British Journal of Pharmacology (2003) 139, 1095–1102
www.nature.com/bjp

Delphinidin, an active compound of red wine, inhibits endothelial cell apoptosis *via* nitric oxide pathway and regulation of calcium homeostasis

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1 Epidemiological studies have suggested that moderate consumption of natural dietary polyphenolic compounds might reduce the risk of cardiovascular disease and also protect against cancer. The present study investigates the effects of delphinidin, an anthocyanin present in red wine, on bovine aortic endothelial cells apoptosis.

2 Based on flow cytometry, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling analysis and detection of mitochondrial cytochrome *c* release, we show that delphinidin (10^{-2} g l^{-1}) alone had no effect either on necrosis or on apoptosis, but it significantly reduced apoptosis elicited by actinomycin D (1 μg ml^{-1}, 24 h) and 7β-hydroxycholesterol (10 μg ml^{-1}, 18 h).

3 The protective effect of delphinidin was abolished by inhibitors of nitric oxide-synthase (NOS) (L-NA, 100 μM and SMT, 100 μM), guanylyl cyclase (ODQ, 100 μM) and MAP kinase (PD98059, 30 μM).

4 Western blot analysis and protein detection by confocal microscopy demonstrate that the antiapoptotic effect of delphinidin was associated with an increased endothelial NOS expression mediated by a MAP kinase pathway.

5 Finally, delphinidin alone had no effect on cytosolic-free calcium ($[Ca^{2+}]_i$), but normalized the changes in $[Ca^{2+}]_i$ produced by actinomycin D towards the control values, suggesting that the antiapoptotic effect of delphinidin is associated with the maintenance of $[Ca^{2+}]_i$ in the physiological range.

6 All of the observed effects of delphinidin may preserve endothelium integrity, the alteration of which lead to pathologies including cardiovascular diseases, such as atherosclerosis, and is often associated with cancers. In conclusion, the protective effect of delphinidin against endothelial cell apoptosis contributes to understand the potential benefits of a consumption rich in polyphenols.
British Journal of Pharmacology (2003) **139,** 1095–1102. doi:10.1038/sj.bjp.0705347

Keywords: Cell death; endothelium; NO; cGMP; MAP kinases; Ca^{2+} handling; delphinidin

Abbreviations: BAECs, bovine aortic endothelial cells; $[Ca^{2+}]_i$, cytosolic free calcium; eNOS, endothelial NO-synthase; iNOS, inducible NO-synthase; L-NA, N^{ω}-nitro-L-arginine; NO, nitric oxide; NOS, NO-synthase; ODQ, 1*H*-[1,2,4] oxadiazolo [4,3a] quinoxalin-1]; RWPC, red wine polyphenolic compounds; SMT, *S*-methyl-isothiourea; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

Introduction

Natural dietary polyphenolic compounds present in fruits, vegetables or red wine have been reported to protect against cardiovascular diseases and cancer because of their multitude biological activities (Hertog *et al*., 1993; Middleton *et al*., 2000). Indeed, polyphenolic compounds have been reported to suppress cell proliferation, prevent platelet aggregation, stabilize immune cells and promote vascular smooth muscle cell relaxation (Middleton *et al*., 2000). The mechanisms by which they act have not been totally elucidated. Polyphenols have also been reported to scavenge reactive oxygen species, inhibit oxidation of low-density lipoproteins and interact with the nitric oxide (NO) generating pathway (Middleton *et al*., 2000). Previous studies from our laboratory have identified the anthocyanin, delphinidin, as a polyphenol presenting an endothelium-dependent relaxant effect. Its action depends on the ability of delphinidin to stimulate NO production, independently of its antioxidant property (Andriambeloson *et al*., 1997). NO has vasorelaxant (Furchgott *et al*., 1984) and antiaggregatory properties and is able to limit the flux of atherogenic plasma proteins into the artery wall (Roberts *et al*., 1997), all of these activities probably contributing to the protective effects of delphinidin and other red wine polyphenolic compounds (RWPC).

Apoptosis, a genetically controlled cell death program, is of fundamental significance for the maintenance of homeostasis in multicellular organisms. Under certain vascular pathological conditions, such as atherosclerosis, hypertension and

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Advance online publication: 19 June 2003

restenosis (Kockx & Knaapen, 2000), activation of cellular pathways leading to endothelial cell apoptosis has been described, and can be either beneficial or detrimental. The main features of apoptotic cells are DNA fragmentation, chromatin condensation and cell fragmentation without increased permeability of the plasma membrane. NO exhibits a double-edged role in apoptosis, depending on its concentration. High amounts of NO produced by the inducible NO-synthase (iNOS) appear to be linked to its proapoptotic effect (Terenzi *et al*., 1995). In contrast, the antiapoptotic effects of NO are mainly mediated by low amounts of NO following the stimulation of the constitutive endothelial NO-synthase (eNOS) (Mannick *et al*., 1994). Studies on the antiapoptotic mechanisms of NO have identified multiple targets that range from direct and indirect interactions with the apoptotic machinery. These include the induction of cytoprotective stress proteins, cGMP-dependent inhibition of apoptotic signal transduction, including modification of Ca^{2+} homeostasis, suppression of caspase activity and inhibition of cytochrome *c* release (Kim *et al*., 1999).

The endothelium is recognized as having a central role in many cardiovascular diseases, and apoptosis is emerging as a determinant process in disease progression. Here, we report the protective effect of delphinidin on endothelial cell apoptosis through NO-guanylyl cyclase-dependent pathway. The maintenance of Ca^{2+} homeostasis in endothelial cells may account for the protective effects of delphinidin.

Methods

Materials

The anthocyanin delphinidin chloride was purchased from Extrasynthèse (Genay, France). Delphinidin chloride dry powder was 99.93% pure, according to the manufacturer. Delphinidin was diluted in 10% DMSO/90% H_2O. After appropriate dilutions, the final concentration of DMSO did not exceed 0.1%. Actinomycin D, 7β-hydroxycholesterol and propidium iodide (PI) were purchased from Sigma (St Louis, MO, U.S.A.). Anti-eNOS and anti-iNOS were from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-cytochrome *c* antibody was from BD Biosciences Pharmingen (San Diego, CA, U.S.A.). Anti-mouse HRP-conjugated was from Promega (Madison, WI, U.S.A.). Alexa Fluor™ 488 was purchased from Molecular Probes (Leiden, Netherlands), PD98059 from Tocris (Ballwin, MO, U.S.A.) and TUNEL assay kit from Roche Diagnostics (Mannheim, Germany).

Cell culture and treatment

Bovine aortic endothelial cells (BAECs) were grown as previously described (Schini *et al*., 1988). Briefly, cells were cultured in plastic flasks precoated with type I collagen (0.06 mg ml^{-1}) in a mixture of DMEM and Ham's F12 (1 : 1, v v^{-1}) with 10% fetal bovine serum, 2 mM L-glutamine, 100 mg ml^{-1} heparin, 100 U ml^{-1} penicillin and streptomycin and 10 μM vitamin C. Cultures were maintained at 37°C in a humidified incubator gassed with 5% CO_2 in air. Cells were used up to five passages. For apoptosis induction, Ca^{2+} experiments and Western blotting, cells were seeded at a suitable density (5×10^5 cells per well in six-well plate, and 6×10^6 cells per 100-mm Petri dishes, respectively) for 4 h and apoptosis was then induced in subconfluent and exponentially growing BAECs. For confocal microscopy, cells were subcultured as described previously at a density of 5×10^5 cells per 35-mm Petri dishes in which a 2 cm diameter hole had been cut in the base and replaced by a thin (0.07 mm) glass coverslip (Martin *et al*., 2002).

Apoptosis was induced by the addition of either actinomycin D (1 μg ml^{-1}) for 24 h or 7β-hydroxycholesterol (10 μg ml^{-1}) for 18 h. Actinomycin D has been used experimentally in models of cell death (Bock *et al*., 2002; Kim *et al*., 2002). 7β-hydroxycholesterol is a pathogenic endogenous agent, known to be responsible at least in part for cytotoxicity of oxidized low-density lipoproteins and induces apoptosis of several cell types (Aupeix *et al*., 1996). Delphinidin was added to cells at the same time as actinomycin D or 7β-hydroxycholesterol. Delphinidin was used at 10^{-2} g l^{-1}, a concentration eliciting maximal endothelium-dependent relaxation of precontracted rat aorta (Andriambeloson *et al*., 1998), a transient elevation of $[Ca^{2+}]_i$ in endothelial cells leading to NO production (Martin *et al*., 2002) and the inhibition of serum- and vascular endothelium growth factor-induced proliferation in endothelial cells (Martin *et al*., 2003). N^{ω}-nitro-L-arginine (L-NA, 100 μM), *S*-methyl-isothiourea (SMT, 100 μM), 1*H*-[1,2,4] oxadiazolo [4,3a] quinoxalin-1] (ODQ, 100 μM) and PD98059 (30 μM), which are respectively inhibitors of NOS, guanylyl cyclase and MEK1/2, were also present during the induction of apoptosis when indicated.

Determination of DNA strand breaks

DNA single-strand breaks occurring during apoptosis were analyzed by using the TUNEL assay according to the manufacturer's instructions. Briefly, cell concentration was adjusted to 30×10^6 cells ml^{-1} and fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) and incubated with TUNEL reaction mixture. Breaks could be identified by labeling free 3′-OH termini with fluorescein-labeled nucleotides in a reaction catalyzed by terminal deoxynucleotidyl transferase (TdT). Incorporated fluorescein was analyzed with a Bio-Rad 1024 MRC confocal microscope with a 40× epifluorescence objective (Nikon). Z-series were collected in 1 μm steps and final images were analyzed after stacking of 22 images corresponding to the total cell volume.

Hypodiploid DNA quantification was performed as described previously (Gidon-Jeangirard *et al*., 1999). Briefly, cell concentration was adjusted to 5×10^5 cells ml^{-1} and fixed in 70% ethanol for at least 1 h at 4°C. Cells were washed in Hank's balanced salt solution before resuspension in a solution containing type I RNAse A (0.5 mg ml^{-1}) and were incubated for 10 min at 37°C. PI (0.1 mg ml^{-1}) was then added and samples were allowed to stand 15 min in the dark at room temperature before flow cytometry analysis using CELLQuest software (Becton Dickinson, San Jose, CA, U.S.A.).

Immunostaining of eNOS and iNOS by confocal microscopy

After incubation with actinomycin D, adherent cells were fixed for 10 min with PBS containing 3% paraformaldehyde on ice. After three washes with PBS, cells were treated 30 min with

0.2% Triton X-100 in PBS and blocked 30 min with PBS/3% BSA. Cells were washed three times with PBS/0.2% BSA and then incubated with either anti-eNOS or anti-iNOS antibodies, diluted 1/200 ($v\,v^{-1}$) for 1 h at room temperature. After three washes, cells were incubated with the Alexa Fluor™ 488-conjugated goat anti-mouse secondary antibody, diluted 1/200 ($v\,v^{-1}$) for 1 h. Cells were observed with a Bio-Rad 1024 MRC confocal microscope as previously described (see TUNEL protocol). The amount of labeling was quantified with NIH-image software.

Western blotting

Cells were harvested and spun down at $1000 \times g$ for 10 min, and were lysed for 1 h in 200 μl of ice-cold lysis buffer (50 mM Tris, 250 mM NaCl, 8 mM $MgCl_2$, 10 $\mu g\,ml^{-1}$ of aprotinin, leupeptin and pepstatin, 1 mM PMSF, 5 mM EDTA, 0.5 mM EGTA, 2 mM $NaVO_3$ and 1% Triton X-100). After quantification by the Bradford method, 50 μg protein was resuspended in Laemmli's buffer, separated on 7% SDS–PAGE and Western blotted. The sample loading was verified by staining membranes with Ponceau red and amidoblack. Blots were probed with anti-eNOS, anti-iNOS and anti-cytochrome *c* antibodies, followed by the HRP-conjugated antibody. Blots were treated with ECL reagents for 5 min, exposed to CL-Xposure films. Blots were scanned and densitometric analysis was performed of the scanning images using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com).

Determination of cytosolic calcium concentration ($[Ca^{2+}]_i$)

Cells were loaded with Fura-2/AM (5 μM) (45 min at 37°C in culture medium), then washed and placed in physiological salt solution (PSS in mM: 119 NaCl, 4.75 KCl, 1.25 $CaCl_2$, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 25 $NaHCO_3$, 11 glucose, 20 HEPES, pH 7.4, 37°C). $[Ca^{2+}]_i$ was determined by fluorimetric readings performed with F-2000 Hitachi spectrofluorimeter system. The maximum and minimum fluorescence were sequentially determined by the addition of 10 μM ionomycin, followed by the addition of 10 mM EGTA. Basal $[Ca^{2+}]_i$ of control cells was 216 ± 19 nM.

Statistical analysis

Data are represented as mean ± s.e.m. In all cases, *n* refers to the number of experiments. Statistical analyses were performed by Student's *t*-test and analysis of variance (ANOVA). Differences were considered significant at a value of $P < 0.05$.

Results

Actinomycin D and 7β-hydroxycholesterol induced apoptosis of BAECs

Staining with PI revealed nuclei with hypodiploid DNA (sub-G1 peak) corresponding to apoptotic cells. Basal apoptosis, that is, in the presence of 0.1% DMSO, never exceed 5%. Exposure of BAECs to actinomycin D, a chemotherapeutic agent, induced DNA fragmentation in a time-dependent manner with a maximal value of 19 ± 1% reached after 24 h of treatment (Figure 1a, $n = 30$). At 18 h, the major part of the cell population was still attached to the culture flask. At 24 h, cells exhibited morphological characteristics of apoptosis including shrinkage, membrane blebbing and detached (Figure 1b). Cells were also treated with the pathogenic agent, known to be responsible, at least in part, for the cytotoxicity of oxidized low-density lipoproteins, 7β-hydroxycholesterol. Treatment of BAECs for 18 h with 10 $\mu g\,ml^{-1}$ 7β-hydroxycholesterol induced 14 ± 2% apoptosis (see Figure 2a). Longer period of incubation time enhanced cell mortality by necrosis determined by trypan blue exclusion, making measurement of the degree of apoptosis almost impossible (not shown).

Delphinidin prevented actinomycin D- and 7β-hydroxycholesterol-induced apoptosis

The effect of delphinidin on the apoptotic process was tested on actinomycin D and 7β-hydroxycholesterol-treated cells. Delphinidin significantly reduced apoptosis induced by actinomycin D in a time-dependent manner with a maximal effect obtained at 24 h. At this time, delphinidin inhibited 30 ± 3% the apoptosis induced by actinomycin D (Figure 2a, $n = 30$). Delphinidin also significantly reduced 7β-hydroxycholesterol-induced apoptosis by 45 ± 6% (Figure 2a, $n = 11$). The protective effect of delphinidin against actinomycin D-induced apoptosis was also confirmed by TUNEL assay. As observed in Figure 2b, delphinidin treatment decreased the labeling in

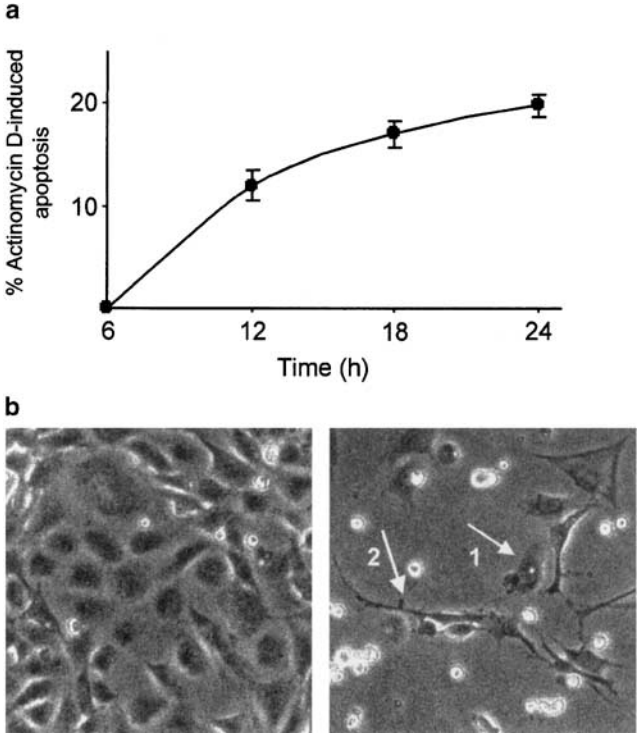

Figure 1 Actinomycin D induces apoptosis of endothelial cells. (a) BAECs were exposed to actinomycin D (1 $\mu g\,ml^{-1}$) for the time indicated, and cell death was assessed by PI staining using flow cytometry. Values showing the apoptosis induced by actinomycin D (apoptosis in the presence of actinomycin D minus basal apoptosis) are mean ± s.e.m. ($n = 30$). (b) Morphological changes observed by light microscopy of actinomycin D-treated cells (right) *versus* control cells (left). Arrows on the right-hand panel point to cell shrinkage (1) and membrane blebbing (2), characteristic features of apoptotic cell death.

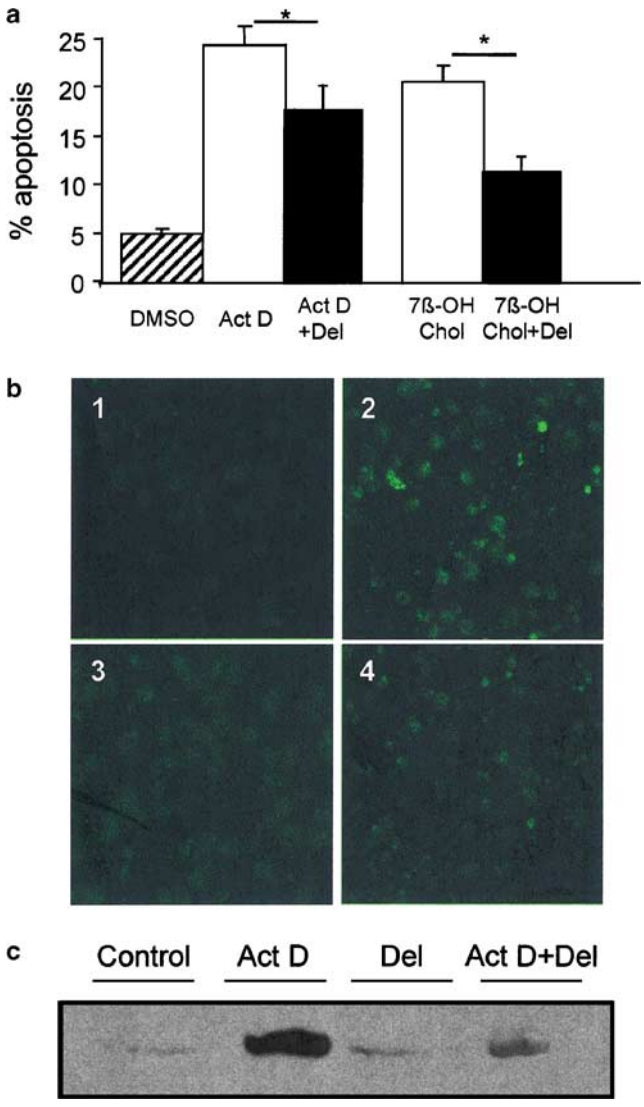

Figure 2 Effect of delphinidin on actinomycin D-and 7β-hydroxycholesterol-induced apoptosis in endothelial cells. (a) Histograms showing the percentage of hypodiploid DNA of cells after 24 h treatment with either DMSO, actinomycin D (1 μg ml^{-1}, Act D) alone ($n=30$), 7β-hydroxycholesterol (10 μg ml^{-1}, 7β-OH Chol) alone ($n=11$) or in combination with delphinidin (10^{-2} g l^{-1}, Del). Values represent the percentage of hypodiploid population for each treatment or control cells, (b) Confocal imaging of TUNEL assay from control (**1**), and cells treated with actinomycin D (**2**), delphinidin (**3**) and actinomycin D + delphinidin (**4**). Apoptotic cells are detected by green fluorescence caused by incorporation of fluorescein-dUTP. (c) Western blot revealing cytochrome *c* release from mitochondria in BAECs exposed to either control medium (lane 1), actinomycin D (1 μg ml^{-1}, Act D) (lane 2), delphinidin (10^{-2} g l^{-1}, Del) (lane 3) or the combination of actinomycin D and delphinidin (lane 4).

the TUNEL assay. We also investigated the effect of delphinidin on the apoptotic process regulated at the level of the mitochondria by quantifying the cytosolic level of cytochrome *c* by Western blot analysis. In fact, the release of the cytochrome *c* from the mitochondria into the cytosol is considered as a marker of apoptosis (Hengartner, 2000). As shown in Figure 2c, actinomycin D treatment increases the cytochrome *c* release from the mitochondria, and this increase was reduced in the presence of delphinidin. These results indicate that the antiapoptotic effect of delphinidin is associated with inhibition of the intrinsec apoptotic pathway. Delphinidin alone did not induce apoptosis (i.e. in the absence of apoptogenic agents, results not shown).

Antiapoptotic effect of delphinidin is mediated through the NO and guanylyl cyclase pathway

To investigate the implication of NO pathway in delphinidin-induced inhibition of apoptosis, the effect of the nonselective NOS inhibitor, L-NA was tested. In Figure 3, this inhibitor reduced significantly the apoptosis evoked by actinomycin D, probably due to a cross-reaction between both actinomycin D and L-NA. Moreover, in the presence of L-NA, delphinidin was not able to inhibit actinomycin D or 7β-hydroxycholesterol-induced apoptosis (Figure 3a, b, $n=10$). These results show that delphinidin acts through the NO pathway to protect cells against apoptosis independently of the inducer. Another nonspecific inhibitor of NOS, SMT (Boer *et al*., 2000), had no effect by itself on actinomycin D-induced apoptosis. In the presence of 100 μM SMT, the antiapoptotic effect of delphinidn was abolished.

To decipher the mechanism(s) by which NO mediated the antiapoptotic effect of delphinidin, actinomycin D-treated cells were incubated with the soluble guanylyl cyclase inhibitor ODQ. Under these conditions, ODQ abolished the inhibitory effect of delphinidin (Figure 3d, $n=4$). The same results were obtained in 7β-hydroxycholesterol-induced apoptosis (not shown). By themselves, L-NA, SMT and ODQ had no effect on cell viability measured by trypan blue exclusion (not shown). Altogether, these results suggest that the antiapoptotic effect of delphinidin is associated with NO production.

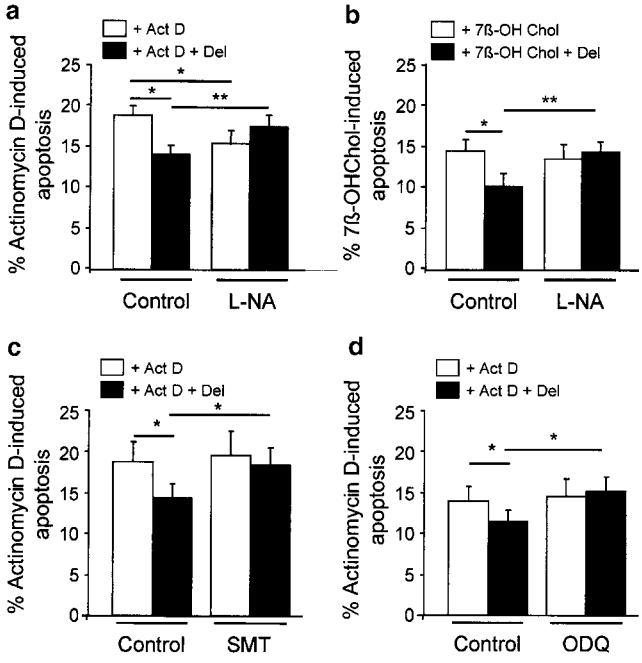

Figure 3 Involvement of the NO/cGMP pathway in the antiapoptotic effect of delphinidin in endothelial cells. BAECs were exposed to either actinomycin D (1 μg ml^{-1}, Act D) or 7β-hydroxycholesterol (10 μg ml^{-1}, 7β-OH Chol) in the absence or presence of delphinidin (Del, 10^{-2} g l^{-1}) alone or in combination with L-NA (100 μM), SMT (100 μM) or ODQ (100 μM). Histograms show the percentage of hypodiploid DNA of cells. Values are the mean $\pm$ s.e.m. of 4–10 experiments. $^{*}P<0.05$, $^{**}P<0.01$.

Antiapoptotic effect of delphinidin is related to eNOS overexpression

To get insight into the mechanism involved in NO-induced protection, we investigated the level of eNOS and iNOS expression. Immunostaining of eNOS showed that the protein level was significantly increased in the presence of delphinidin alone (Figure 4a). However, immunostaining of eNOS by confocal microscopy cannot be easily visualized after actinomycin D treatment because a high proportion of cells was detached from the matrix after treatment with actinomycin D. In order to avoid this problem, Western blot analysis was performed. eNOS expression measured by Western blot analysis was dramatically reduced after treatment with actinomycin D (Figure 4b, $n=4$). Delphinidin increased the level of eNOS expression, compensating the reduction induced by actinomycin D, such that eNOS expression was not significantly different from that of control. No iNOS expression was observed under any conditions (not shown).

Antiapoptotic effect of delphinidin involves the MEK1/2 pathway

The MEK1/2 pathway has been shown to play an important role as a prosurvival element (Xia *et al*., 1995). In order to determine whether this pathway is involved in delphinidin-induced inhibition of apoptosis, cells were treated with the specific inhibitor of MEK1/2, PD98059. By itself, PD98059 had no effect on actinomycin D-induced apoptosis, but this inhibitor abolished the antiapoptotic effect of delphinidin (Figure 5a, $n=6$). The involvement of MEK1/2 pathway in the regulation of eNOS expression was also studied. As shown in Figure 5b, treatment of BAECs by PD98059 significantly altered the overexpression of eNOS induced by delphinidin. It should be noted that PD98059 by itself had no effect on eNOS expression.

Antiapoptotic effect of delphinidin requires the maintenance of $[Ca^{2+}]_i$ in the physiological range

Basal $[Ca^{2+}]_i$ values ($t=0$ h) were not significantly different throughout the experimental condition used (Figure 6, $n=5$). In control cells (i.e. in the presence of DMSO), the changes in $[Ca^{2+}]_i$ were not significantly different at any time tested. The

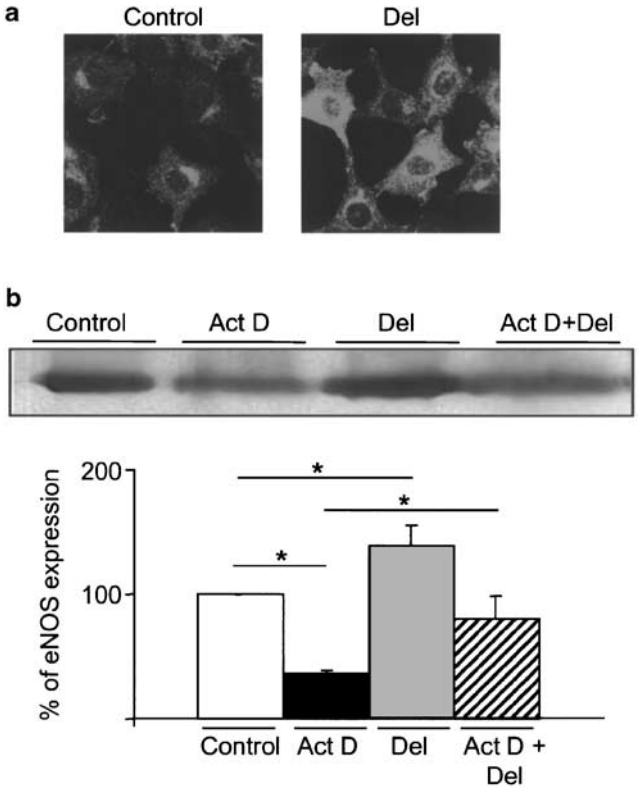

Figure 4 Quantification of eNOS expression (a) Confocal imaging of eNOS expression in adherent BAECs exposed to either control medium or delphinidin (Del) for 24 h. (b) Western blot revealing eNOS expression in BAECs exposed to either control medium (lane 1), actinomycin D (1 μg ml^{-1}, Act D) (lane 2), delphinidin (10^{-2} g l^{-1}, Del) (lane 3) or the combination of actinomycin D and delphinidin (lane 4). Histograms show densitometric analysis of eNOS expression. Data represent the mean$\pm$s.e.m. of four separate experiments. *$P<0.05$.

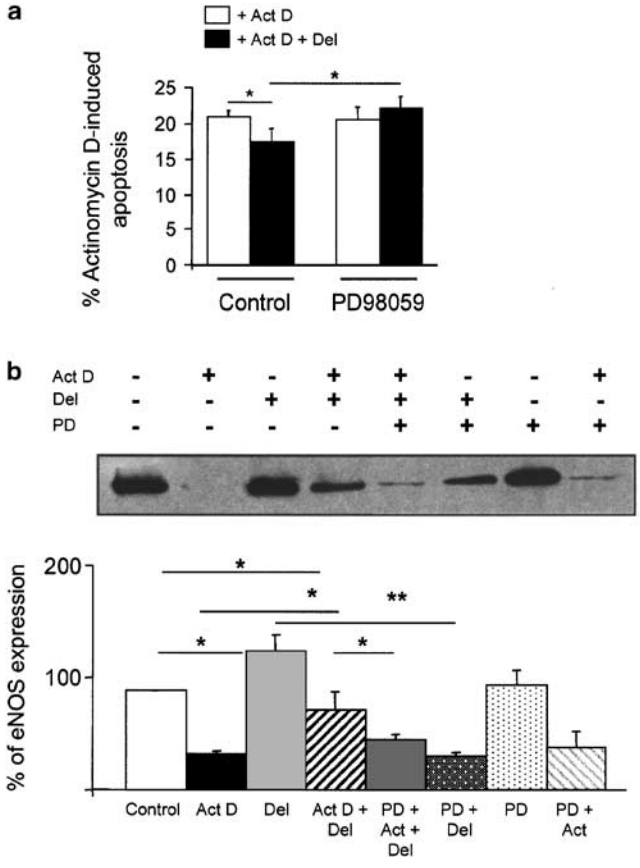

Figure 5 Involvement of MEK1/2 pathway in the antiapoptotic effect of delphinidin in endothelial cells (a) BAECs were exposed to actinomycin D (1 μg ml^{-1}, Act D) alone or in combination with delphinidin (10^{-2} g l^{-1}, Del). Involvement of MEK1/2 pathway was investigated with a MEK1/2 inhibitor, PD98059 (30 μM). Histograms show the percentage of hypodiploid DNA of cells. Values are the mean$\pm$s.e.m. of six experiments. *$P<0.05$. (b) Western blot revealing eNOS expression in BAECs exposed to either control medium, actinomycin D (1 μg ml^{-1}, Act D), delphinidin (10^{-2} g l^{-1}, Del), PD 98059 (30 μM) or the combination of actinomycin D, delphinidin and PD98059. Histograms show densitometric analysis of eNOS expression. Data represent the mean$\pm$s.e.m. of four separate experiments. *$P<0.05$.

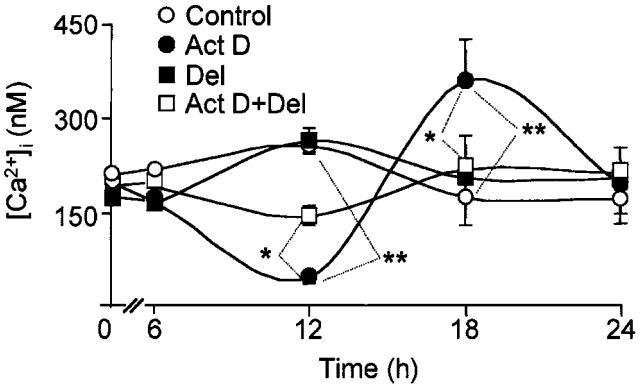

Figure 6 Involvement of calcium variations in endothelial cells in the antiapoptotic effect of delphinidin. BAECs were exposed to actinomycin D (1 μg ml^{-1}, Act D), delphinidin (10^{-2} g l^{-1}, Del) or in combination of actinomycin D plus delphinidin at indicated times (i.e. 6, 12, 18, and 24 h). $[Ca^{2+}]_i$ values are the mean $\pm$ s.e.m. of five experiments. $^*P<0.05$, $^{**}P<0.01$.

effect of actinomycin D treatment on $[Ca^{2+}]_i$ was biphasic. Indeed, $[Ca^{2+}]_i$ was significantly decreased during the early phase of apoptosis (12 h), it was significantly greater at 18 h when apoptosis was near to maximum (see Figure 1), and then it returned to control values after 24 h. Delphinidin alone had no effect on $[Ca^{2+}]_i$ at any time tested, but normalized the changes in $[Ca^{2+}]_i$ produced by actinomycin D towards the control values. Thus, delphinidin significantly attenuated the decrease in $[Ca^{2+}]_i$ induced by actinomycin D at 12 h, and it completely prevented the increase in $[Ca^{2+}]_i$ observed at 18 h. No difference in Ca^{2+} homeostasis was observed at 6 and 24 h at any experimental conditions used.

Discussion

In the present study, we demonstrate that delphinidin, an anthocyanin contained in natural diet, is able to protect endothelial cells against apoptosis. Of particular interest is the finding that the antiapoptotic effect of delphinidin results from increased eNOS expression *via* MEK1/2 inhibitor-sensitive pathway. The effect of delphinidin also involves the NO and guanylyl cyclase-dependent pathway and is associated with the maintenance of endothelial $[Ca^{2+}]_i$ level in a physiological range and the decrease of cytochrome *c* release from the mitochondria.

The concentration of delphinidin (10^{-2} g l^{-1}) used in the present study is identical to that able to promote endothelium-dependent relaxation of rat aortic rings (Andriambeloson *et al*., 1998), to stimulate the increase in $[Ca^{2+}]_i$ in endothelial cells (Martin *et al*., 2002), to inhibit epidermal growth-factor receptor in human vulva carcinoma cell line A431 (Meiers *et al*., 2001) and to inhibit serum and vascular endothelial growth factor-induced proliferation in BAECs (Martin *et al*., 2003). Recent studies concerning bioavailability of polyphenols are in agreement with their potential therapeutic effects. Anthocyanins are present in fruits, beans, cereals, vegetables, or red wines, and are therefore ingested in considerable amounts as a constituent of the human diet (180–215 mg daily) (Kuhnau *et al*., 1976). According to the fact that the absorption rate of anthocyanins, such as delphinidin and cyanidin, was about 1% after oral administration, the concentration of delphinidin used in these study should be reached *in vivo* (i.e. 10 μg/ml) (Matsumoto *et al*., 2001). Also recently, we found that daily feedings of rats with RWPC for 1 week with 20 mg kg^{-1} or for 3 weeks with 40 mg kg^{-1} (a concentration 10–20 times greater than that producing maximal relaxation of rat aortic rings *ex vivo*) results in sufficient circulating concentration of polyphenols including delphinidin to improve cardiovascular functions (Diebolt *et al*., 2001; Bernatova *et al*., 2002). Altogether, this suggests that the concentration of delphinidin used in this study might be reached in plasma after dietary ingestion of polyphenols and may therefore be physiologically relevant.

A therapeutically relevant mechanism by which natural dietary polyphenolic compounds may exert their beneficial effects against cardiovascular diseases and the thrombotic tendency associated with cancer is the protection of endothelial cell against apoptosis. Endothelial injury, mainly by oxysterols, is commonly considered as playing a pivotal role in atherosclerosis both in the early stages of lesion formation, and later in the disease development process by inducing atherosclerotic plaque unstability. Thus, inhibition of endothelial apoptosis might prevent or reduce thrombosis. In the present study, it was observed that delphinidin protected endothelial cells against apoptosis. Interestingly, delphinidin treatment led to a significant reduction of both oxysterol and actinomycin D toxicity.

Our previous studies showed that NO participates strongly in the effects of delphinidin. In fact, polyphenols, including delphinidin, have been shown to induce endothelial NO production without any increase in the oxidative stress (Andriambeloson *et al*., 1998; Diebolt *et al*., 2001; Bernatova *et al*., 2002; Martin *et al*., 2002). Here, no iNOS expression could be detected in endothelial cells treated with actinomycin D alone or in combination with delphinidin. Thus, NO formed from iNOS expression cannot account for the protective effect of delphinidin even though overexpression of iNOS could be able to suppress lipopolysaccharide-induced endothelial cell apoptosis (Tzeng *et al*., 1997). In contrast, the nonselective NOS inhibitors, L-NA and SMT, prevented the effect of delphinidin on actinomycin D- or 7β-hydroxycholesterol-induced apoptosis. The protective effect of delphinidin in endothelial cells against actinomycin D-induced apoptosis was prevented by the guanylyl cyclase inhibitor, ODQ. Thus, it is likely that NO through the cGMP, resulting from guanylyl cyclase hydrolysis of guanosine triphosphate, plays a significant role in this process. In addition, whereas actinomycin D alone dramatically reduced eNOS protein level within endothelial cells, delphinidin restored eNOS expression at control level. Although actinomycin D is an inhibitor of mRNA synthesis, it was used at low concentrations in this study (i.e. 10–25 times lower as that effective to abolish gene transcription) (Inoue *et al*., 1995; Hisamoto *et al*., 2001), and this may explain the fact that delphinidin is still able to increase protein expression, even in its presence. This is consistent with the findings demonstrating that actinomycin D induces an upregulation of several protein expression (Roulston *et al*., 1998; Kleeff *et al*., 2000; Kim *et al*., 2002). Taken together, the results obtained in the present study strongly suggest that NO formed by eNOS isoform, whose expression is considerably enhanced by delphinidin, plays a significant role in protecting endothelial cell against apoptosis. These data are consistent with the hypothesis that levels of NO generated by

eNOS can inhibit apoptosis (Dimmeler *et al*., 1997) and allow maintenance of endothelial cell survival, in contrast to that released from iNOS which is generally described to possess a proapoptotic effect. Our results also shed light on the capacity of delphinidin to reglulate eNOS expression in endothelial cells. The fact that delphinidin counteracts the reduction of eNOS expression in actinomycin D-treated cells suggests that delphinidin probably acts, at least in part, by increasing the transcriptional rate of this enzyme. Cieslik *et al*. (2001) have recently reported that lysophosphatidylcholine is able to upregulate eNOS *via* the MEK1/2-dependent pathway. In accordance with these observations, both the protective effect and the overexpression of eNOS induced by delphinidin were abolished in the presence of the MEK inhibitor, PD98059. Hence, these data are consistent with the hypothesis that delphinidin protects endothelial cells against apoptosis through the upregulation of eNOS expression at least *via* MEK inhibitor-sensitive pathway. The mechanisms by which endothelial NO, upon stimulation by delphinidin, mediates its antiapopotic effects were further investigated. One of the molecular mechanisms by which NO protects endothelial cells against apoptosis is the blocking of the intrinsic/mitochochondrial apoptotic pathway. In this sudy, we reported that delphinidin is able to inhibit the release of cytochrome *c* into the cytosol induced by actinomycin D. It was recently described that in isolated mitochondria, NO effectively inhibits permeability transition and subsequent cytochrome *c* release (Brooks *et al*., 2000). NO can also inhibit cytochrome *c* release indirectly through upregulation of Bcl-2 (Rossig *et al*., 2000). We can therefore hypothesize that blocking the release of cytochrome *c* from the mitochondria could be a mechanism by which NO mediates the antiapoptotic effect of delphinidin. Another mechanism by which NO-cGMP pathway protects endothelial cells against apoptosis is the maintenance of $[Ca^{2+}]_i$ homeostasis. The increase of $[Ca^{2+}]_i$ has been reported to be one of the key signals leading to apoptotic cell death (Thornberry & Lazebnik, 1998). In the present study, actinomycin D induced an initial reduction, followed by a later elevation of $[Ca^{2+}]_i$. Delphinidin alone did not modify $[Ca^{2+}]_i$ values, but it prevented the effect of actinomycin D, so that, delphinidin restores $[Ca^{2+}]_i$ in physiological range. The early effect might be explained by the capacity of delphinidin to stimulate an increase of $[Ca^{2+}]_i$ in endothelial cells (Martin *et al*., 2002) which therefore counterbalances the reduction of $[Ca^{2+}]_i$ produced by actinomycin D. Once, after delphinidin treatment, the $[Ca^{2+}]_i$ level is high enough in the cells, it could stimulate the NO-guanylyl cyclase pathway *via* the activation of eNOS. In its turn, cGMP could decrease $[Ca^{2+}]_i$ through either inhibition of Ca^{2+} entry, enhancement of Ca^{2+} extrusion, activation of Ca^{2+} sequestration into intracellular store or inhibition of inositol triphosphate generation (Kim *et al*., 1997). The reduction of $[Ca^{2+}]_i$ by cGMP may account for the latter effect of delphinidin on the actinomycin D-induced increase in $[Ca^{2+}]_i$ and apoptosis. The fact that delphinidin abolished the effects of actinomycin D treatment on $[Ca^{2+}]_i$ changes, but only partially reduced actinomycin D-induced apoptosis suggests that other mechanisms might be involved in the delphinidin effect such as the reduction of cytochrome *c* release from the mitochondria. Nevertheless, the present work shows that delphinidin facilitates the maintenance of $[Ca^{2+}]_i$ within the physiological range upon stimulation by actinomycin D, explaining, at least in part, its protective effect against endothelial apoptosis.

In conclusion, we have shown that, delphinidin exerts an antiapoptotic effect in endothelial cells through the NO-guanylyl cyclase pathway, probably by upregulating eNOS expression *via* MEK1/2 pathway. Its preventive effect against cell death is associated with a strict control of Ca^{2+} homeostasis and the inhibition of the release of cytochrome *c* from the mitochondria. Endothelial protection by delphinidin may contribute to the beneficial effects of natural dietary polyphenolic compounds, including those contained in red wine, against cardiovascular disease. This antiapoptotic potential of delphinidin may be of importance in atherosclerosis in which the balance between survival and apoptosis is a major determinant of atherosclerotic plaque development.

We are grateful to Dr Jean-Marie Freyssinet for helpful suggestions during the preparation of this manuscript. We also thank Dr Gillian Gray for the careful reading of the manuscript. Sophie Martin is in recipient of a Fellowship from ONIVIN. This study was in part supported by grants from the Société Française de Distilleries (00/903/14/026), the EEC (Fair Program CT97-3261-DG12-SSMI) and ONIVIN (00/903/14/013).

References

ANDRIAMBELOSON, E., KLESCHYOV, A.L., MULLER, B., BERETZ, A., STOCLET, J.C. & ANDRIANTSITOHAINA, R. (1997). Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *Br. J. Pharmacol.,* **120,** 1053–1058.

ANDRIAMBELOSON, E., MAGNIER, C., HAAN-ARCHIPOFF, G., LOBSTEIN, A., ANTON, R., BERETZ, A., STOCLET, J.C. & ANDRIANTSITOHAINA, R. (1998). Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. *J. Nutr.,* **128,** 2324–2333.

AUPEIX, K., TOTI, F., SATTA, N., BISCHOFF, P. & FREYSSINET, J.M. (1996). Oyxsterols induce membrane procoagulant activity in monocytic THP-1 cells. *Biochem. J.,* **314,** 1027–1033.

BERNATOVA, I., PECHANOVA, O., BABAL, P., KYSELA, S., STVRTINA, S. & ANDRIANTSITOHAINA, R. (2002). Wine polyphenols improve cardiovascular remodeling and vascular function in NO-deficient hypertension. *Am. J. Physiol. (Heart Circ. Physiol.),* **282,** H942–H948.

BOCK, J., SZABO, I., JEKLE, A. & GULBINS, E. (2002). Actinomycin D-induced apoptosis involves the potassium channel Kv1.3. *Biochem Biophys. Res. Commun.,* **295,** 526–531.

BOER, R., ULRICH, WR., KLEIN, T., MIRAU, B., HAAS, S. & BAUR, I. (2000). The inhibitory potency and selectivity of arginine substrate site nitric-oxide synthase inhibitors is solely determined by their affinity toward the different isoenzymes. *Mol. Pharmacol.,* **58,** 1026–1034.

BROOKS, P.S., SALINAS, E.P., DARLEY-USMAR, K., EISERICH, J.P., FREEMAN, B.A., DARLEY-USMAR, V.M. & ANDERSON, P.G. (2000). Concentration-dependent effects of nitric oxide on mitochondrial permeability transition and cytochrome *c* release. *J. Biol. Chem.,* **275,** 20474–20479.

CIESLIK, K., ABRAMS, C.S. & WU, K.K. (2001). Up-regulation of endothelial nitric-oxide synthase promoter by the phosphatidylinositol 3-kinase gamma /Janus kinase 2/MEK-1-dependent pathway. *J. Biol. Chem.,* **276,** 1211–1219.

DIEBOLT, M., BUCHER, B. & ANDRIANTSITOHAINA, R. (2001). Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression. *Hypertension,* **38,** 159–165.

DIMMELER, S., HAENDELER, J., NEHLS, M. & ZEIHER, A.M. (1997). Suppression of apoptosis by nitric oxide *via* inhibition of interleukin-1 beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J. Exp. Med.,* **185,** 601–607.

FURCHGOTT, R.F., JOTHIANANDAN, D. & CHERRY, P.D. (1984). Endothelium-dependent responses: the last three years. *Bibl. Cardiol.,* **24,** 1–15.

GIDON-JEANGIRARD, C., HUGEL, B., HOLL, V., TOTI, F., LAPLANCHE, J.L., MEYER, D. & FREYSSINET, J.M. (1999). Annexin V delays apoptosis while exerting an external constraint preventing the release of CD4+and PrPc+membrane particles in a human T lymphocyte model. *J. Immunol.,* **162,** 5712–5718.

HENGARTNER, M.O. (2000). The biochemistry of apoptosis. *Nature,* **407,** 770–776.

HERTOG, M.G., FESKENS, E.J., HOLLMAN, P.C., KATAN, M.B. & KROMHOUT, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet,* **342,** 1007–1011.

HISAMOTO, K., OHMICHI, M., KANDA, Y., ADACHI, K., NISHIO, Y., HAYAKAWA, J., MABUCHI, S., TAKAHASHI, K., TASAKA, K., MIYAMOTO, Y., TANIGUCHI, N. & MURATA, Y. (2001). Induction of endothelial nitric-oxide synthase phosphorylation by the raloxifene analog LY117018 is differentially mediated by Akt and extracellular signal-regulated protein kinase in vascular endothelial cells. *J. Biol. Chem.,* **276,** 47642–47649.

INOUE, N., VENEMA, R.C., SAYEGH, H.S., OHARA, Y., MURPHY, T.J. & HARRISON, D.G. (1995). Molecular regulation of the bovine endothelial cell nitric oxide synthase by transforming growth factor-beta 1. *Arterioscler. Thromb. Vasc. Biol.,* **15,** 1255–1261.

KIM, P.K., WANG, Y., GAMBOTTO, A., KIM, Y.M., WELLER, R., ZUCKERBRAUN, B.S., HUA, Y., WATKINS, S.C. & BILLIAR, T.R. (2002). Hepatocyte Fas-associating death domain protein/mediator of receptor-induced toxicity (FADD/MORT1) levels increase in response to pro-apoptotic stimuli. *J. Biol. Chem.,* **277,** 38855–38862.

KIM, Y.M., BOMBECK, C.A. & BILLIAR, T.R. (1999). Nitric oxide as a bifunctional regulator of apoptosis. *Circ. Res.,* **84,** 253–256.

KIM, Y.M., TALANIAN, R.V. & BILLIAR, T.R. (1997). Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity *via* two distinct mechanisms. *J. Biol. Chem.,* **272,** 31138–31148.

KLEEFF, J., KORNMANN, M., SAWHNEY, H. & KORC, M. (2000). Actinomycin D induces apoptosis and inhibits growth of pancreatic cancer cells. *Int. J. Cancer,* **86,** 399–407.

KOCKX, M.M. & KNAAPEN, M.W. (2000). The role of apoptosis in vascular disease. *J. Pathol.,* **190,** 267–280.

KUHNAU, J. (1976). The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet.,* **24,** 117–191.

MANNICK, J.B., ASANO, K., IZUMI, K., KIEFF, E. & STAMLER, J.S. (1994). Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein–Barr virus reactivation. *Cell,* **79,** 1137–1146.

MARTIN, S., ANDRIAMBELOSON, E., TAKEDA, K. & ANDRIANTSITOHAINA, R. (2002). Red wine polyphenols increase calcium in bovine aortic endothelial cells: a basis to elucidate signalling pathways leading to nitric oxide production. *Br. J. Pharmacol.,* **135,** 1579–1587.

MARTIN, S., FAVOT, L., MATZ, R., LUGNIER, C. & ANDRIANTSITOHAINA, R. (2003). Delphinidin inhibits endothelial cell proliferation and cell cycle progression through a transient activation of ERK-1/-2. *Biochem. Pharmacol.,* **65,** 669–675.

MATSUMOTO, H., INABA, H., KISHI, M., TOMINAGA, S., HIRAYAMA, M. & TSUDA, T. (2001). Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rates and humans and appear in the blood as the intact forms. *J. Agric. Food chem.,* **49,** 1546–1551.

MEIERS, S., KEMENY, M., WEYAND, U., GASTPAR, R., VON ANGERER, E. & MARKO, D. (2001). The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *J. Agric. Food Chem.,* **49,** 958–962.

MIDDLETON JR, E., KANDASWAMI, C. & THEOHARIDES, T.C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.,* **52,** 673–751.

ROBERTS, K.A., WOO, M.M. & RUTLEDGE, J.C. (1997). Nitric oxide mediates LDL uptake in the artery wall in response to high concentrations of 17 beta-estradiol. *Arterioscler. Thromb. Vasc. Biol.,* **17,** 2123–2131.

ROSSIG, L., HAENDELER, J., HERMANN, C., MALCHOW, P., URBICH, C., ZEIHER, A.M. & DIMMELER, S. (2000). Nitric oxide down-regulates MKP-3 mRNA levels: involvement in endothelial cell protection from apoptosis. *J. Biol. Chem.,* **275,** 25502–25507.

ROULSTON, A., REINHARD, C., AMIRI, P. & WILLIAMS, L.T. (1998). Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor alpha. *J. Biol. Chem.,* **273,** 10232–10239.

SCHINI, V., GRANT, N.J., MILLER, R.C. & TAKEDA, K. (1988). Morphological characterization of cultured bovine aortic endothelial cells and the effects of atriopeptin II and sodium nitroprusside on cellular and extracellular accumulation of cyclic GMP. *Eur. J. Cell Biol.,* **47,** 53–61.

TERENZI, F., DIAZ-GUERRA, M.J., CASADO, M., HORTELANO, S., LEONI, S. & BOSCA, L. (1995). Bacterial lipopeptides induce nitric oxide synthase and promote apoptosis through nitric oxide-independent pathways in rat macrophages. *J. Biol. Chem.,* **270,** 6017–6021.

THORNBERRY, N.A. & LAZEBNIK, Y. (1998). Caspases: enemies within. *Science,* **281,** 1312–1316.

TZENG, E., KIM, Y.M., PITT, B.R., LIZONOVA, A., KOVESDI, I. & BILLIAR, T.R. (1997). Adenoviral transfer of the inducible nitric oxide synthase gene blocks endothelial cell apoptosis. *Surgery,* **122,** 255–263.

XIA, Z., DICKENS, M., RAINGEAUD, J., DAVIS, R.J. & GREENBERG, M.E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science,* **270,** 1326–1331.

(Received March 20, 2003
Accepted April 30, 2003)