

Induction of Apoptosis by Pyrrolidinedithiocarbamate and N-Acetylcysteine in Vascular Smooth Muscle Cells*

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Pyrrolidinedithiocarbamate (PDTC) and N-acetylcysteine (NAC) have been used as antioxidants to prevent apoptosis in lymphocytes, neurons, and vascular endothelial cells. We report here that PDTC and NAC induce apoptosis in rat and human smooth muscle cells. In rat aortic smooth muscle cells, PDTC induced cell shrinkage, chromatin condensation, and DNA strand breaks consistent with apoptosis. In addition, overexpression of Bcl-2 suppressed vascular smooth muscle cell death caused by PDTC and NAC. The viability of rat aortic smooth muscle cells decreased within 3 h of treatment with PDTC and was reduced to 30% at 12 h. The effect of PDTC and NAC on smooth muscle cells was not species specific because PDTC and NAC both caused dose-dependent reductions in viability in rat and human aortic smooth muscle cells. In contrast, neither PDTC nor NAC reduced viability in human aortic endothelial cells. The use of antioxidants to induce apoptosis in vascular smooth muscle cells may help prevent their proliferation in arteriosclerotic lesions.

Apoptosis or programmed cell death is characterized by cell shrinkage, membrane blebbing, and chromatin condensation that culminate in cell fragmentation (1). Stimuli as diverse as hyperthermia, growth factor withdrawal, chemotherapeutic agents, radiation, and oxidative stress induce apoptosis in many cell types (2–5), and several cellular proteins have been identified that activate or suppress it (6–13). The B-cell leukemia/lymphoma-2 (Bcl-2)¹ protein has been shown to prevent apoptosis induced by diverse stimuli (14–17), perhaps by acting as an antioxidant (14, 18). This hypothesis is consistent with observations that antioxidants such as pyrrolidinedithiocarbamate (PDTC) and N-acetylcysteine (NAC) prevent apo-

ptosis in lymphocytes (14, 19–21), neurons (18, 22), and vascular endothelial cells (23).

Proliferation of vascular smooth muscle cells is one of the most important features of arteriosclerosis (24). Rao and Berk (25) have shown that hydrogen peroxide stimulates proliferation of vascular smooth muscle cells but inhibits proliferation of vascular endothelial cells. However, the effect of antioxidants on smooth muscle cells has been unclear. PDTC and NAC are two structurally different thio-containing agents. Although NAC at low concentrations (<1 mM) has been reported to cause oxidative stress (5), both agents have been shown to function as effective antioxidants and to prevent oxidant-induced apoptosis (14, 19–21) and activation of the transcription factors NF- κ B and AP-1 (26–28) and vascular cell adhesion molecule-1 (29). In this study, we tested the effects of PDTC and NAC on vascular smooth muscle and endothelial cells and show that both agents, in dose ranges at which they are used as antioxidants, induced apoptosis in rat and human aortic smooth muscle cells but not in human aortic endothelial cells. This induction of apoptosis in smooth muscle cells occurred in a dose- and time-dependent manner. Furthermore, overexpression of Bcl-2 blocked PDTC- and NAC-induced apoptosis in rat aortic smooth muscle cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Rat aortic smooth muscle cells (RASMC) were harvested from male Sprague-Dawley rats (200–250 g) by enzymatic dissociation according to the method of Gunther *et al.* (30) with modification (31). Fetal RASMC (A7r5) were obtained from ATCC. RASMC were cultured in Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS) and supplemented with 10% fetal calf serum (HyClone, Logan UT), penicillin (100 units/ml), streptomycin (100 μ g/ml), and 25 mM Hepes (pH 7.4) (Sigma). Human aortic smooth muscle cells (HASMC) and human aortic endothelial cells (HAEC) were purchased from Clonetics Corp. (San Diego). HASMC were cultured in MCDB 131 medium supplemented with 5% fetal calf serum, 10 ng/ml recombinant human epidermal growth factor, 2 ng/ml basic fibroblast growth factor, and 5 μ g/ml insulin. HAEC were also grown in MCDB 131 medium supplemented with 2% fetal calf serum, 10 ng/ml epidermal growth factor, 12 μ g/ml bovine brain extract, and 10 μ g/ml heparin. PC-12 cells were obtained from ATCC and cultured in RPMI 1640 medium (Mediatech, Washington D. C.) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (32). Cells were passaged every 3–5 days, and experiments were performed on cells five to eight passages from primary culture. We purchased PDTC, deferoxamine, D-penicillamine, and bathocuproinedisulfonic acid (BPSA) from Sigma; NAC (L form) from Chiron Therapeutics (Emeryville, CA); Hoechst 33258 from Molecular Probes Inc.; terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP from Boehringer Mannheim; and anti-human Bcl-2 polyclonal antibody from Pharmingen (San Diego, CA).

Induction and Morphologic Study of Apoptosis—PDTC and NAC, at various concentrations, were added to subconfluent and exponentially

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¹ The abbreviations used are: Bcl-2, B-cell leukemia/lymphoma-2 protein; PDTC, pyrrolidinedithiocarbamate; NAC, N-acetylcysteine; RASMC, rat aortic smooth muscle cell(s); HASMC, human aortic smooth muscle cell(s); HAEC, human aortic endothelial cell(s); BPSA, bathocuproinedisulfonic acid; TdT, terminal deoxynucleotidyl transferase; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

growing RASMC, HASMC, and HAEC. Apoptotic cells were identified by inverted light, fluorescence, and electron microscopy. For fluorescence microscopy, cells grown on coverslips were washed with Dulbecco's phosphate-buffered saline and then fixed in 4% paraformaldehyde in Dulbecco's phosphate-buffered saline. After fixation, the cells were stained for 10 min with Hoechst 33258 at 8 $\mu\text{g}/\text{ml}$. Cells containing apoptotic nuclei were examined by fluorescence microscopy at 600 \times magnification. For electron microscopy, cells in 60-mm Petri dishes were fixed in 2% glutaraldehyde in Dulbecco's phosphate-buffered saline for 30 min at room temperature followed by fixation with 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 min at room temperature, essentially as described (33). TdT-mediated dUTP-biotin nick end labeling was used to detect DNA breaks in apoptotic cells *in situ* (34). Cells grown on coverslips were rinsed three times with phosphate-buffered saline, incubated in avidin (25 $\mu\text{g}/\text{ml}$ in phosphate-buffered saline and 0.4% Triton-X 100) for 30 min at room temperature to block endogenous biotin, rinsed three times with phosphate-buffered saline, and then incubated in 3% H_2O_2 at room temperature for 10 min. After another three rinses with phosphate-buffered saline, the cells were rinsed with TdT buffer and incubated in 50 μl of TdT buffer (10 units of TdT and 0.5 nmol of biotinylated dUTP) at 37 $^{\circ}\text{C}$ for 60 min. Biotinylated dUTP incorporated into DNA breaks in the nuclei was detected by an avidin-biotin complex method (DAB/nickel chromogen). RASMC were counterstained with eosin.

Viability Assay—Subconfluent, exponentially growing RASMC, HASMC, HAEC, and PC-12 cells in 24-well plates were incubated with PDTC or NAC for the indicated times. Cell viability was determined by a modified 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which is based on the conversion of the tetrazolium salt 3-(4,5-dimethyl thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-H-tetrazolium by mitochondrial dehydrogenase to a formazan product (35), as measured at an absorbance of 490 nm.

Plasmids and Transfection—Plasmid pC Δ j-bcl-2 (36), containing the human Bcl-2 coding region under control of the simian virus 40 enhancer and promoter and carrying the neomycin resistance marker, and plasmid pC Δ j-SV2, identical but for the lack of the Bcl-2 coding region, were used to create stably transfected RASMC. Fetal RASMC (A7r5) were transfected with 2 μg of pC Δ j-bcl-2 or pC Δ j-SV2 by using Lipofectin (Life Technologies, Inc.) and selected by growth in G418 (0.5 mg/ml). We screened 24 pC Δ j-bcl-2 transformants and 12 pC Δ j-SV2 transformants for Bcl-2 expression by SDS-polyacrylamide gel electrophoresis and Western blot analysis, according to a published procedure (37) and using a commercial Bcl-2 antiserum (Pharmingen) at a dilution of 1:800. Of the 24 pC Δ j-bcl-2 transformants, 3 expressed high levels of Bcl-2; no expression was detected in the 12 pC Δ j-SV2 transformants.

RESULTS AND DISCUSSION

After 6 h of exposure to 150 μM PDTC, normal RASMC (Fig. 1A) underwent cell shrinkage characteristic of apoptosis (Fig. 1B); fluorescent staining of the DNA revealed chromatin condensation in PDTC-treated RASMC (Fig. 1D) but not in untreated RASMC (Fig. 1C). Another hallmark of apoptosis is DNA strand breaks caused by endonuclease, which can be detected *in situ* by nick end labeling tissue sections with dUTP-biotin by terminal deoxynucleotidyl transferase (34, 38). In contrast with untreated cells (Fig. 1E), positive staining was visible in most of the nuclei in RASMC that had been treated with PDTC (Fig. 1F). Finally, electron microscopy revealed highly condensed chromatin localized to the inner side of an intact nuclear membrane in PDTC-treated RASMC. The state of the treated nucleus (Fig. 1H) was in sharp contrast with that of the normal (untreated) nucleus (Fig. 1G). In addition to being a thio-containing antioxidant, PDTC is a metal chelator. To exclude the possibility that the effect of PDTC depended solely on its ability to chelate metals, we treated RASMC with NAC. NAC is another thio-containing antioxidant that does not have the ability to chelate metals. RASMC treated with 10 mM NAC manifested morphologic changes identical to those observed in PDTC-treated RASMC (not shown), indicating that two different antioxidants induce apoptosis in vascular smooth muscle cells.

We also used a modified MTT assay of cell viability (35, 39) to measure antioxidant-induced apoptosis. The viability of

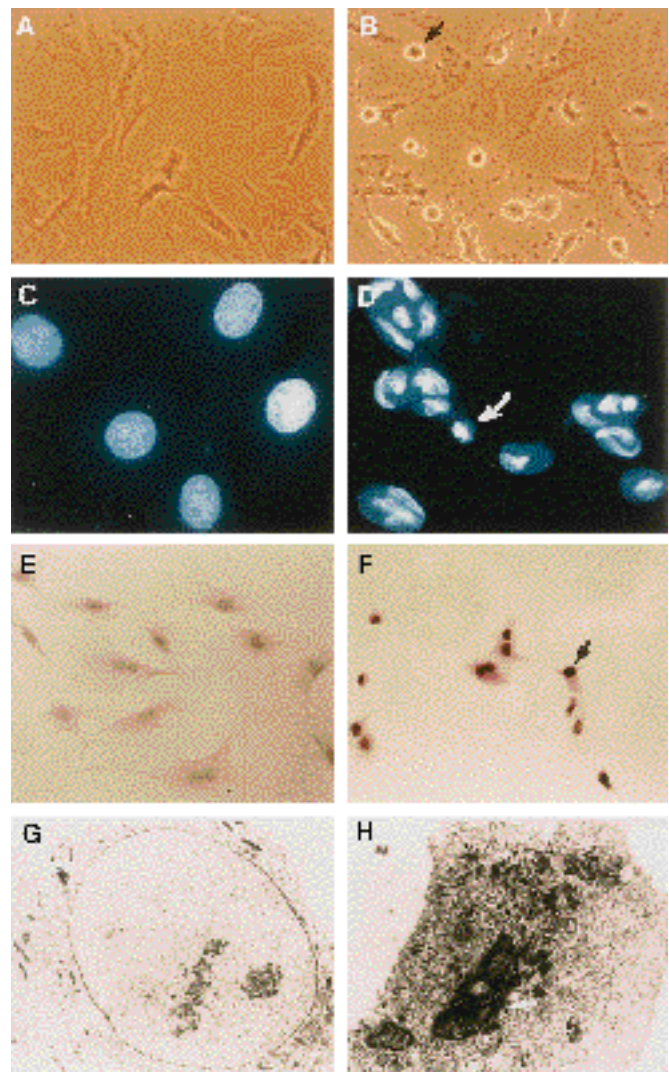


FIG. 1. Morphology and DNA fragmentation after PDTC-induced apoptosis in RASMC. RASMC were not treated (left) or treated (right) with 150 μM PDTC. A and B, light micrographs of growing RASMC (100 \times). After 6 h of treatment, cytoplasmic condensation and cell shrinkage are visible in some RASMC (B). Arrow indicates a representative apoptotic cell. C and D, micrographs of fluorescently stained RASMC DNA (600 \times). The homogeneous, lightly stained nuclear chromatin in untreated cells (C) is in sharp contrast to the chromatin condensation accompanying PDTC-induced apoptosis (D). Arrow indicates an apoptotic body. E and F, DNA breaks stained *in situ* by the TdT-mediated dUTP-biotin nick end labeling method (34) (200 \times). Arrow (F) indicates a representative apoptotic nucleus. G and H, electron micrographs of RASMC (3000 \times). RASMC in 60-mm Petri dishes were treated with PDTC for 6 h and processed as described (33). The untreated RASMC (G) has large nucleoli, and the heterochromatin is scanty, whereas the PDTC-treated RASMC (H) shows marked chromatin condensation within an intact nuclear envelope. Arrow (H) marks dense aggregation of chromatin in the periphery of the nucleus.

RASMC decreased within 3 h of treatment with PDTC (Fig. 2A) and was reduced to approximately 30% of base line at 12 h. PDTC also decreased the viability of RASMC in a dose-dependent manner (Fig. 2B). As little as 25 μM PDTC reduced rat aortic smooth muscle cell viability by 25%, whereas 150 μM PDTC reduced viability by 73%. This decrease in vascular smooth muscle cell survival was not specific to rats; PDTC (Fig. 2C) and NAC (Fig. 2D) both caused dose-dependent reductions in survival in HASMC at 24 h. In contrast, neither PDTC nor NAC reduced survival in HAEC (Fig. 2, C and D).

The concentrations of NAC that induced apoptosis in vascular smooth muscle cells (Fig. 2D) have been shown to prevent

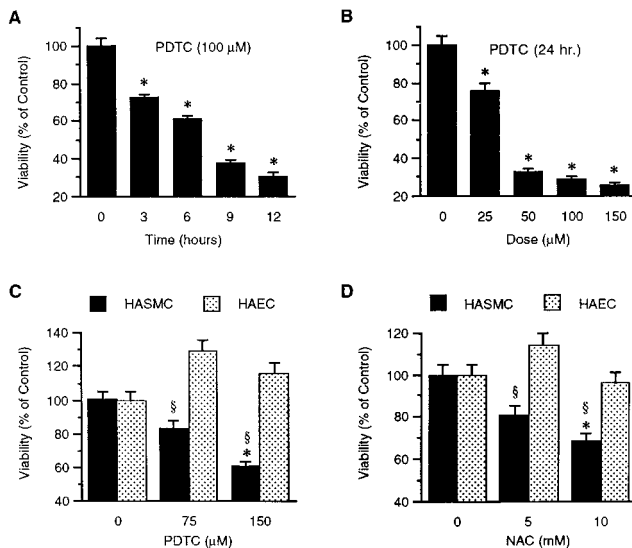


FIG. 2. Viability of vascular cells after treatment with PDTC and NAC. Subconfluent, exponentially growing RASMC, HASMC, and HAEC in 24-well plates were incubated with PDTC or NAC for the indicated times. Cell viability was determined by a modified MTT assay (35). *A*, time course of the effect of PDTC on rat aortic smooth muscle cell viability. *B*, dose response of the effect of PDTC on rat aortic smooth muscle cell viability. *C*, Differential effect of PDTC on the viability of HASMC and HAEC. *D*, differential effect of NAC on the viability of HASMC and HAEC. Values represent mean \pm S.E. from four samples. A factorial analysis of variance was applied to the values, followed by Fisher's least significant difference test. Significance was accepted at $p < 0.05$. *, treated group different from control group. §, HASMC group different from HAEC group.

apoptosis in lymphocytes, neurons, and endothelial cells (14, 19–21). To confirm that an antioxidant could prevent apoptosis under our culture conditions, we performed experiments in PC-12 neuronal cells. Serum deprivation induced apoptosis in PC-12 cells (Fig. 3), as Greene (40) has also shown. PDTC inhibited this apoptosis in a dose-dependent manner (Fig. 3), and 100 μ M PDTC completely prevented apoptosis in PC-12 cells. This observation suggests that the induction of apoptosis by antioxidants in RASMC and HASMC is cell type specific.

To determine whether Bcl-2 inhibited antioxidant-induced apoptosis in vascular smooth muscle cells, we transfected into fetal RASMC expression plasmids that did or did not contain the human Bcl-2 coding region. Several stably transfected clones were isolated, and Bcl-2 expression was confirmed by Western blotting with an antibody against human Bcl-2 (Fig. 4A). As in adult RASMC (Fig. 2A), PDTC (Fig. 4B) and NAC (not shown) both induced dose-dependent apoptosis in fetal RASMC. Cells that overexpressed Bcl-2, however, were resistant to apoptosis induced by PDTC (Fig. 4B) and NAC (data not shown).

Reactive oxygen species (superoxide anion (O_2^-), hydroxyl radical ($\text{OH}\cdot$), and hydrogen peroxide (H_2O_2)) have been implicated in causing cell damage and cell death (14, 18, 41). Yet, we have observed that PDTC and NAC, two structurally different antioxidants that prevent apoptosis in other cell types *in vitro*, induced apoptosis in human and rat aortic smooth muscle cells (Figs. 1 and 2). The unique susceptibility to antioxidants of vascular smooth muscle cells indicates that they respond differently than other cell types to changes in the reduction-oxidation state. Consistent with this view, Rao and Berk (25) have shown that H_2O_2 increases proliferation of vascular smooth muscle cells but inhibits proliferation of vascular endothelial cells.

In the presence of iron or copper, antioxidants such as ascorbic acid act as prooxidants via the Fenton reaction (42, 43). To

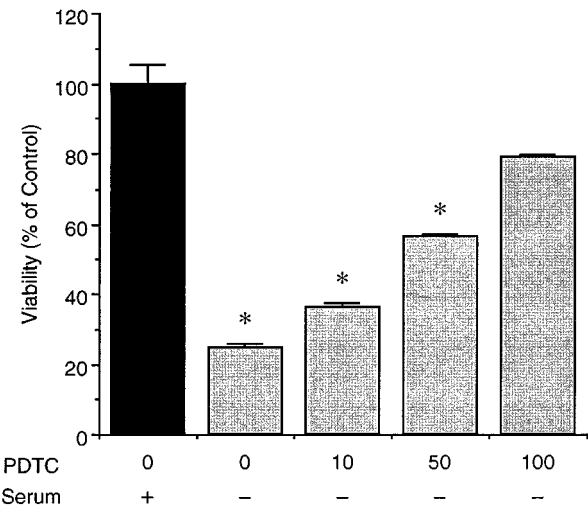


FIG. 3. Prevention by PDTC of apoptosis induced by serum deprivation in PC-12 cells. PC-12 cells were plated at a density of 1×10^5 cells/well in 24-well, collagen-coated plates and cultured in medium containing 10% horse serum and 5% fetal bovine serum. After 24 h, cells were extensively washed and placed in serum-free medium. Cell viability was determined as described for Fig. 2. *Black bar* represents control cells maintained for 36 h in medium containing serum. *Shaded bars* represent cells maintained for 36 h in serum-free medium and treated with the indicated concentrations (in μ M) of PDTC. Values represent mean \pm S.E. from three samples. Comparisons were subjected to ANOVA followed by Fisher's least significant difference test, and significance was accepted at $p < 0.05$. *, significant decrease in survival in cells depleted of serum versus those replete with serum.

rule out the possibility that the antioxidant-induced apoptosis we observed was caused simply by autooxidation and subsequent generation of free radicals, we treated RASMC with PDTC in the presence or absence of 50 μ M deferoxamine, D-penicillamine, or BPSA (this concentration was chosen because it was sufficient to allow chelation of 100 times the iron (deferoxamine) or copper (D-penicillamine and BPSA) actually present in the culture medium). Neither deferoxamine nor D-penicillamine affected PDTC-induced apoptosis, and BPSA prevented only ~20% of PDTC-induced apoptosis. Thus, the antioxidant-induced apoptosis we observed in RASMC cannot be ascribed simply to autooxidation by the thio compounds added during the experiment.

Although the dose-response curve for the PDTC-induced decrease in RASMC survival is rather linear (Fig. 2B), this pattern is not observed universally for antioxidants in other cell types. For example, McCord and co-workers (44–46) published a bell-shaped dose-response curve for the protective effect of superoxide dismutase against ischemia-reperfusion injury in rat and rabbit hearts. High doses of superoxide dismutase were less effective than low doses, and very high doses had a deleterious effect.

Bcl-2 has been shown to protect cell types of diverse lineage from apoptosis induced by many stimuli (14–17). Our finding that Bcl-2 suppresses antioxidant-induced cell death (Fig. 4B) supports our conclusion from morphological studies that vascular smooth muscle cell death caused by PDTC and NAC is apoptosis. Bcl-2 has been shown to prevent apoptosis in lymphocytes and neurons by regulating an antioxidant pathway (14, 18). However, Bcl-2 has also been shown to function as a prooxidant (47). Furthermore, recent reports indicate that Bcl-2 prevents apoptosis induced by hypoxia (48) or by staurosporine under very low oxygen conditions (49). Thus, Bcl-2 may also prevent apoptosis via a mechanism unrelated to its effect on reactive oxygen species. Our observation that Bcl-2 overexpression rescues RASMC from apoptosis induced by PDTC (Fig. 4B) and NAC also suggests that Bcl-2 may prevent apo-

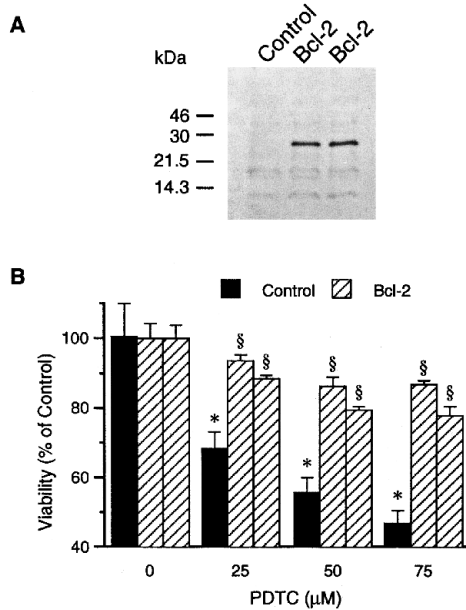


FIG. 4. Overexpression of Bcl-2 rescues RASM from PDTC-induced apoptosis. Protein overexpression was obtained by stably transfecting fetal RASM (A7r5 cells, ATCC) with a Bcl-2 expression plasmid (36) or control plasmid. Clones were selected in medium containing G418 (Geneticin) (500 μ g/ml). A, Bcl-2 protein expression was assessed by Western blot analysis as described (50). Protein was extracted from cells transfected with plasmid pCΔj-SV2 (control) and pCΔj-bcl-2 (Bcl-2; two different cell lines), and 20 μ g of protein was loaded in each lane. A polyclonal antibody to human Bcl-2 (1:800 dilution, Pharmingen) identified a 26-kDa protein in the Bcl-2 lanes but not in the control lane. B, viability of control and Bcl-2 cell lines exposed to PDTC. Because fetal RASM are very sensitive to antioxidants, they were treated with 25–75 μ M PDTC for 24 h. Viability was determined by the MTT assay. PDTC reduced the viability of control RASM (black bars) in a dose-dependent manner. Viability in the two lines of RASM that overexpressed Bcl-2 (hatched bars) was significantly greater than that in the control line. *, $p < 0.05$, treated versus untreated control. §, $p < 0.05$, Bcl-2 groups versus control.

ptosis in vascular smooth muscle cells through a pathway unrelated to its antioxidant activity.

Arteriosclerosis and its complications, heart attack and stroke, are the major causes of death in developed countries (24). Since proliferation of vascular smooth muscle cells is one of the key features of arteriosclerosis (atherosclerosis, restenosis after balloon angioplasty or coronary bypass surgery, and transplant arteriosclerosis), our finding that antioxidants promote apoptosis in vascular smooth muscle cells, but not in vascular endothelial cells, may provide a new therapeutic strategy for the treatment of arteriosclerosis.

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