

Nitric Oxide Mediates Natural Polyphenol-induced Bcl-2 Down-regulation and Activation of Cell Death in Metastatic B16 Melanoma*

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Intravenous administration to mice of *trans*-pterostilbene (t-Pter; 3,5-dimethoxy-4'-hydroxystilbene) and quercetin (QUER; 3,3',4',5,6-pentahydroxyflavone), two structurally related and naturally occurring small polyphenols, inhibits metastatic growth of highly malignant B16 melanoma F10 (B16M-F10) cells. t-Pter and QUER inhibit *bcl-2* expression in metastatic cells, which sensitizes them to vascular endothelium-induced cytotoxicity. However, the molecular mechanism(s) linking polyphenol signaling and *bcl-2* expression are unknown. NO is a potential bio-regulator of apoptosis with controversial effects on Bcl-2 regulation. Polyphenols may affect NO generation. Short-term exposure (60 min/day) to t-Pter (40 μ M) and QUER (20 μ M) (approximate mean values of the plasma concentrations measured within the first hour after intravenous administration of 20 mg of each polyphenol/kg) down-regulated inducible NO synthetase in B16M-F10 cells and up-regulated endothelial NO synthetase in the vascular endothelium and thereby facilitated endothelium-induced tumor cytotoxicity. Very low and high NO levels down-regulated *bcl-2* expression in B16M-F10 cells. t-Pter and QUER induced a NO shortage-dependent decrease in cAMP-response element-binding protein phosphorylation, a positive regulator of *bcl-2* expression, in B16M-F10 cells. On the other hand, during cancer and endothelial cell interaction, t-Pter- and QUER-induced NO release from the vascular endothelium up-regulated neutral sphingomyelinase activity and ceramide generation in B16M-F10 cells. Direct NO-induced cytotoxicity and ceramide-induced mitochondrial permeability transition and apoptosis activation can explain the increased endothelium-induced death of Bcl-2-depleted B16M-F10 cells.

Different natural polyphenols show potent antioxidant effects and may have therapeutic applications in oxidative stress-related diseases such as cancer (1–3). The cancer-che-

mopreventive activity of *trans*-resveratrol (t-RESV²; *trans*-3,5,4'-trihydroxystilbene) was first reported by Jang *et al.* (4). However, the potential anticancer properties of t-RESV are strongly limited because of its low bioavailability (5). Thus, structural modifications of the t-RESV molecule appeared to be necessary to increase its bioavailability while preserving its biological activity. Recently, we found that *trans*-pterostilbene (t-Pter; *trans*-3,5-dimethoxy-4'-hydroxystilbene) and quercetin (QUER; 3,3',4',5,6-pentahydroxyflavone) have longer *in vivo* half-lives compared with t-RESV (6). *In vitro* growth of highly malignant B16 melanoma F10 (B16M-F10) cells is inhibited (56%) by short-term exposure (60 min/day) to t-Pter (40 μ M) and QUER (20 μ M) (6). Intravenous administration of t-Pter and QUER (20 mg/kg/day) to mice inhibits (73%) metastatic growth of B16M-F10 cells in the liver, a common site for metastasis development (6). The antimetastatic mechanism involves (a) t-Pter-induced inhibition of vascular adhesion molecule 1 (VCAM-1) expression in the hepatic sinusoidal endothelium (HSE), which decreases B16M-F10 cell adhesion to the endothelium via very late activation antigen 4, and (b) QUER- and t-Pter-induced inhibition of *bcl-2* expression in metastatic cells, which sensitizes them to vascular endothelium-induced cytotoxicity (6).

Analysis of the *bcl-2* family of genes revealed that B16M-F10 cells (high metastatic potential), compared with B16M-F1 cells (low metastatic potential), overexpress *bcl-2* preferentially (7). t-Pter increases expression of pro-death *bax* (~2.2-fold) and decreases expression of anti-death *bcl-2* (~2.0-fold) (6), whereas QUER increases expression of different pro-death genes (*bax*, *bak*, *bad*, and *bid*; 1.5–2.5-fold) and decreases expression of all anti-death genes analyzed (*bcl-2* (~7.3-fold), *bcl-w* (~1.5-fold), and *bcl-x_L* (~2-fold)) (6). *bcl-2* overexpression prevents the QUER- and t-Pter-dependent increase in metastatic B16M-F10 cell death caused by the HSE *in vivo* (6), thus suggesting that Bcl-2 by itself plays a critical role in regulating B16M-F10 resistance against vascular endothelium-in-

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² The abbreviations used are: t-RESV, *trans*-resveratrol; t-Pter, *trans*-pterostilbene; QUER, quercetin; B16M-F10, B16 melanoma F10; VCAM-1, vascular adhesion molecule 1; HSE, hepatic sinusoidal endothelium; iNOS, inducible nitric-oxide synthetase; eNOS, endothelial nitric-oxide synthetase; DMEM, Dulbecco's modified Eagle's medium; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; CREB, cAMP-response element-binding protein; siRNA, small interfering RNA; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; SMase, sphingomyelinase; AMG, aminoguanidine; HPLC, high pressure liquid chromatography; SNAP, S-nitroso-N-acetyl-DL-penicillamine.

duced damage. In agreement with this idea, we also observed that antisense *bcl-2* therapy potentiates tumor necrosis factor- α -induced oxidative stress and death in B16M-F10 cells (7). However, the molecular mechanisms that link polyphenol signaling with *bcl-2* expression are unclear.

In vitro, t-RESV-driven apoptosis of chronic leukemic B cells has been shown to correlate with activation of caspase-3, a drop in the mitochondrial membrane potential, and reduction in the expression of inducible nitric-oxide synthetase (iNOS) (8, 9). NO is a potential bioregulator of apoptosis because high concentrations of NO or peroxynitrite can induce apoptotic death in different cells types, including tumor cells (10), although, on the other hand, NO may also act as an anti-apoptotic signal associated with, for example, suppression of mitochondrial cytochrome *c* release, ceramide generation, and caspase activation (10). Indeed, NO donors can elevate *bcl-2* expression both at the mRNA and protein levels and prevent apoptotic cell death *in vitro* (11), whereas, paradoxically, also *in vitro*, NO-induced apoptosis of K-1735 melanoma cells (12) or human myeloid leukemia U937 cells (13) is associated with down-regulation of Bcl-2 and caspase activation. Therefore, NO, which may either prevent or induce apoptosis, can also increase or decrease Bcl-2 levels. These apparently controversial facts suggest that different intracellular NO levels may likely determine opposite effects. Whether natural polyphenols such as t-PTER and QUER also cause reduction in iNOS gene expression in metastatic cells (and consequently a decrease in their intracellular NO levels) and whether this is linked to changes in *bcl-2* expression is unknown.

On the other hand, t-RESV, as well as other polyphenols (e.g. black tea polyphenols), can increase endothelial nitric-oxide synthetase (eNOS) activity and induce accumulation of p53 and p21^{WAF1/CIP1} in cultured pulmonary artery endothelial cells (14, 15). Thus, it is possible that natural polyphenols may also alter NO levels in the metastatic microenvironment during interaction of cancer and endothelial cells, which is important because endothelial NO generation was found to be essential in the mechanism of tumor cytotoxicity during B16M-F10 cell adhesion to the vascular endothelium (16).

The aim of this study was to investigate the possible relationship between NO and the effect t-PTER and/or QUER (at *in vivo* bioavailable concentrations) on *bcl-2* expression in B16M-F10 cells. Our results show that this polyphenolic association decreases NO production in isolated B16M-F10 cells and increases NO release from the vascular endothelium during B16M-F10/endothelial cell interaction. At both steps, changes in NO levels trigger Bcl-2 down-regulation and activation of death mechanisms in metastatic B16M-F10 cells.

EXPERIMENTAL PROCEDURES

Culture of B16M-F10 Cells—Murine B16M-F10 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), pH 7.4, supplemented with 10% fetal calf serum (Invitrogen), 10 mM HEPES, 40 mM NaHCO₃, 100 units/ml penicillin, and 100 μ g/ml streptomycin (17). Cell integrity was assessed by trypan blue exclusion and leakage of lactate dehydrogenase activity (17).

Measurement of H₂O₂, Nitrite, and Nitrate—The assay of H₂O₂ production was based, as reported previously (16), on the H₂O₂-dependent oxidation of homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid) to a highly fluorescent dimer (2,2'-dihydroxydiphenyl-5,5'-diacetic acid), which is mediated by horseradish peroxidase (18). Nitrite and nitrate determinations were performed as described previously (16) and based on the methodology of Braman and Hendrix (19). Total NO_x (NO₂⁻ plus NO₃⁻) determinations were made by monitoring NO evolution from a measured sample placed into a boiling VCl₃/HCl solution (which will reduce both NO₂⁻ and NO₃⁻ to NO). Quantitation was accomplished using a standard curve made up of known amounts of NO₂⁻ and NO₃⁻.

Isolation, Identification, and Culture of the HSE—Male C57BL/6J mice (10–12 weeks old) were from Charles River Laboratories, Inc. (Barcelona, Spain). The HSE was separated and identified as described previously (20). Sinusoidal cells were separated in a 17.5% (w/v) metrizamide gradient. HSE cultures were established and maintained in pyrogen-free DMEM supplemented as described above for the B16M-F10 cells. Differential adhesion of endothelial cells to the collagen matrix and washing allowed complete elimination of other sinusoidal cell types (Kupffer and stellate cells and lymphocytes) from the culture flasks.

B16M/Endothelial Cell Adhesion and Cytotoxicity Assays—B16M-F10 cells were loaded with 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR). (10⁶ cells were incubated in 1 ml of HEPES-buffered DMEM containing 50 μ g of BCECF-AM and 5 μ l of Me₂SO for 20 min at 37 °C.) After washing, BCECF-AM-containing cells were resuspended in HEPES-buffered DMEM without phenol red at a concentration of 2.5 \times 10⁶ cells/ml and added (0.2 ml/well) to endothelial cells (plated 24 h before) and also to plastic- or collagen-precoated control wells. The plates were then incubated at 37 °C, and 20 min later, the wells were washed three times with fresh medium and read for fluorescence using a Fluoroskan Ascent FL (LabSystems, Manchester, UK). The number of adherent tumor cells was quantified by arbitrary fluorescence units based on the percentage of the initial number of B16M cells added to the HSE culture (21). Damage to B16M cells during their *in vitro* adhesion to the HSE was measured as described previously (22) using tumor cells loaded with calcein acetoxymethyl ester (Molecular Probes). Other reagents used in experiments of tumor cytotoxicity were from Sigma.

Reverse Transcription-PCR and Detection of mRNA Expression—Total RNA was isolated using the TRIzol kit (Invitrogen) following the manufacturer's instructions. cDNA was obtained using a random hexamer primer and a MultiScribe reverse transcription kit (TaqMan reverse transcription reagents, Applied Biosystems, Foster City, CA) as described by the manufacturer. A PCR Master Mix and AmpliTaq Gold DNA polymerase (Applied Biosystems) containing specific primers (synthesized by Integrated DNA Technologies according to published sequences available from the GenBank™ Data Bank) were then added: iNOS, 5'-CGGATATCTCTTGCAA-GTCCAAA (forward) and 5'-AAGTATGTGTCTGCAG-ATATG (reverse); eNOS, 5'-CACCAGGAAGAAGACCTT-

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TAAAGGA (forward) and 5'-CACACGCTTCGCCATCAC (reverse); *bcl-2*, 5'-CTCGTCGCTACCGTCGTGACTTCCG (forward) and 5'-CAGATGCCGGTTCAGGTACTCAGTC (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5'-CCTGGAGAAACCTGCCAAGTATG (forward) and 5'-GGTCTCAGTGTAGCCCAAGATG (reverse). Real-time quantitation of the mRNA relative to glyceraldehyde-3-phosphate dehydrogenase was performed with a SYBR Green I assay and a iCycler detection system (Bio-Rad). Target cDNA was amplified as follows: 10 min at 95 °C and then 40 cycles of amplification (denaturation at 95 °C for 30 s and annealing and extension at 60 °C for 1 min per cycle). The increase in fluorescence was measured in real time during the extension step. The threshold cycle (C_T) was determined, and then the relative gene expression was expressed as follows: -fold change = $2^{-\Delta(\Delta C_T)}$, where $\Delta C_T = C_T \text{ target} - C_T \text{ glyceraldehyde-3-phosphate dehydrogenase}$ and $\Delta(\Delta C_T) = \Delta C_T \text{ treated} - \Delta C_T \text{ control}$.

Bcl-2 Analysis—Bcl-2 protein was quantitated in the soluble cytosolic fraction by enzyme immunoassay (23) using a monoclonal antibody-based assay from Sigma (1 unit of Bcl-2 is defined as the amount of Bcl-2 in 1000 non-transfected B16M-F10 cells).

eNOS-deficient Mice—Generation of eNOS-deficient mice was carried out as described previously (24). We interbred heterozygous (+/-) eNOS-deficient mice to generate eNOS^{+/+} and eNOS^{-/-} mice. We used eNOS^{+/+} and wild-type C57BL/6J mice as controls. Genotyping of the animals was performed by Southern blotting DNA from tail biopsies. The identification of eNOS^{+/+} and eNOS^{-/-} mice was as described previously (24). Briefly, 20- μ g samples were digested with BamHI, separated on 1.0% agarose gels, and transferred to nylon-supported nitrocellulose. The blots were then hybridized using a random primer-labeled 1.4-kb eNOS cDNA probe (24). A 5.3-kb fragment was diagnostic of the endogenous eNOS locus, and a 6.4-kb fragment was diagnostic of the targeted allele.

Measurement of iNOS Activity in B16M-F10 Cells—Conversion of L-arginine to L-citrulline was measured by a modification of a previously described methodology (25). The assay was carried out by adding 100 μ g of sample protein to 150 μ l of assay buffer (50 mM HEPES, 1 mM dithiothreitol, 1 mM MgCl₂, 5 mg/liter pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 3 mg/liter aprotinin, pH 7.4) containing 70 μ M arginine, 250,000 dpm L-[³H]arginine, (GE Healthcare, Little Chalfont, UK), 2 mM NADPH, 5 μ M tetrahydrobiopterin, 5 μ M FAD, and 0.5 mM CaCl₂ to measure total NOS activity or in the presence of 1 mM EGTA (without calcium) to determine Ca²⁺-independent iNOS activity. After 30 min at 37 °C, the reaction was stopped with 100 μ l of 1 M trichloroacetic acid. The samples were adjusted to pH 4.6 by adding 500 μ l of 20 mM HEPES and applied to Dowex AG 50W-X8 resin columns. L-[³H]Citrulline was eluted and separated by thin-layer chromatography. Radioactivity was counted with an AR-2000 scanner detector (Bioscan, Inc., Washington, D. C.). The results are expressed as pmol of L-[³H]citrulline/mg of protein/min.

Western Blot Analysis—Cultured cells were harvested as indicated above and then washed twice with ice-cold Krebs-Henseleit bicarbonate buffer, pH 7.4. Whole cell extracts were

made by freeze-thaw cycles in buffer containing 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin, pH 7.4. Fifty μ g of protein (as determined by the Bradford assay (68)) were boiled in Laemmli buffer and resolved by 12.5% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and subjected to Western blotting with anti-cAMP-response element-binding protein (CREB) or anti-phospho-CREB (Ser¹³³) monoclonal antibody (Chemicon International Inc., Temecula, CA). Blots were developed using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL system, Amersham Biosciences).

Gene Silencing—Small interfering RNA (siRNA) transfection experiments were performed with double-stranded RNA designed and synthesized by Eurogentec (Seraing, Belgium). A CREB-specific siRNA sense orientation strand (5'-UACAGCUG-GCUAACA AUGGdTdT-3') was used. Cells were transfected with the siRNA delivery reagent jetSITM (Eurogentec) at 3 μ l/ μ g of siRNA according to the manufacturer's instructions. Transfection efficiency in cells plated on coverslips was determined with fluorescein isothiocyanate-labeled siRNA and determined by cell counting using a TCS-SP2 confocal microscope (Leica Microsystems, Bensheim, Germany) to be 90–95% after 24 and 48 h.

Guanylate Cyclase Assay—Enzyme activity (soluble and particulate) was determined as described previously (26, 27). Briefly, the reaction mixture contained 7.5 pmol of Tris-HCl, pH 7.6, 0.75 μ mol of creatine phosphate, 0.5 μ mol of cGMP, 0.45 μ mol of MgCl₂, 1.2 μ mol of theophylline, 0.6 units of creatine kinase, and 37.5 nmol of [8-³H]GTP (10–12 mCi/mmol; PerkinElmer Life Sciences) in a total reaction volume of 150 μ l. The radioactive cGMP produced was isolated as described previously (28) and measured using a Packard Tri-Carb 2700TR Varisette analyzer (PerkinElmer Life Sciences).

Assay of Sphingomyelinases—This procedure was a modification of a previously reported methodology (29). B16M-F10 cells (5×10^6) were separated from the co-cultured HSE as described previously (30), washed twice with ice-cold phosphate-buffered saline, and homogenized in 0.4 ml of lysis buffer (10 mM HEPES/KOH, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 100 μ M pepstatin, 0.15 units/ml aprotinin, and 50 mg/ml leupeptin) containing 0.1% Triton X-100. The homogenate was passed through a 25-gauge needle and then centrifuged at 100,000 \times g for 1 h at 4 °C. The supernatant was used as an enzyme source. The mixture used to assay magnesium-dependent neutral sphingomyelinase (SMase) contained 0.1 M Tris-HCl, pH 7.5, 50 nmol of [*N*-methyl-¹⁴C]sphingomyelin (specific activity, 55 mCi/mmol; GE Healthcare), 10 mM MgCl₂, 0.1% Triton X-100, and 200 μ g of protein in a final volume of 0.2 ml. For magnesium-independent neutral SMase, MgCl₂ was removed from the reaction mixture. For acid SMase, 0.1 M sodium acetate, pH 5.0, was used instead of Tris-HCl. Incubation was carried out at 37 °C for 30 min. The reaction was stopped by adding 1.25 ml of chloroform/methanol (2:1). Then, 0.25 ml of double-distilled water were added to the tubes and vortexed. The tubes were centrifuged at 1000 \times g for 6 min to separate the two phases. The clear upper phase was removed, placed in a glass scintilla-

tion vial, and counted with a scintillation counter (Packard Tri-Carb 2700TR Varisette).

C_{16} -ceramide and C_{16} -dihydroceramide Analysis—The amounts of ceramide and dihydroceramide in B16M-F10 cells were measured as described previously (31). Briefly, cancer cell suspensions (5×10^6 cells in 200 μ l of DMEM) were vigorously mixed with 4 ml of chloroform/methanol (2:1, v/v) for 20 min. Then, 0.8 ml of distilled water were added to the mixture, and the sample was vortexed and centrifuged. The lower layer was collected, and the chloroform was allowed to evaporate. The residue was dissolved in a solvent and subjected to liquid chromatography-mass spectrometry analysis using a Quattro micro triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Shimadzu LC-10ADV pump and SCL-10AVP controller system with an SIL-10ADV autoinjector. Samples were analyzed by reverse-phase high pressure liquid chromatography using a Phenomenex ODS column (35 \times 2 mm) with 5- μ m particle size. In all cases, 40 μ l were injected onto the column. The temperature of the column was maintained at 25 $^{\circ}$ C. Mobile phases were as follows: mobile phase A, 5 mM ammonium formate/methanol/tetrahydrofuran (5:2:3, v/v); and mobile phase B, 5 mM ammonium formate/methanol/tetrahydrofuran (1:2:7, v/v) containing 0.01% formic acid. Elution was carried out at a flow rate of 0.2 ml/min with 70% mobile phase A and 100% mobile phase B for 6.3 min in a linear gradient mode.

Cell Death Analysis—Apoptotic and necrotic cell death were distinguished by fluorescence microscopy (32). For this purpose, isolated cells were incubated with Hoechst 33342 (10 μ M; which stains all nuclei) and propidium iodide (10 μ M; which stains nuclei of cells with disrupted plasma membranes) for 3 min and analyzed using a Nikon Diaphot 300 fluorescence microscope with excitation at 360 nm. Nuclei of viable, necrotic, and apoptotic cells were observed as blue round nuclei, pink round nuclei, and fragmented blue or pink nuclei, respectively. About 1000 cells were counted each time. DNA strand breaks in apoptotic cells were assayed using a direct terminal transferase dUTP nick end labeling assay (Roche Applied Science) and fluorescence microscopy following the manufacturer's methodology.

Assay for *in Vitro* Invasion of the Hepatic Endothelial Cell Monolayer by B16M-F10 Cells—Invasion of the endothelial cell monolayer by B16M-F10 cells was assayed following the method by Ohigashi *et al.* (33) with some modifications. HSE cells were seeded on 1% gelatin-coated tissue culture dishes with grids. When the cells reached confluency, the culture medium was replaced with fresh medium. After a 2-h incubation, the cultures were washed with DMEM, and then HSE cells were overlaid with B16M-F10 cells and cocultured for 5 days. The invasion capacity of B16M-F10 cells was measured by counting the number of colonies/1 cm^2 formed under the HSE monolayer using a phase-contrast microscope.

Statistical Analysis—Data are presented as the means \pm S.D. for the indicated number of different experiments. Statistical analyses were performed using Student's *t* test, and *p* values <0.05 were considered significant.

TABLE 1

Effect of t-PTER and QUER on NO and H_2O_2 generation by B16M-F10 cells growing *in vitro*

B16M-F10 cells were cultured as described under "Experimental Procedures." t-PTER (40 μ M) and QUER (20 μ M) were added at 23 h after seeding and were present for only 60 min. After the 60-min period in the presence of polyphenols, the culture flasks were washed out three times with phosphate-buffered saline, and the medium was renewed. Data represent the total amount of nitrite plus nitrate (NO_x) and H_2O_2 that accumulated in the culture medium during a 3-h period starting 12 h after removal of the polyphenols. During the incubation period, B16M-F10 cells maintained a viability of $>95\%$ (data not shown). Values represent the means \pm S.D. for five to six different experiments.

Addition	NO_x nmol/ 10^6 cells	H_2O_2 nmol/ 10^6 cells
Basal medium	0.43 \pm 0.08	12.6 \pm 2.9
t-PTER	0.27 \pm 0.06 ^a	10.5 \pm 1.6
QUER	0.15 \pm 0.04 ^a	9.4 \pm 1.7
t-PTER + QUER	0.05 \pm 0.02 ^a	8.3 \pm 1.4 ^b

^a *p* <0.01 comparing each value *versus* the control (basal medium).

^b *p* <0.05 .

RESULTS

Effect of t-PTER and QUER on NO and H_2O_2 Generation by Melanoma Cells—Growing B16M-F10 cells generate NO and H_2O_2 (16). Constitutive production of endogenous NO by iNOS has an anti-apoptotic role in human melanoma cells (34), whereas endogenous H_2O_2 , as well as other reactive oxygen species, acts as an intracellular messenger regulating cell growth and differentiation (35). Natural polyphenols are potential iNOS inhibitors (8, 9) and antioxidants (36). Thus, we tested the effect of t-PTER and QUER on NO and H_2O_2 production by B16M-F10 cells. To mimic *in vivo* conditions, we incubated B16M-F10 cells for a limited period (60 min) in the presence of t-PTER (40 μ M) and/or QUER (20 μ M) (approximate mean values of the concentrations of PTER and QUER measured in plasma during the first hour after intravenous administration of 20 mg of each polyphenol/kg) (6). As shown in Table 1, t-PTER and QUER decreased NO, and their effect was synergic when both were present (reaching almost 90% inhibition of NO generation). Reverse transcription-PCR expression analysis revealed that the t-PTER- and/or QUER-induced decrease in NO generation by B16M-F10 cells was associated with inhibition of iNOS gene expression (Fig. 1). However, H_2O_2 production was only significantly decreased ($\sim 35\%$) when both polyphenols were present (Table 1).

Endogenous NO may also be synthesized in melanoma cells by other NOS activities (endothelial (*e.g.* Ref. 34) and mitochondrial (37)). However, the effect t-PTER and QUER on NO generation by B16M-F10 cells (Table 1) was similar to that obtained when these cells were cultured for 12 h in the presence of a specific iNOS inhibitor (2 mM aminoguanidine (AMG)) added to 24-h cultured cells ($\text{NO}_x = 0.08 \pm 0.03$ nmol/ 10^6 cells, calculated as described in the legend to Table 1). iNOS activity was 3.0 ± 0.5 or <0.1 pmol of [^3H]citrulline/mg of protein/min in control and AMG-treated B16M-F10 cells, respectively. These results indicate that endogenous NO in B16M-F10 cells is derived mainly from iNOS and that this enzyme is a main target for the polyphenols.

Furthermore, QUER undergoes rapid systemic metabolism once it is absorbed in the body. Its major metabolite, QUER 3'-sulfate, can be found in substantially higher concentrations than the parent compound in the plasma (38). However, it

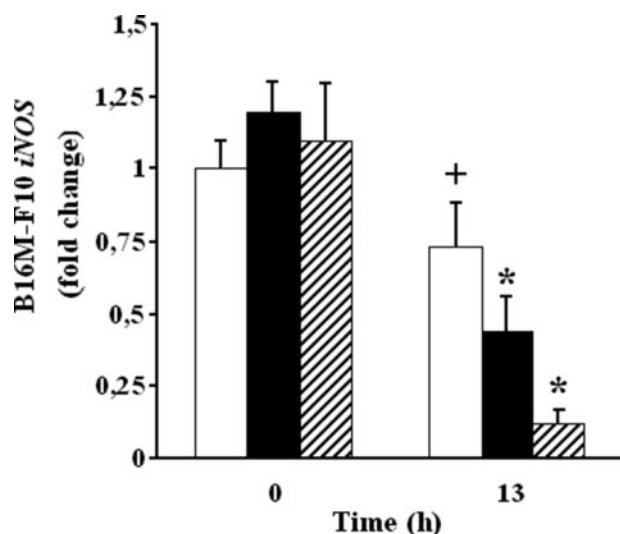


FIGURE 1. Expression of the iNOS gene in B16M-F10 cells treated *in vitro* with t-PTER and/or QUER. B16M-F10 cells were cultured and treated as described in the legend to Table 1. iNOS gene expression was measured at 23 h after seeding and at 12 h after removal of the polyphenols. The data are expressed as -fold change and are the means \pm S.D. for four to five different experiments. White bars, t-PTER; black bars, QUER; hatched bars, t-PTER and QUER. +, $p < 0.05$; *, $p < 0.01$ comparing 0 and 13 h after polyphenol addition.

remains unclear whether QUER 3'-sulfate exhibits any activity upon NO production. To clarify this, QUER metabolites were analyzed in plasma after administration of 20 mg of QUER/kg to B16M-F10-bearing mice as described (6). Extraction of QUER metabolites from plasma and HPLC analysis were performed exactly as described by De Pascual-Teresa *et al.* (38). The HPLC profile showed a main peak that was identified as QUER 3'-sulfate by its tandem mass spectra. A calibration curve was obtained using a standard of QUER 3'-sulfate (0.01–50 μM ; Extrasynthèse S.A., Bordeaux, France), which was found to be linear with a correlation coefficient of >0.99 . The limits of detection and quantitation for our method were 0.02 μM . QUER 3'-sulfate levels in plasma were <0.1 , 1 ± 0.2 , 3 ± 0.6 , 10 ± 1.7 , 6 ± 0.9 , 1 ± 0.3 , and <0.1 μM at 5, 10, 20, 30, 60, 90, and 120 min, respectively, after intravenous administration of QUER. Cultured B16M-F10 cells were exposed to 8 μM QUER 3'-sulfate (approximate mean value of the plasma concentrations measured at 30–60 min after intravenous administration of 20 mg of QUER/kg) for 30 min using the same approach used for QUER and t-PTER that respects bioavailable concentrations (see Table 1). However, in the presence of QUER 3'-sulfate, NO_x and H_2O_2 values were not significantly different from controls ("Basal medium" in Table 1). Therefore, we conclude that, although bioavailable concentrations of QUER decrease NO generation by the cancer cells, its main metabolite does not.

Effect of t-PTER and QUER on NO and H_2O_2 Generation during Melanoma Cell Adhesion to the Vascular Endothelium— Interaction of cancer and endothelial cells in capillary beds is a critical step in the initiation of metastasis (39). The mechanism of B16M-F10/HSE interaction includes mannose receptor-mediated melanoma cell attachment to the HSE, which subsequently causes cytokine (tumor necrosis factor- α , interleukin-1B, and interleukin-18) and NO/ H_2O_2 release, VCAM-1-dependent adherence,

TABLE 2

Effect of t-PTER and QUER on *in vitro* NO and H_2O_2 generation during B16M-F10 cell interaction with the HSE

24-h cultured HSE cells (\pm polyphenol(s) added at 12 h after plating and removed by washing 60 min later) were co-cultured with 72-h cultured B16M cells (\pm polyphenol(s)) as follows. t-PTER (40 μM) and QUER (20 μM) were added at 6, 30, and 54 h of culture time and were present each time for only 60 min. After the 60-min period in the presence of polyphenols, the culture flasks were washed out three times with phosphate-buffered saline, and the medium was renewed. At 20 min after B16M-F10 cell addition to the HSE, the plates were washed as described under "Experimental Procedures," and the measured ratio of tumor cells adhering to the HSE was $\sim 1:1$. Data represent the total amount of nitrite plus nitrate (NO_x) and H_2O_2 that accumulated in the culture medium during a 3-h period. During this incubation period, both HSE and B16M-F10 cells maintained a viability of $>95\%$ (data not shown). Values represent the means \pm S.D. for five to six different experiments.

Addition	NO_x		H_2O_2	
	nmol/ 10^6 cells	HSE	nmol/ 10^6 cells	HSE
Basal medium	2.5 ± 0.6	90 ± 8	38 ± 7	66 ± 12
t-PTER	3.7 ± 0.8	86 ± 11	45 ± 8	70 ± 10
QUER	5.0 ± 1.0^a	89 ± 14	41 ± 7	73 ± 11
t-PTER + QUER	7.2 ± 1.2^a	84 ± 10	42 ± 10	69 ± 13

^a $p < 0.01$ comparing each value with the control (basal medium).

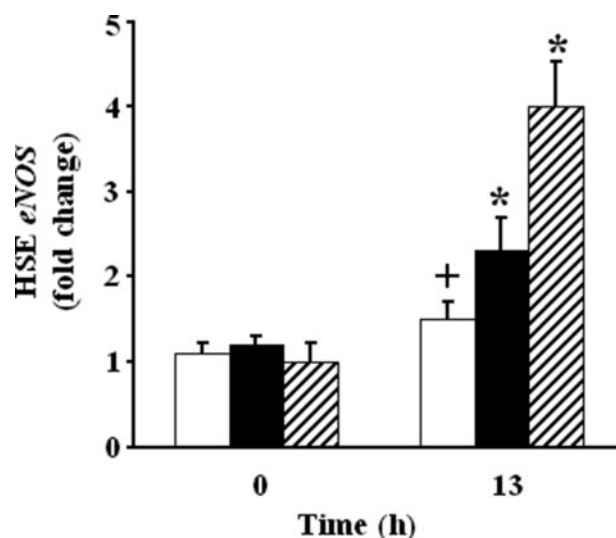


FIGURE 2. Expression of the eNOS gene in HSE cells treated *in vitro* with t-PTER and/or QUER. HSE cells were cultured in the presence or absence of t-PTER (40 μM) and QUER (20 μM) as described in the legend to Table 2. eNOS gene expression was measured at 23 h after seeding and at 12 h after removal of the polyphenols. The data are expressed as -fold change and are the means \pm S.D. for four to five different experiments. White bars, t-PTER; black bars, QUER; hatched bars, t-PTER and QUER. +, $p < 0.05$; *, $p < 0.01$ comparing 0 and 13 h after polyphenol addition.

and melanoma growth factor release by the HSE (e.g. Ref. 40). Endothelium-derived reactive nitrogen and oxygen species and OONO radicals in particular are harmful and lethal for a high percentage of adherent melanoma cells (16). Thus, because t-RESV can increase eNOS activity (14), in the second set of experiments, we used an *in vitro* B16M-F10/endothelial cell adhesion assay to investigate the effect of t-PTER and QUER on NO and H_2O_2 production during the process of tumor cell attachment to the HSE. As shown in Table 2, during B16M-F10/HSE interaction, QUER and t-PTER/QUER significantly increased NO release to the extracellular medium, whereas H_2O_2 generation was not affected. Reverse transcription-PCR expression analysis revealed that the t-PTER- or t-PTER/QUER-induced increase in NO generation by B16M-F10 cells was associated with an increase in eNOS gene expression (Fig. 2). This is important because, as

TABLE 3

Effect of t-PTER and QUER on the rates of B16M-F10 cell adhesion, cytotoxicity, and invasion during interaction with the HSE

HSE and B16M-F10 cells were co-cultured in the presence or absence of t-PTER (P; 40 μM) and QUER (Q; 20 μM) as described in the legend to Table II. In melanoma/endothelial cell adhesion experiments, at 20 min after B16M-F10 cell addition to the HSE, the plates were washed as described under "Experimental Procedures." In endothelium-induced B16M-F10 cell cytotoxicity assays, tumor cytotoxicity (expressed as the percentage of tumor cells that lost viability within the 4–6-h period of incubation; see "Experimental Procedures") was determined after 6 h of incubation. During the 6-h period of incubation, HSE cell viability was 99–100% in all cases. Values represent the means \pm S.D. for five to six different experiments in each case.

HSE	Tumor cell adhesion		NO _x		H ₂ O ₂		Tumor cell cytotoxicity		Apoptotic cells		No. of penetrated colonies/cm ²	
	Control	P + Q	Control	P + Q	Control	P + Q	Control	P + Q	Control	P + Q	Control	P + Q
	%		nmol/10 ⁶ cells		nmol/10 ⁶ cells		%		%			
eNOS ^{+/+}	65 \pm 12	31 \pm 7 ^a	2.2 \pm 0.4	7.7 \pm 1.0 ^a	36 \pm 5	42 \pm 7	10 \pm 3	56 \pm 8 ^a	5 \pm 2	42 \pm 7 ^a	77 \pm 9	13 \pm 5 ^a
eNOS ^{-/-}	61 \pm 11	34 \pm 5 ^a	0.2 \pm 0.1 ^b	0.4 \pm 0.1 ^b	35 \pm 6	39 \pm 6	1 \pm 0.5 ^b	3 \pm 1 ^b	1 \pm 0.5 ^b	1 \pm 0.5 ^b	152 \pm 16 ^b	132 \pm 17 ^b

^a $p < 0.01$ comparing t-PTER and QUER versus no polyphenol addition.

^b $p < 0.01$ comparing eNOS^{-/-} versus eNOS^{+/+} cells.

reported previously (16), just an increase in the metastatic microenvironment NO levels is enough to increase cancer cell death.

To calculate the percentage of NO_x produced by the HSE during the adhesion process, in parallel experiments, we used HSE cells precultured for 12 h in the presence of 1 mM N^G-nitro-L-arginine methyl ester, which blocks NO synthesis activity (41). (Under these conditions, after being washed with medium and cultured in the absence of tumor cells, HSE cells do not produce detectable amounts of NO₂⁻ or NO₃⁻ in the following 4 h.) Similar results were obtained when NO_x produced by the HSE was calculated as follows: NO_x produced by co-cultured HSE and B16M cells minus NO_x produced by cultured B16M-F10 cells alone (data not shown). The percentage of H₂O₂ generated by the HSE was calculated by subtracting H₂O₂ produced by co-cultured HSE and B16M-F10 cells minus H₂O₂ produced by cultured B16M-F10 cells alone (data not shown). Our data show that, during the adhesion process, most of the NO and H₂O₂ were generated by the HSE (Table 2). Preculture of endothelial cells in the presence of N^G-nitro-L-arginine methyl ester did not alter the percentage of B16M cell adhesion (data not shown) compared with controls (see the legend to Table 2).

Effect of t-PTER and QUER on Melanoma Cell Adhesion, Viability, and Invasive Growth upon Interaction with the Vascular Endothelium—The potential increase in the rate of metastatic cell death induced by the polyphenols was further investigated and correlated with NO and H₂O₂ generation, the percentage of tumor cell adhesion to the HSE, and the rate of invasion through HSE monolayers. These experiments were performed using control HSE cells and HSE cells isolated from eNOS-deficient (eNOS^{-/-}) mice to abolish eNOS-dependent NO production. Because the effects of t-PTER and QUER on NO_x generation either by the B16M-F10 cells (Table 1) or during cancer and endothelial cell interaction (Table 2) were practically additive, in the next experiments, we focused on the polyphenol association. As shown in Table 3, the percentage of B16M-F10 cell adhesion to the endothelium decreased in the presence of polyphenols, in agreement with the previously reported PTER-induced inhibition of VCAM-1 expression in the HSE (6). In the presence or absence of polyphenols, NO generation was almost abolished in eNOS^{-/-} HSE cells (Table 3), implying that the t-PTER- and QUER-induced increase in NO production is fully dependent on eNOS. The t-PTER- and QUER-induced increase (5–6-fold) in the percentage of B16M-

F10 cell death (mainly apoptotic) during cancer and endothelial cell interaction was also NO-dependent (Table 3). Furthermore, when assaying the *in vitro* invasion of hepatic endothelial cell monolayers by B16M-F10 cells, we found a marked decrease (~83%) in the number of penetrated colonies in the presence of t-PTER and QUER (Table 3); however, when eNOS^{-/-} HSE cells instead of control eNOS^{+/+} HSE cells were used, the number of colonies increased by ~2-fold, and the inhibition elicited by the polyphenols almost disappeared (Table 3). To further prove that NO is indeed involved in the mechanism of tumor cell killing, we added a NO scavenger (300 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt, Affiniti Research Products, Mamhead, UK) (42) to the co-culture medium. During HSE and B16M-F10 cell interaction in either the presence or absence of t-PTER and QUER (as in Table 3), NO_x values remained <0.1 nmol/10⁶ cells in the presence of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt. Under these conditions, tumor cell cytotoxicity was ~1% in either the presence or absence of t-PTER and QUER.

NO Regulates bcl-2 Expression in B16M-F10 Cells—We know from previously reported data that t-PTER- and QUER-induced inhibition of *bcl-2* expression sensitizes B16M-F10 cells to vascular endothelium-induced cytotoxicity (6). In addition, the results reported above show that HSE-induced B16M-F10 cell cytotoxicity is potentiated by t-PTER and QUER in a NO-dependent fashion (Table 3). However, we do not know if exposure to high NO levels (as those expected in the metastatic environment) (Table 3) also affects *bcl-2* expression and/or Bcl-2 levels in those cancer cells that survive the interaction with the vascular endothelium. To investigate this, we manipulated NO generation by incubating B16M-F10 cells in the presence of AMG and/or the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP). As shown in Table 4, AMG-induced iNOS inhibition was associated with a marked decrease in NO_x, *bcl-2* expression, and Bcl-2 levels. However, when 0.3 mM SNAP elicited recovery of NO_x levels to practically control values, *bcl-2* expression and Bcl-2 levels were found to be also similar to their respective controls (Table 4). In addition, when 3.0 mM SNAP promoted higher NO generation, a decrease in *bcl-2* expression and Bcl-2 levels was also measured (Table 4). Moreover, SNAP (0.3 mM) reversed the effects of t-PTER and QUER (Table 4), thus revealing a direct role of NO in t-PTER/QUER-induced Bcl-2 down-regulation. These results suggest that very low and high NO levels (compared with controls) may

NO Mediates Polyphenol-induced Melanoma Cell Death

TABLE 4

Effect of NO levels on *bcl-2* expression and Bcl-2 levels in B16M-F10 cells

AMG and/or SNAP was added to 12- or 24-h cultured B16M-F10 cells, respectively. t-PTER and QUER were added as indicated in the legend to Table I. NO_x levels were determined at 30 h after seeding. Reverse transcription-PCR analysis of *bcl-2* expression was performed at 36 after seeding, and measurement of Bcl-2 levels at 48 h after seeding. Values represent the means ± S.D. for five to six different experiments.

Addition	NO _x	<i>bcl-2</i>	Bcl-2
	nmol/10 ⁶ cells	-fold change	units/mg protein
Basal medium	0.39 ± 0.07	1.1 ± 0.2	32 ± 6
AMG (2 mM)	0.05 ± 0.02 ^a	0.1 ± 0.03 ^a	4 ± 1 ^a
SNAP (0.3 mM)	0.62 ± 0.12 ^a	0.9 ± 0.2	30 ± 5
SNAP (3 mM)	7.15 ± 0.26	0.2 ± 0.07 ^a	7 ± 2 ^a
AMG (2 mM) + SNAP (0.3 mM)	0.33 ± 0.08	1.0 ± 0.2	30 ± 7
AMG (2 mM) + SNAP (3.0 mM)	6.08 ± 0.17 ^a	0.3 ± 0.09 ^a	9 ± 3 ^a
t-PTER (40 μM) + QUER (20 μM)	0.04 ± 0.01 ^a	0.05 ± 0.02 ^a	5 ± 1 ^a
t-PTER (40 μM) + QUER (20 μM) + SNAP (0.3 mM)	0.36 ± 0.07	1.0 ± 0.1	35 ± 4
t-PTER (40 μM) + QUER (20 μM) + SNAP (3 mM)	6.44 ± 0.23 ^a	0.3 ± 0.05 ^a	10 ± 4 ^a

^a *p* < 0.01 comparing each value with the control (basal medium).

have similar effects on *bcl-2* expression and Bcl-2 levels. In this sense, t-PTER and QUER administration may represent a double advantage *in vivo*. Polyphenol-induced down-regulation of the iNOS gene (and consequently of *bcl-2*) in B16M-F10 cells may render metastatic cells more susceptible to immune- and endothelium-elicited cytotoxicity, and on the other hand, polyphenol-induced up-regulation of endothelial eNOS can facilitate cytotoxic NO-dependent cancer cell death.

t-PTER- and QUER-induced NO Shortage in B16M-F10 Cells Down-regulates the Activity of CREB—The transcription factor CREB mediates survival in many cells, including cancer cells (43). CREB and its associated proteins act as survival factors for human melanoma cells and hence contribute to the acquisition of their malignant phenotype (44). CREB proteins function as positive regulators of the *bcl-2* gene via direct binding of CREB and activating transcription factor 1 family members to a cAMP-response element within the *bcl-2* promoter (45). Recently, it was shown that sustained inhibition of NO production triggers apoptosis in differentiated cerebellar granule neuron cultures, a fact that is associated with down-regulation of important survival factors such as Akt/protein kinase B and CREB (46). Ciani *et al.* (46) identified *bcl-2* as one of the anti-apoptotic genes down-regulated by decreased CREB phosphorylation.

The mechanism of CREB transcriptional activation depends on phosphorylation of Ser¹³³, which may be mediated by different kinases (*e.g.* Ref. 47). Thus, we investigated this possibility in B16M-F10 cells and found that t-PTER and QUER caused inhibition of CREB phosphorylation (Fig. 3). This effect was similar to the NO shortage-dependent inhibition of CREB phosphorylation observed in the presence of AMG (Fig. 3). The t-PTER- and QUER-induced decrease in phospho-CREB was prevented in the presence of 0.3 mM SNAP (Fig. 3), thus indicating a direct link between NO generation and CREB phosphorylation. Therefore, a NO shortage-dependent decrease in phospho-CREB could be, at least in part, the mechanism involved in the polyphenol-induced decrease in *bcl-2* expression. To test this hypothesis further, B16M-F10 cells were loaded by electroporation (Bio-Rad system, 1.0 kV/cm with a time constant of 50 ms) with anti-phospho-CREB monoclonal antibody (see “Experimental Procedures” and Ref. 48 for technical details). A decrease in *bcl-2* expression (~80% compared with controls; data not shown), not significantly different from that induced

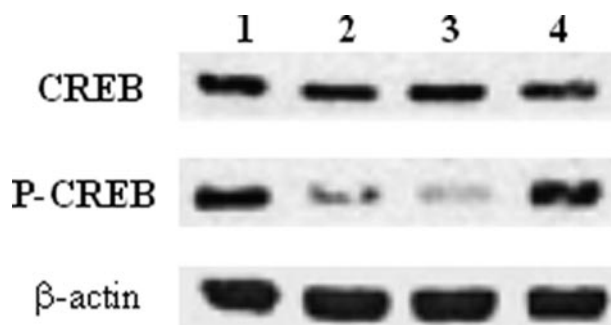


FIGURE 3. Effect of t-PTER and QUER on CREB phosphorylation at Ser¹³³ in B16M-F10 cells. AMG (2 mM) and SNAP (0.3 mM) were added to 24- and 36-h cultured B16M-F10 cells, respectively. t-PTER (40 μM) and QUER (20 μM) were added at 23 h after seeding and were present for only 60 min. Whole cell extracts obtained 24 h after AMG or polyphenol addition were subjected to Western blotting. Proteins were probed with antibody specific for CREB phosphorylated at Ser¹³³ (P-CREB), CREB, or β-actin. Lane 1, control; lane 2, AMG; lane 3, t-PTER and QUER; lane 4, t-PTER, QUER, and SNAP.

by 2 mM AMG (Table 4), was observed in the presence of anti-CREB antibody. Moreover, silencing of CREB using specific siRNA was associated with lower *bcl-2* expression in B16M-F10 cells (Fig. 4). In addition, PTER/QUER-induced *bcl-2* down-regulation was reversed by a guanylate cyclase activator (YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole, Yung Shin Pharmaceutical Industry Co., Ltd., Taichung, Taiwan) (Fig. 5). These results prove the direct connection of PTER/QUER to CREB and to *bcl-2* expression. *bcl-2* expression and phospho-CREB levels returned to control values (as in Fig. 4) (data not shown) in surviving B16M-F10 cells after 12 h of co-culture with endothelial cells, in agreement with the data showing that the effect of t-PTER and QUER could be reversed by NO directly (Table 4).

t-PTER- and QUER-induced NO Release from the Vascular Endothelium Up-regulates Neutral SMase Activity in B16M-F10 Cells—During interaction of metastatic and endothelial cells, t-PTER- and QUER-induced up-regulation of the eNOS gene (Fig. 2) also promoted an increase in NO levels in the metastatic microenvironment (Table 2). Consequently, t-PTER and QUER further potentiated the NO-dependent tumor cytotoxicity elicited by the endothelium (Table 3). NO-induced tumor cytotoxicity is due mainly to the formation of potent oxidants such as [•]OONO radicals via a trace metal-dependent process (16). However, during B16M-F10/HSE interaction, H₂O₂ levels were not altered in the presence of t-PTER and

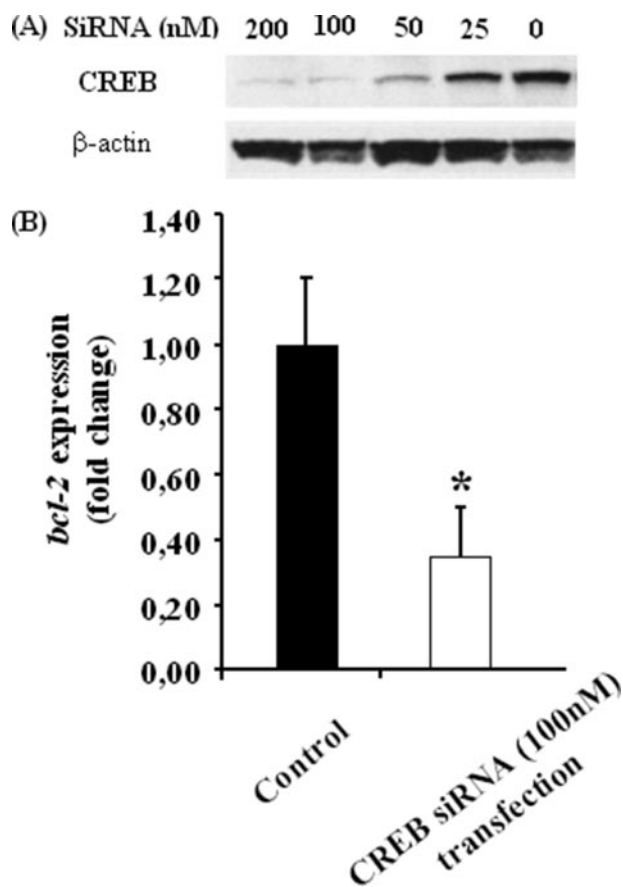


FIGURE 4. Silencing of CREB using specific siRNA is associated with *bcl-2* down-regulation in B16M-F10 cells. Gene silencing experiments were performed as described under "Experimental Procedures." *A*, whole cell extracts obtained from control and CREB siRNA-transfected B16M-F10 cells at 24 h after seeding were subjected to Western blotting. Proteins were probed with specific antibodies as described in the legend to Fig. 3. *B*, *bcl-2* expression was analyzed as described in the legend to Table 4. Values represent the means \pm S.D. for four different experiments. *, $p < 0.01$.

QUER (Table 2). Therefore, a higher generation of highly reactive oxidants cannot be argued as a mechanism to explain the increased tumor cell death.

Experiments in human leukemia HL-60 cells have shown that NO-induced apoptosis is associated with an increase in ceramide generation (29). This lipid second messenger plays an important role in regulating cell growth, differentiation, and death (49). Thus, we investigated whether NO, at the level released during B16M-F10/HSE interaction in the presence of t-PTER and QUER, is linked to increased B16M-F10 cell death via an increase in ceramide generation. As shown in Table 5, co-culture of B16M-F10 and eNOS^{+/+} cells indeed caused an increase (~2-fold) in ceramide within the cancer cells.

Ceramide can be generated through different mechanisms. First, it can be produced by dihydroceramide desaturase as dihydroceramide formed on microsome surfaces by *N*-acylation of sphinganine catalyzed by dihydroceramide synthase (50). However, control dihydroceramide levels measured in B16M-F10 cells (20 ± 6 pmol/mg of protein, $n = 5$) after co-culture with the HSE were not significantly changed in the presence of t-PTER and QUER. Second, ceramide is also formed by hydrolysis of sphingomyelin catalyzed by different SMase activities. Hence, we measured magnesium-dependent and -inde-

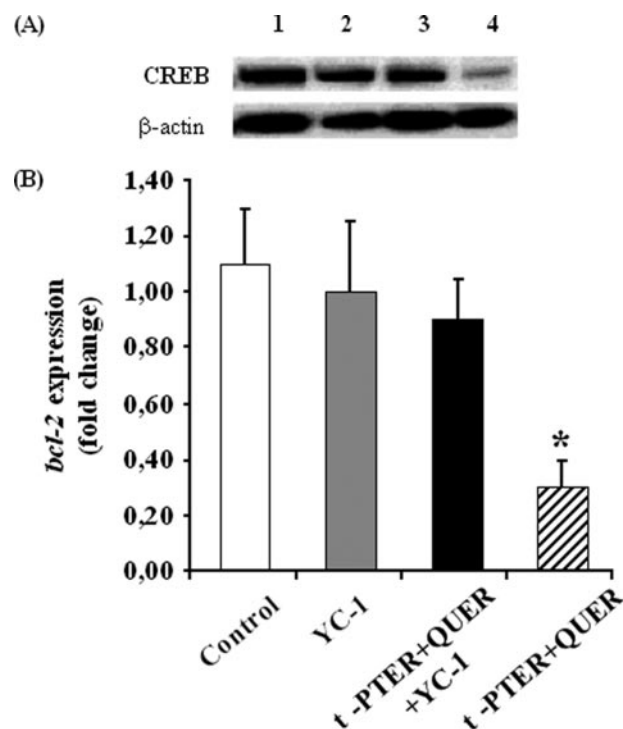


FIGURE 5. Guanylate cyclase activation reverses t-PTER/QUER-induced *bcl-2* down-regulation. YC-1 was added to 23-h cultured B16M-F10 cells. t-PTER (40 μ M) and QUER (20 μ M) were added at 23 h after seeding and were present for only 60 min. *A*, whole cell extracts obtained at 24 h after YC-1 or polyphenol addition were subjected to Western blotting. Proteins were probed with antibody specific for CREB phosphorylated at Ser¹³³ or β -actin. Lane 1, control; lane 2, YC-1; lane 3, t-PTER, QUER, and YC-1; lane 4, t-PTER and QUER. *B*, expression of *bcl-2* was measured as described in the legend to Table 4. Values are the means \pm S.D. for four to five different experiments. *, $p < 0.01$. Guanylate cyclase activity was 53 and 117 pmol of cGMP formed per min/mg of protein in control and YC-1-treated B16M-F10 cells, respectively ($n = 5$).

pendent neutral SMase and acid SMase activities in B16M-F10 cells after co-culture with the HSE and found that the magnesium-dependent neutral SMase activity was increased in the presence of polyphenol association (Table 5). Ceramide may promote apoptosis by interacting with apoptotic proteases or by signaling mitochondrial permeability transition (51). Kroemer and co-workers (52) showed that Bcl-2 is a highly efficient inhibitor of ceramide-induced mitochondrial permeability transition and release of apoptosis-inducing factor. Thus, t-PTER- and QUER-induced ceramide generation may be the mechanism that explains, at least in part, the increased death of Bcl-2-depleted B16M-F10 cells during their interaction with the vascular endothelium (Table 3). To test this hypothesis further, t-PTER- and QUER-pretreated B16M-F10 cells (as in Table 1) were incubated for 6 h in the presence of cell-permeable C₆-ceramide (Sigma) (53) at 75 pmol/mg of protein (Table 5). *In vitro* added exogenous ceramide caused $30 \pm 6\%$ ($n = 5$) apoptotic B16M-F10 cell death, which is significantly different from the data reported in Table 3 during interaction with eNOS^{+/+} HSE cells in the presence of t-PTER and QUER ($p < 0.05$). Therefore, although HSE-released NO has direct cytotoxic effects on B16M-F10 cells (due mainly to the formation of potent oxidants such as ⁻OONO) (16), the majority of cancer cell death appears to be directly associated with the polyphenol-induced increase in ceramide generation.

NO Mediates Polyphenol-induced Melanoma Cell Death

TABLE 5

Effect of t-PTER and QUER on SMase activities and ceramide levels in B16M-F10 cells during interaction with the HSE

SMase activities and ceramide levels in B16M-F10 cells are expressed as nmol/mg of protein/h and as pmol/mg of protein, respectively. These measurements were performed in isolated cancer cells after 3 h of interaction with the HSE. During this incubation period, both HSE and B16M-F10 cells maintained a viability of >90% (data not shown). NSMase, neutral SMase.

	B16M-F10			
	+eNOS ^{+/+}		+eNOS ^{-/-}	
	Control	t-PTER + QUER	Control	t-PTER + QUER
Mg-dependent NSMase	1.26 ± 0.34	2.35 ± 0.55 ^a	1.05 ± 0.23	1.17 ± 0.30 ^b
Mg-independent NSMase	0.50 ± 0.17	0.55 ± 0.14	0.47 ± 0.12	0.52 ± 0.20
Acid SMase	7.44 ± 2.10	6.82 ± 1.58	7.59 ± 1.41	7.12 ± 2.00
Ceramide	36 ± 12	75 ± 20 ^a	30 ± 8	34 ± 10 ^b

^a *p* < 0.01 comparing t-PTER + QUER versus no polyphenol addition.

^b *p* < 0.01 comparing eNOS^{-/-} versus eNOS^{+/+} cells.

However, despite the cytotoxic effect of ceramide reported above, the same ceramide concentration did not cause tumor cell death in viable B16M-F10 cells surviving 12 h after co-culture with endothelial cells (data not shown). To test whether this is directly linked to Bcl-2, we used an antisense *bcl-2* oligodeoxynucleotide to deplete Bcl-2 levels in B16M-F10 cells as described (7). *In vitro* treatment of B16M-F10 cells with the antisense *bcl-2* oligodeoxynucleotide (50 μM) decreased *bcl-2* expression and Bcl-2 levels to values that were not significantly different from those reported in Table 4 in the presence of AMG (2 mM). When antisense *bcl-2* oligodeoxynucleotide-treated cells were incubated for 6 h in the presence of 75 pmol of the cell-permeable C₆-ceramide (as described above) (Table 5), we observed 28 ± 5% (*n* = 4) apoptotic B16M-F10 cell death. Therefore, ceramide at the levels generated during B16M-F10 cell interaction with the vascular endothelium is not cytotoxic for cancer cells unless it is associated with Bcl-2 down-regulation.

DISCUSSION

Short-term exposure of B16M-F10 cells (high metastatic potential) to t-PTER and QUER at a concentration that is relevant under *in vivo* conditions decreases iNOS gene expression (Fig. 1) and NO generation (Table 1). NO shortage decreases CREB phosphorylation (Fig. 3) and *bcl-2* expression (Table 4). In parallel, polyphenol association increases eNOS gene expression in the vascular endothelium (Fig. 2), leading, during cancer and endothelial cell interaction, to higher endothelial NO release (Tables 2 and 3) and to higher tumor cytotoxicity (Table 3). Furthermore, during B16M-F10/endothelial cell adhesion, t-PTER and QUER induce a NO-dependent increase in magnesium-dependent neutral SMase activity in cancer cells and thereby in ceramide generation (Table 5). NO and ceramide act as molecular inducers of apoptosis in Bcl-2-depleted B16M-F10 cells (see "Results").

As stated in the Introduction, paradoxically, NO can promote apoptosis in some cells, whereas it inhibits apoptosis in others (see Ref. 10 for a review). NO-induced apoptosis in cells has been linked to high (supraphysiological) concentrations of NO (10) and appears to be regulated by the redox state and by formation of transition metal complexes (16, 54, 55). This concept is in agreement with the idea that activation of iNOS gene expression may facilitate cancer cell death and inhibition of metastases (56). On the other hand, anti-apoptotic signaling has been generally correlated with low or physiological NO lev-

els (10). However, it was reported recently that AMG-induced inhibition of endogenous NO synthesis does not affect cell cycle progression of human melanoma cells, but leads to cell death by apoptosis (34). In addition, it has been shown that, *in vitro*, t-RESV induces the apoptosis of leukemic B cells and simultaneously inhibits the production of NO through iNOS gene down-regulation (57). Nevertheless, the effects of t-RESV on 48-h cultured leukemic B cells are unrealistic: e.g. 3–50 μM t-RESV, constantly present in the culture medium, increases the number of fluorescein isothiocyanate-labeled annexin V-positive cells by 10–50%; 10–50 μM t-RESV decreases iNOS gene expression by 50–90%; and 50 μM t-RESV decreases Bcl-2 content by 25–60% (57). Bioavailability of natural polyphenols is a critical issue that must be investigated before correlating *in vitro* observations and biochemical effects (2). After intravenous administration to rabbits of 20 mg of t-RESV/kg (a dose that represents, for an adult human weighing 70 kg, ~140 times the maximum amount of total RESV found in 1 liter of red wine), its highest concentration in plasma (42.8 ± 4.4 μM at 5 min after administration) decreases very rapidly to 0.9 ± 0.2 μM (0.2 mg/liter) at 60 min and to <0.1 μM at 120 min (5). Following a similar protocol, we calculated a half-life of t-RESV in mouse plasma of ~10.2 min (6) and in human plasma of ~11.5 min.³ If the same amount of t-RESV (20 mg/kg) is administered orally, the highest concentration in plasma (2–3 μM in mice and ~1 μM in rabbits or rats) is found within the first 5 min after administration, followed by a decrease to <0.1 μM at 60 min (5). On the other hand, after intravenous administration to mice of 20 mg of t-PTER or QUER/kg (a dose that represents, for an adult human weighing 70 kg, ~1000 times the maximum amount of PTER found in 1 kg of dark grapes and ~20 times the maximum daily intake of QUER), their mean values of plasma concentrations measured within the first hour are 40 and 20 μM, respectively (6). Both t-PTER and QUER decrease *bcl-2* expression (6). Moreover, both polyphenols show synergic effects on NO generation by B16M-F10 and endothelial cells (Tables 1–3). Therefore, it is obvious that the association has advantages over each single polyphenol. Our present findings show that a link between iNOS down-regulation in malignant cells and polyphenols can be firmly established using the association of t-PTER and QUER at bioavailable concentrations (short-term exposure at mean plasma concentrations). How-

³ J. M. Estrela, M. Asensi, and P. Ferrer, unpublished data.

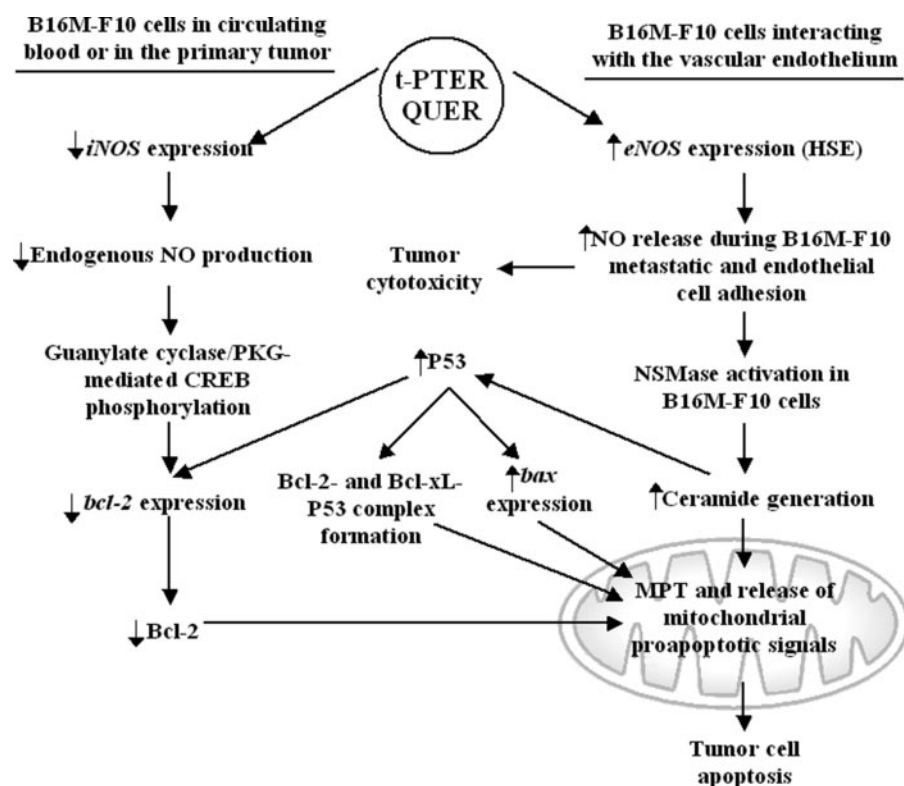


FIGURE 6. Molecular mechanisms in NO/Bcl-2-dependent metastatic cell death activation induced by t-PTER and QUER. A polyphenol-induced decrease in iNOS gene expression leads to Bcl-2 depletion in B16M-F10 cells (at steps previous to the interaction with the vascular endothelium). A t-PTER- and QUER-induced increase in eNOS gene expression increases NO release in the metastatic microenvironment. Reactive nitrogen species can directly damage the cancer cells, whereas NO can also promote apoptosis by increasing ceramide generation. The biochemical mechanisms described here take place in B16M-F10 cells with only one exception: the polyphenol-induced increase in eNOS gene expression and NO release from HSE cells. The multiple molecular inter-relationships are explained in detail under "Results" and "Discussion." PKG, cGMP-dependent protein kinase; NSMase, neutral sphingomyelinase; MPT, mitochondrial permeability transition.

ever, for this purpose, concentrations of t-PTER and QUER obtained after intravenous administration were necessary.

t-PTER, a naturally occurring analog of t-RESV but ~60–100 times stronger as an antifungal agent, shows similar anti-carcinogenic properties (58). t-PTER is present, for example, in extracts of the heartwood of *Pterocarpus marsupium*, used in Ayurvedic medicine for the treatment of diabetes, and in dark-skinned grapes (although quantitative studies have shown that, for every 10 parts t-RESV, there are only 1–2 parts t-PTER) (Ref. 58 and references therein). Human intake of flavones and flavonols (the most common flavonoids) is ~3–70 mg/day (mainly QUER, 60–75%; major sources include tea, wine, berries, apples, and onions) (2). Flavonoids are among the most potent antioxidants because they show one or more of the following structural elements: an *o*-diphenolic group, a 2,3-double bond conjugated with 4-oxo function, and OH groups at positions 3 and 5. QUER combines three properties, and previous research has confirmed that it also exhibits anti-tumor properties (2, 59). However, all bioactive polyphenols studied so far undergo conjugation in the intestinal tract of man and rodents. This conjugation is rapid and extensive. It is in this form that they are absorbed, with very little of the free polyphenol gaining access to the blood (60–63); in fact, the latter is so small that, theoretically, it could even arise from limited hydrolysis of the conjugates because blood is quite rich in glucuronidase, sul-

fatase, and other deconjugating enzymes. Orally administered polyphenols are unlikely to be biologically effective unless their biological effects are not inactivated by conjugation and/or the free polyphenol can be released by hydrolysis of conjugates and can be taken up by target tissues in amounts to generate pharmacologically active concentrations. There is no evidence so far that either of the above possibilities is true. These facts imply the need for intravenous administration to obtain pharmacologically active concentrations of natural polyphenols, at least for therapy applications in the case of systemically disseminated cancers. Nevertheless, doses required to inhibit metastatic growth *in vivo* may possibly depend on the tumor cell type.

CREB and its associated proteins act as survival factors for human melanoma cells (44). A NO shortage-induced decrease in CREB activity (Fig. 3) interferes with the cGMP-dependent protein kinase, the most important intracellular signaling pathway activated by NO (64). In addition, high levels of NO (released by the endothelium) promote an increase in ceramide for-

mation (Table 5), a potential apoptotic activator. Moreover, in cells expressing functional Bcl-2, the mechanism of death action for ceramide may also involve, at least in part, a p53-dependent suppression of *bcl-2* expression (65, 66). Recently, Mihara *et al.* (67) also showed that p53 promotes permeabilization of the outer mitochondrial membrane by forming complexes with the protective Bcl-x_L and Bcl-2 proteins. Moreover, the tumor suppressor p53 may also transactivate the expression of pro-apoptotic genes such as *bax* and of the cyclin-dependent kinase inhibitor p21 (65). Thus, theoretically, t-PTER and QUER may act against the tumor under *in vivo* conditions at different steps and by different mechanisms. Fig. 6 summarizes these mechanisms and their possible inter-relationships.

In conclusion, the more relevant and novel findings reported in this study are as follows. (a) At bioavailable concentrations, polyphenol-induced Bcl-2 down-regulation (via guanylate cyclase/cGMP-dependent protein kinase-mediated CREB phosphorylation) in pre-invasive B16M-F10 cells is NO-dependent; (b) by enhancing eNOS expression (HSE), polyphenols increase NO release from the vascular endothelium upon interaction with metastatic B16M-F10 cells; and (c) in the presence of polyphenols, NO-mediated neutral SMase activation and increased ceramide generation are the main cell death-activated mechanisms in Bcl-2-depleted metastatic B16M-F10 cells. Our results demonstrate that, at different steps, very low and high NO lev-

els act in parallel polyphenol-activated mechanisms, leading to metastatic cell death.

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