■). B, cis-inhibition experiments. Colon cancer cell lines were incubated at 37°C (black bars) and at 4°C (white bars) for 6 minutes with [3H]-resveratrol alone (R*) or with a 20-fold excess of unlabeled resveratrol (R20). C, Uptake of [3H]-resveratrol (R30: 30 μmol/L; 37°C) by SW480, HT29 colon cancer cells, and U937 leukemic cells with (●) or without (○) monensin (5 μmol/L) for the indicated times. D, flow cytometric analysis of Sulforhodamine-101 endocytosis (25 μg/mL, 30 minutes) and the effect of an endocytosis inhibitor, monensin (5 μmol/L), on Sulforhodamine-101 uptake. SW480 and HT29 cells were also left untreated or treated with resveratrol (R30: 30 μmol/L for 6 minutes). Red line, control Ab; black line, Sulforhodamine-101; blue line, Sulforhodamine-101 with monensin (5 μmol/L). One representative of 3 independent experiments is shown. E, SW480 cells were treated with [3H]-resveratrol (R30: 30 μmol/L; 37°C) or combinations of [3H]-resveratrol and inhibitors of endocytosis as described. F, time-dependent uptake of [3H]-resveratrol in SW480 cells treated with [3H]-resveratrol alone (30 μmol/L; 37°C) (●) or the combination of both [3H]-resveratrol and filipin (1 μg/mL; ▲), nystatin (12 ng/mL; ■), and MβCD (2 mg/mL; △). G, SW480 cells were treated with [3H]-resveratrol (R30: 30 μmol/L; 0.5 μCi/mL) or a combination of [3H]-resveratrol and MβCD at various concentrations (2; 6; 12 mg/mL) during 6 minutes. The uptake of tracer in A, B, C, E, and F was determined and expressed in dpm per 10^6 cells (mean ± SD of a representative experiment from 3 independent experiments).
Figure 3. Resveratrol accumulates into lipid rafts. A, time-dependent uptake and distribution of [3H]-resveratrol (30 μmol/L; 37°C) in SW480 cells after subcellular fractionation. Resveratrol content was measured in whole-cell lysates (■), cytosolic and membrane-fraction (○), and nuclear fraction (▲). B, Top, lipid composition of SW480 cell lysate fractions after resveratrol treatment (30 μmol/L for 6 minutes). Black bars, cholesterol; white bars, sphingomyelin. Bottom, distribution of [3H]-resveratrol into cell fractions obtained on sucrose gradient after resveratrol treatment (30 μmol/L, for 6 minutes), in the absence (■) or presence (○) of MβCD (6 mg/mL). Fractions 3 to 4 represent detergent-resistant rafts characterized by Western blot analysis of proteins rafts markers. C, distribution of resveratrol (DAPI filter set) and LysoSensor-positive acidic vesicles (FITC filter set) in SW480 cells untreated (Co) or treated with resveratrol (R30: 30 μmol/L for 6 minutes). D, percentages of viable SW480 cells mock treated (Co) or treated with 30 μmol/L resveratrol (R30) for 24 and 48 hours in different growth medium acidity conditions (pH 7.4 or 6.0). E, time course of radiolabeled resveratrol uptake in SW480 cells (30 μmol/L) grown at 37°C in different pH conditions. Data in A, B, C, D, and E are means ± SD of a representative experiment from 3 independent experiments.
cells exposed to 30 μmol/L resveratrol (Fig. 6A). Moreover, occlusion of the RGD (an arginine-glycine-aspartate peptide inhibitor) binding site in the integrin extracellular domain diminished the resveratrol uptake (Fig. 6B) and resveratrol-induced apoptosis (Fig. 6C) in cancer cells.

**Discussion**

The present study shows that resveratrol, which binds serum proteins such as albumin (26), can enter the cells through both a passive diffusion and a carrier-facilitated
and monensin-sensitive process. Resveratrol accumulates in DRMs known as lipid rafts and promotes the redistribution of various protein kinases as well as components of the integrin signaling complex in these membrane fractions. All these events seem to be required for activation of downstream signaling pathways, leading to cell death in tumor cells.

Resveratrol has been proposed to function as a cancer chemopreventive agent through inhibition of promutagen bioactivation and stimulation of carcinogen detoxification.
Resveratrol also has direct cytostatic (6, 7) or cytotoxic effects (4, 10, 12, 15). The later effect can be mediated by apoptosis through activation of both the extrinsic and the intrinsic pathways to caspase activation, oncoproteins, and kinase pathways (4, 10, 12, 15, 24). Mechanisms responsible for the resveratrol chemopreventive and chemosensitization properties are poorly known in tumor cells, but evidence indicates that resveratrol uptake and the cancer microenvironment plasma membrane play a key role in these processes.

Here, we show that resveratrol activates MAPK pathways to induce apoptosis both in colon carcinoma cell lines and in leukemic lymphoma cells but not in normal cells such as human normal monocytes or rat nontransformed intestinal cells in accordance with recent phase I/II trials showing the absence of resveratrol toxicity (28). The inhibition of resveratrol-induced apoptosis by monensin in tumor cells suggests a clathrin-independent endocytosis of the compound. A variety of clathrin-independent endocytic pathways are responsible for taking up large particles or small solutes, together with membrane into cells, and some of them depend on lipid rafts (29–31), including endocytosis of glycosylphosphatidylinositol (GPI)-anchored proteins (32, 33), interleukin-2 linked to its receptors (34), and several ether lipids (e.g., alkyl lysophospholipid; refs. 35–37). After initial insertion in the outer leaflet of the plasma membrane lipid bilayer, resveratrol accumulates in lipid rafts, as described for epigallocatechin (38), and is taken up by lipid raft–dependent, clathrin-independent endocytosis. In addition to endocytosis, disruption of lipid rafts affects resveratrol-induced activation of downstream pathways, including kinase activation and cell death by caspase-3–mediated apoptosis. Interestingly, resveratrol seems to promote the redistribution of kinases, including ERKs and JNKs, in lipid rafts, which may facilitate their activation and downstream induction of apoptosis (9, 24, 39–41).

Resveratrol also induces the redistribution of integrin \( \alpha_\text{v}\beta_3 \) in lipid rafts. Resveratrol binding to integrin \( \alpha_\text{v}\beta_3 \) (8) could account for the activation of ERK1/2 (42). As resveratrol induced a redistribution of the integrin \( \alpha_\text{v}\beta_3 \), as well as Akt, FAK, Fyn, and Ras proteins in lipid rafts, our data support that these microdomains may function as microcompartments for the assembly of signaling complexes (43). On integrin ligation, Fyn is activated and binds to...
SHC, via the SH3 domain of Fyn. It has been previously reported that this sequence of events is necessary to couple integrins to the Ras/ERK pathway (44).

Taken together, these findings show that accumulation of resveratrol in lipid rafts and active endocytosis are required for resveratrol to activate kinase-dependent signaling pathways (ERK/JNK/Akt) and to trigger caspase-dependent apoptosis in cancer cells. These results confirm the importance of lipid rafts in the biological effects of resveratrol. Initial reports have shown the essential role played by rafts in the initiation of Fas-mediated apoptosis with ether lipids (36, 45). In a similar manner, we have previously shown that lipid rafts play a role in clustering or aggregating surface receptors and adaptor molecules into membrane complexes at specific sites and are shown to be essential for initiating signaling from a number of receptors, particularly in the initiation of Fas-mediated apoptosis during resveratrol treatment (4). These micro-domains sequester the polyphenol, as described recently for autocrine motility factor (AMF)/phosphoglucone isomerase (PGI)-paclitaxel (46), and therefore function as a platform that finely tunes up the tumor cell response to this agent, either directly by inducing cell-cycle arrest or cell death (47) or indirectly by sensitizing the cells to chemotherapeutic agents (11, 15). This sensitization could involve the inhibition of the H+−ATP synthase by resveratrol (48). Similarly to the proton pump inhibitor omeprazole (49), resveratrol could affect the lysosomal function and reduce cell environment acidity, thereby facilitating the drug activity (50). Interestingly, Parolini and colleagues have described that acidity may also change the lipid composition of tumor cell membrane and consequently play an important role in the internalization of exosomes in tumor cells (51). In the models of colon carcinoma used in this study, an acidification of the medium does not affect the antiproliferative effect of resveratrol and its uptake. In addition, a part of polyphenol uptake involves a passive process that could precede intracellular specific interaction (e.g., aryl hydrocarbon and estrogen receptors). Further insights in the uptake mechanisms of resveratrol at the cytoplasmic membrane level will provide a better understanding of the capability of tumor cells to accumulate the molecule and to detail the relationships between accumulation and early events leading to apoptosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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