

JPET #143172

Opposite effects of two resveratrol tetramers, vitisin A and hopeaphenol on apoptosis of myocytes isolated from adult rat heart.

Kazuhiko Seya, Kouta Kanemaru, Chiharu Sugimoto, Megumi Suzuki, Teruko Takeo, Shigeru Motomura, Haruo Kitahara, Masatake Niwa, Yoshiteru Oshima and Ken-Ichi Furukawa

Department of Pharmacology, Hirosaki University Graduate School of Medicine (K.S., K.K., S.M., K.F.), Faculty of Education, Hirosaki University (C.S., H.K.), Department of Medical Technology, Hirosaki University Graduate School of Health Science (M.S., T.T.), Faculty of Pharmacy, Meijo University (M.N.) and Graduate School of Pharmaceutical Science, Tohoku University (Y.O.)

JPET #143172

Running title: **Opposite effects of resveratrol tetramers**

Address correspondence to: Dr. Ken-Ichi Furukawa, Department of Pharmacology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, JAPAN, Tel: +81-172-39-5022, Fax: +81-172-39-5023, E-mail: furukawa@cc.hirosaki-u.ac.jp

The number of text pages: 29

The number of figures: 7

The number of tables: 0

The number of references: 34

The number of words in the Abstract: 230

The number of words in the Introduction: 631

The number of words in the Discussion: 869

A list of nonstandard abbreviations

3, 3'-Dipropylthiocarbonyl iodide (diS-C3(5))

Respiratory control ratio (RCR)

4-Diisothiocyano-2, 2-disulfonic acid stilbene (DIDS)

A recommended section

Cardiovascular or Toxicology

JPET #143172

Abstract

It has been reported that resveratrol from *Vitis* plants has various cardioprotective effects. *Vitis* plants also include various resveratrol tetramers. The aim of our study is to clarify the pharmacological properties of resveratrol tetramers. We isolated two resveratrol tetramers as major products of *Vitis* plants. One is vitisin A, a complex of two resveratrol dimers, (+)- ϵ -viniferin and ampelopsin B, and the other is hopeaphenol composed of two moles of ampelopsin B. Unexpectedly, vitisin A (30-300 nM) dose-dependently facilitated swelling and depolarization of mitochondria, and cytochrome c release from mitochondria, that are indices of cardiomyocyte apoptosis. Furthermore, vitisin A induced apoptosis in the primary culture of adult rat ventricular myocytes. On the other hand, hopeaphenol (1-10 μ M) dose-dependently inhibited Ca^{2+} (30 μ M)-induced mitochondrial depolarization and cytochrome c release from mitochondria but had not affected mitochondrial swelling. Moreover, hopeaphenol inhibited vitisin A-induced apoptosis. In structural and functional studies, we further confirmed that vitisin B, one of the resveratrol tetramers having (+)- ϵ -viniferin unit, induces mitochondrial swelling and cytochrome c release from mitochondria like as vitisin A, and that vitisifuran A, one of the resveratrol tetramers having ampelopsin B unit, inhibits Ca^{2+} -induced cytochrome c release from mitochondria like as hopeaphenol. These results show that resveratrol tetramers have at least two opposite effects on cardiomyocytes: the one having (+)- ϵ -viniferin unit induces cardiomyocyte apoptosis, and the other having ampelopsin B but not (+)- ϵ -viniferin unit inhibits it.

JPET #143172

Introduction

The family Vitaceae is primarily known for the commercial table grapes, raisins and wine production (Bavaresco et al., 1999). These grapevines can synthesize antifungal compounds, phytoalexins (Dercks and Creasy, 1989; Hain et al., 1993), in response to elicitation such as an infection by *Botrytis cinerea*, the causal agent for gray mold (Liswidowati et al., 1991). Their chemical structures are based on a polyphenol-type stilbene, resveratrol (trans-3, 5, 4'-trihydroxystilbene) (Wu et al., 2001) (Fig. 1A). Resveratrol has been credited with mediating a number of beneficial effects in the cardiovascular system. Especially, resveratrol's beneficial effects on cardiomyocytes occur in myocardial infarction. Resveratrol has anti-apoptotic effect through the reduction of JNK phosphorylation (Abdalla et al., 1999), the increase in Akt phosphorylation (Kaga et al., 2006), and the upregulation of inducible nitric oxide synthase (Imamura et al., 2002) in rat myocardial infarction model, preventing secondary myocardial infarction. These findings depict that resveratrol is a potential therapeutic agent in a multitude of cardiovascular diseases.

In addition to resveratrol, numerous oligostilbenes, consisting of oxidative oligomers of resveratrol, are biosynthesized as phytoalexins and polyphenols are believed to be active principles of a wide range of medicinal plants used to produce cardioprotection, but their mechanisms of action are not fully elucidated. In the last three decades, we have extracted and purified various novel oligostilbenes that are resveratrol tetramers from the genus *Vitis* (Oshima et al., 1995a; Ito et al., 1998, 1999). Some pharmacological properties of resveratrol tetramers such as anti-inflammatory and/or hepatoprotective effects have been reported (Oshima et al., 1993, 1995b; Huang et al., 2001). Recently, we reported that vitisin C induced vasorelaxation in the rabbit aorta by increasing nitric oxide production (Seya et al., 2003). In addition, Andriambeloson et al. (1997) have demonstrated that polyphenolic extracts, including stilbenes, from red wines induce endothelium-dependent vasorelaxation. However,

JPET #143172

there is no direct information about the cardioprotective effects of resveratrol tetramers.

It is known that cardiomyocyte apoptosis is induced by myocardial infarction and reperfusion therapy of this disease. Olivetti et al. (1996) reported that, within 10 days of the onset of acute myocardial infarction in humans, 12% of cardiomyocytes were TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling)-positive, indicating the induction of apoptosis, in the border zone of the infarction. Although reperfusion therapy for acute myocardial infarction is known to prevent the extension of infarct size, these beneficial effects are limited by acceleration of damage by reperfusion, myocardial inflammation, which activates inducible nitric oxide (NO) synthase, followed by cardiomyocyte apoptosis (Moens et al., 2005; Frangogiannis et al., 2002). Recently, we demonstrated that ODQ, an NO-sensitive guanylyl cyclase inhibitor, inhibited cardiomyocyte apoptosis in the ischemia-reperfusion condition (Seya et al., 2007). Apoptosis evoked by reperfusion after myocardial infarction may play an indispensable role in the development of cardiac remodeling. Although it is well known that resveratrol has an anti-apoptotic effect on the cardiomyocytes in the ischemic condition described as below, this effect of resveratrol tetramers is yet unknown.

The aim of our present study is to demonstrate cardioprotective effects of resveratrol tetramers using adult rat left ventricular mitochondria and primary cultured cardiomyocytes. We isolated two resveratrol tetramers as major products of *Vitis* plants, vitisin A (Fig. 1D), a complex of two resveratrol dimers, (+)- ϵ -viniferin (Fig. 1B) and ampelopsin B (Fig. 1C), and hopeaphenol (Fig. 1E) composed of two ampelopsin B units and investigated the effects of these natural products on cytochrome c release from mitochondria, activation of mitochondrial membrane permeable transition, and induction of apoptosis in cardiac mitochondria and primary cultured adult cardiomyocytes. Unexpectedly, we found that vitisin A and hopeaphenol have opposite effects on cardiomyocyte apoptosis. To clarify the structural

JPET #143172

correlation between two natural products' pharmacological effects, we further investigated using vitisin B (Fig. 1F), having (+)- ϵ -viniferin but not ampelopsin B unit, and vitisifuran A (Fig. 1G), having ampelopsin B but not (+)- ϵ -viniferin unit.

JPET #143172

Methods

Materials

Cyclosporin A, 4-diisothiocyano-2, 2-disulfonic acid stilbene (DIDS), Ru360, dimethyl sulfoxide (DMSO), annexin V-FITC and propidium iodide were obtained from Wako Pure Chemical Industries (Japan). 3, 3'-Dipropylthiodicarbocyanide iodide (diS-C3(5)) was obtained from LAMBDA Fluoresztechnologie (Austria). All chemicals used were of the highest purity commercially available. All chemicals were made fresh at sufficiently high concentrations that only very small aliquots had to be added to the assay tubes or culture medium. All natural products were freshly dissolved in DMSO. The final concentration of DMSO in the experimental tubes never exceeded 0.4% and had no effect on the cells or assays. The final concentration of DMSO was 0.1% in the experiments using mitochondrial suspension or 0.11% in those using primary cultured cardiomyocytes. Primary antibody against cytochrome c and Alexa Fluor 680 Goat Anti-mouse IgG as secondary antibody were purchased from Invitrogen (Carlsbad, CA)

This study was carried out in accordance with the Guidelines for Animal Experimentation, Hirosaki University.

Isolation of resveratrol tetramers

Isolation of resveratrol tetramers was performed using methods described previously (Oshima et al., 1995a; Ito et al., 1999). Briefly, dried corks of *Vitis vinifera* and stems of *Vitis coignetiae* (Vitaceae) were extracted with methanol and acetone, respectively, at room temperature to yield methanol and acetone extracts. The methanol extract was partitioned with ethyl acetate, and water to give ethyl acetate-soluble fraction. This fraction was repeatedly chromatographed over silica gel, and was eluted with n-hexane/ethyl acetate (1:4, v/v). The n-hexane/ethyl acetate eluting fraction was chromatographed over silica gel, and was eluted

JPET #143172

with chloroform/methanol (10:1, v/v) to obtain vitisin B and vitisifuran A as an amorphous powder. On the other hand, the acetone extract was partitioned with ethyl acetate, and water to give ethyl acetate-soluble fraction. This fraction was repeatedly chromatographed over silica gel to obtain vitisin A, vitisin B, and hopeaphenol. The yield of vitisin A, vitisin B, hopeaphenol, and vitisifuran A is 0.003, 0.84, 0.003, and 0.00006%, respectively. The structures of these tetramers were determined by IR, UV, fast-atom bombardment MS, and ^1H and ^{13}C -NMR spectra (see Fig. 1). The resveratrol tetramers were completely pure by ^1H - and ^{13}C -NMR spectra.

Isolation of mitochondria

Left ventricular mitochondria were isolated as described in a previous paper (Seya et al 2007). Briefly, left ventricular mitochondria were isolated in a buffer consisting of 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) and 1 mM EGTA by differential centrifugation of heart homogenates from male Wistar rats (350-400 g; CLEA Japan). The isolated mitochondria were resuspended in 250 mM sucrose and 10 mM Tris-HCl (pH 7.4) and stored in ice. The viability of the cardiac mitochondria was assessed by measuring the respiratory control ratio (RCR) using a Clark-type oxygen electrode (Gilson, Middleton, WI). We used mitochondria maintaining RCR value, more than 6. The protein concentration in the mitochondrial suspension was determined by the Bradford method using bovine albumin fraction V as a standard (Bradford, 1976). The protein concentration was determined and adjusted to 10 mg/ml with the homogenization buffer.

Determination of various mitochondrial functions

Cytochrome c release from mitochondria was measured by a western blot method (Seya et al., 2007). Specifically, initial mitochondrial suspension (0.05-0.1 mg protein/ml)

JPET #143172

was incubated in a medium consisting of 0.15 M KCl, 5 mM KH₂PO₄, 5 mM succinic acid and 10 mM HEPES (adjusted to pH 7.4 with 1 M Tris) at 30°C. At 10 min after drug administration, the reaction medium was centrifuged at 7,000 g for 10 min at 4°C. The supernatant was treated with trichloroacetic acid and the cytochrome c level in the precipitate obtained following centrifugation at 20,000 g was measured by western blot. The protein concentration of supernatant was measured to assess the mitochondrial outer membrane integrity. We regarded supernatant maintaining protein concentration up to 10 µg/ml as intact mitochondria and used it for the next step. Precipitates were separated on 14% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes, Immobilon-FL (Millipore, Billerica, MA). After blocking with PBST buffer (10 mM Na₂HPO₄-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) plus 3% skim milk, membranes were incubated with anti-cytochrome c antibodies at room temperature for 1 h. Membranes were then washed three times with PBST buffer (10 min each) and incubated with Alexa Fluor 680 goat anti-mouse secondary antibody for 1 h at room temperature. After three washes with PBST buffer, proteins were detected using Odyssey® Imaging System (LI-COR Biosciences, Lincoln, NE).

Mitochondrial swelling was determined in the above-mentioned medium containing initial mitochondrial suspension (0.05 mg protein/ml) by measuring the decrease in absorbance at 540 nm (Smith, 1967).

The mitochondrial membrane potential was measured in the above-mentioned medium containing 0.5 µg/ml of diS-C3(5) and initial mitochondrial suspension (0.1-0.2 mg protein/ml) by recording the fluorescence intensity at 670 nm in a fluorescence spectrophotometer (FP-750, Jasco, Japan) following excitation at 620 nm (Okimasu et al., 1979).

JPET #143172

Cell culture and detection of apoptotic cells

Ventricular myocytes were isolated from adult Wistar rats (350-400 g) by collagenase perfusion (Eppengerger-Eberhardt et al., 1990). Each intact rectangular cardiomyocyte was very gently sucked up and down with a micro-tip to another dish, and total number of intact cardiomyocytes isolated in this way was about 40 in each dish. After 12 h of culture, the culture medium consisting of Medium-199 supplemented with 14.3 mM NaHCO₃ (pH 7.4), 5 mM creatine, 5 mM taurine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 0.2% BSA was exchanged for medium containing vitisin A, hopeaphenol and other reagents. To inhibit fibroblast growth, cytosine arabinofuranoside (10 μM) was added to the culture medium.

Fluorescein isothiocyanate (FITC)-linked annexin V/propidium iodide staining was performed essentially as described previously (Vermes et al., 1995). In detail, an aliquot of binding buffer (80 μl) containing 25 μg/ml annexin V-FITC and 1 μg/ml propidium iodide was added to the cells and the mixture was incubated for 10 min at room temperature in the dark. After formaldehyde fixation, cells were analyzed under a fluorescence microscope (IX71; OLYMPUS, Japan). Cells treated with 1 mM H₂O₂ served as a positive control. Apoptotic cells were stained with annexin V-FITC, which binds with high affinity to phosphatidylserine, but excluded propidium iodide, a DNA dye that is unable to pass through the plasma membrane. In contrast, necrotic cells were stained with both annexin V-FITC and propidium iodide, since they had lost the physical integrity of their plasma membrane. The number of apoptotic cells was calculated by comparing the rate of apoptotic cells relative to the total cells. In each experiment, the average values of 50-100 cells were taken as the experiment's results.

To perform in situ labeling and quantitative analysis of late phase apoptotic cells, the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method (Gavrieli et al., 1992) was used for detection of fragmented DNA of adult rat primary

JPET #143172

cultured cardiomyocytes after 1 to 3 days using a TaKaRa in situ apoptosis detection kit (TaKaRa, Kusatsu, Japan). Apoptotic cells were visualized with 3, 3'-diaminobenzidine, and detected by light microscopy. Non-apoptotic cells were counterstained by methyl green. The value of apoptotic cells was calculated with a comparison of the rate of apoptotic cells to the total cells.

Statistical analysis

Comparisons between two groups were carried out using the 2-tailed Student t-test. Multiple group comparisons were performed by 1-way ANOVA, followed by Tukey's procedure for the comparison of means. Values are presented as mean \pm SEM, and $p < 0.05$ was considered to indicate statistical significance.

JPET #143172

Results

Apoptogenic effects of vitisin A

As indices of cardiomyocyte apoptosis, we monitored swelling and depolarization of mitochondria, and cytochrome c release from mitochondria. For monitoring of mitochondrial swelling, the decrease in absorbance of an initial mitochondrial suspension (0.05 mg of protein/ml) was measured at 540 nm in high-potassium buffer. In the left panel of Fig. 2A, bolus administration of 30 μM Ca^{2+} as a positive control induced a marked swelling of mitochondria (trace b), and this was completely inhibited in the presence of cyclosporin A, a specific inhibitor of mitochondrial membrane permeable transition pore (trace c). Vitisin A induced mitochondrial swelling in a dose-dependent manner (Fig. 2A, center panel). In the right panel of Fig. 2A, the swelling (trace b) induced by vitisin A (300 nM) was diminished in the presence of 0.2 mM EGTA (trace c), a Ca^{2+} chelator or 1 μM Ru360 (trace d), an inhibitor of Ca^{2+} uniporter but not cyclosporin A (trace e).

The mitochondrial membrane potential was measured using the cyanine dye diS-C3(5) in high-potassium buffer. In the left panel of Fig. 2B, bolus administration of 30 μM Ca^{2+} as a positive control induced depolarization of the mitochondrial membrane potential (trace b), and this was completely inhibited in the presence of cyclosporin A (trace c). Vitisin A also evoked depolarization of the mitochondrial membrane potential, dose-dependently (Fig. 2B, center panel), but its change was inhibited in the presence of 0.2 mM EGTA or 1 μM Ru360 (Fig. 2B, right panel, trace c and d, respectively), but not cyclosporin A (trace e).

The levels of cytochrome c release from cardiac mitochondria induced by resveratrol tetramers were determined by Western blot. Cytochrome c release induced by bolus administration of 30 μM Ca^{2+} was completely inhibited in the presence of cyclosporin A (Seya et al., 2007). Vitisin A also increased cytochrome c release from mitochondria in a dose-dependent manner (Fig. 3A). Cytochrome c release reached 2.2 ± 0.3 -fold of the control

JPET #143172

level at 300 nM (n=5). These increases were significantly inhibited in the presence of 1 μ M Ru360 (1.2 ± 0.04 -fold of the control level, $p < 0.05$) or 100 μ M 4-diisothiocyano-2,2-disulfonic acid stilbene (DIDS) (1.3 ± 0.1 -fold of the control level, $p < 0.05$), an inhibitor of voltage dependent anion channel (VDAC) but not cyclosporin A (2.2 ± 0.4 -fold of the control level) (Fig. 3B).

Vitisin A-induced apoptosis in adult rat cardiomyocytes

In the left panel of Fig. 4A shows representative images of annexin V-FITC staining of cardiomyocytes after 4 h treatment with vitisin A (300 nM). Cells in the early phase of apoptosis are stained by annexin V-FITC (Vermes et al., 1995). Vitisin A significantly increased the number of apoptotic cells, and this increase was significantly inhibited in the presence of Ru360 but not cyclosporin A. As shown in the right panel of Fig. 4A, the control ratio of apoptotic cells before incubation of rat isolated cardiomyocytes with vitisin A was $16.3 \pm 2.6\%$ (n=7). The ratio of apoptotic cells in the presence of vitisin A (300 nM) markedly increased and reached $54.7 \pm 1.9\%$ (n=7, $p < 0.01$ vs control). These increases were significantly inhibited in the presence of 1 μ M Ru360 ($42.2 \pm 1.2\%$, n=4, $p < 0.05$ vs vitisin A) but not cyclosporin A ($56.3 \pm 3.1\%$, n=3).

To investigate the late phase of apoptosis in cardiomyocytes induced by vitisin A, we detected target cells by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. As shown in Fig. 4B, vitisin A (30-300 nM) caused the late phase of apoptosis in cardiac myocytes. Control ratios of apoptotic cells for 1 day incubation of rat isolated cardiomyocytes were $2.4 \pm 0.5\%$ (n=5). The ratio of apoptotic cells in the presence of vitisin A increased in a dose-dependent manner and reached $28.0 \pm 3.0\%$ at 300 nM (n=5). Increment of late apoptotic cells induced by vitisin A was significantly inhibited in the presence of Ru360 ($21.5 \pm 2.8\%$, n=3, $p < 0.01$ vs vitisin A (300 nM)), but not cyclosporin A

JPET #143172

($37.3 \pm 4.4\%$, $n=3$). Ru360 alone did not affect the ratio of apoptotic cells (data not shown).

Anti-apoptotic effects of hopeaphenol

Hopeaphenol (1-10 μM) did not induce mitochondrial swelling (Fig. 5A, left graph) nor affect Ca^{2+} -induced mitochondrial swelling (Fig. 5A, right graph). The sudden increase in absorbance at 540 nm just after the addition of resveratrol tetramers was caused by their self-absorbencies.

On the other hand, hopeaphenol inhibited Ca^{2+} -induced mitochondrial depolarization (Fig. 5B, trace c), although hopeaphenol did not affect mitochondrial membrane potential (Fig. 5B, trace d).

Although hopeaphenol (10 μM) could not stimulate the cytochrome c release from mitochondria (1.3 ± 0.7 -fold of the control level, $n=4$), it (1-10 μM) dose-dependently inhibited Ca^{2+} -induced cytochrome c release from mitochondria (Fig. 6A). Cytochrome c release induced by Ca^{2+} reached 2.7 ± 0.3 -fold of the control level ($n=3$, $p < 0.01$ vs control). This increase was significantly and dose-dependently inhibited in the presence hopeaphenol (1.5 ± 0.2 -fold of the control level for 3 μM hopeaphenol, $p < 0.05$ vs 30 μM Ca^{2+} and 0.5 ± 0.1 -fold of the control level for 10 μM , $p < 0.01$ vs 30 μM Ca^{2+}). Furthermore, hopeaphenol (10 μM) also inhibited SNAP (1 mM)-induced cytochrome c release (Fig. 6B, Seya et al. 2007).

Protective effect of hopeaphenol on vitisin A-induced apoptosis

Effect of hopeaphenol on vitisin A-induced cardiomyocyte apoptosis was investigated (Fig. 6C). The control ratio of apoptotic cells before incubation with vitisin A was $13.1 \pm 7.2\%$ ($n=3$). However, the ratio after for 4 hr incubation with vitisin A (300 nM) reached $43.9 \pm 2.0\%$ ($n=7$, $p < 0.01$ vs control). These increases were significantly inhibited

JPET #143172

in the presence of 1 μM hopeaphenol ($32.7 \pm 3.0\%$, $n=3$, $p < 0.05$ vs vitisin A).

Effects of vitisin B and vitisifuran A on cardiomyocyte apoptosis

Vitisin B (Fig. 1F), one of the resveratrol tetramers having (+)- ϵ -viniferin unit like as vitisin A, induced mitochondrial swelling in a dose-dependent manner (Fig. 7A). The swelling induced by vitisin B (10 μM) was inhibited in the presence of 0.2 mM EDTA, a Ca^{2+} chelator and 1 μM Ru-360, an inhibitor of Ca^{2+} unipoter but not cyclosporin A (data not shown). Vitisin B (10 μM) also increased cytochrome c release from mitochondria like as vitisin A (Fig. 7C), and these increases were significantly inhibited in the presence of 1 μM Ru360 (data not shown).

Vitisifuran A (Fig. 1G), one of the resveratrol tetramers having ampelopsin B unit, did not induce mitochondrial swelling (Fig. 7B, trace c). The sudden increase in absorbance at 540 nm just after the addition of resveratrol tetramers was caused by their self-absorbencies. Furthermore, it did not affect Ca^{2+} - induced mitochondrial swelling (Fig. 7B, trace d). Although vitisifuran A (10 μM) alone did not induce cytochrome c release, it markedly inhibited Ca^{2+} -induced cytochrome c release like as hopeaphenol (Fig. 7D).

JPET #143172

Discussion

We demonstrated that two regioisomers of resveratrol tetramers, vitisin A and hopeaphenol have two opposite effects on cardiomyocyte apoptosis: vitisin A facilitates adult rat cardiomyocyte apoptosis, and hopeaphenol prevents it induced by various apoptotic stimulations. The former effects are inhibited by two distinct reagents, Ru360, an inhibitor of Ca^{2+} uniporter in mitochondrial inner membrane, and 4-diisothiocyano-2, 2-disulfonic acid stilbene (DIDS), an inhibitor of voltage dependent anion channel (VDAC) in mitochondrial outer membrane. The later effects are induced by inhibition of opening of VDAC like as DIDS.

Resveratrol is known as an anti-cancer substance. For example, in human tumor cell lines, resveratrol inhibited cell growth and DNA synthesis in a dose dependent manner (Huang et al., 1998; Clement et al., 1999). Furthermore, resveratrol induces CD95-dependent and/or mitochondria/caspase-9-dependent apoptosis in human tumor cells. On the other hand, there is a report that in the non-proliferative cells, resveratrol prevents apoptosis by preventing Bax activation (Kaga et al., 2006). Unexpectedly, some resveratrol tetramers especially having (+)- ϵ -viniferin unit including vitisin A were able to induce cardiomyocyte apoptosis. We confirmed that (+)- ϵ -viniferin also induced mitochondrial swelling and cytochrome c release from cardiac mitochondria (unpublished data). Although resveratrol selectively inhibits apoptosis of non-proliferative cells, resveratrol tetramers having (+)- ϵ -viniferin unit may induce apoptosis of all species of cells because of their effect of Ca^{2+} uniporter activation in mitochondrial inner membrane.

It is known that DIDS also inhibit mitochondrial inner membrane anion channel (IMAC) which has a wide variety anion selectivity and is inactivated in the physiological condition (Beavis and Davatol-Hag, 1996). We consider that IMAC does not participate in the apoptogenic effects of vitisin A because Ru360, completely inhibits the mitochondrial

JPET #143172

swelling, depolarization of mitochondrial membrane potential, and cytochrome c release from mitochondria induced by vitisin A. However, cyclosporin A, an inhibitor of mitochondrial adenine nucleotide translocase failed to inhibit them. Although it is necessary to measure the inhibitory effect of DIDS on vitisin A-induced cardiomyocyte apoptosis, DIDS prohibited the detection of apoptotic cells because of its high self-absorbance.

In this investigation, we measured various mitochondrial functions with the nominal Ca^{2+} free buffer. Although mitochondrial Ca^{2+} uniporter is the Ca^{2+} selective ion channel, in the low Ca^{2+} buffer, this uniporter is permeable to other various cations, which is significantly inhibited by Ru360 (Kirichok et al. 2004). The results that cytochrome c release induced by vitisin A from mitochondria was inhibited in the presence of Ru360 (1 μM) suggests the possibility that vitisin A activates mitochondrial Ca^{2+} uniporter. We further confirmed that cardiomyocyte apoptosis induced by vitisin A was significantly but small inhibited by Ru360 (1 μM) (Fig. 4A). Higher than 3 μM of Ru360 caused the cell injury and increased necrotic cells. In the TUNEL method for measurement of late phase apoptotic cells, Ru360 (1 μM) strongly inhibited the cardiomyocyte apoptosis induced by vitisin A.

Hopeaphenol composed of two ampelopsin B units significantly inhibited Ca^{2+} - and NO donor-induced cytochrome c release from cardiac mitochondria, and its mechanism may be to inhibit the VDAC activity on the mitochondrial outer membrane. Although common inhibitors DIDS and dicyclohexyl carbodiimide of VDAC are directly binding to VDAC and inhibit its activity, they spontaneously induce mitochondrial swelling unlike hopeaphenol (Bernardes et al. 1994; Yamamoto et al. 2005). From these observations, we hypothesize that hopeaphenol and its derivatives having ampelopsin B are new type VDAC inhibitor, which also inhibited cardiomyocyte apoptosis induced by vitisin A and ischemia-reperfusion condition. We confirmed that ampelopsin A, which is a dehydroxy derivative of ampelopsin B, also inhibited Ca^{2+} -induced cytochrome c release from mitochondria (unpublished data).

JPET #143172

However, vitisin A also has ampelopsin B unit which was not affected by the inhibition of VDAC activity. We consider that the binding activity of (+)- ϵ -viniferin unit to Ca^{2+} uniporter may be stronger than that of ampelopsin B unit to VDAC. The more precise mechanism by which resveratrol tetramers having ampelopsin B unit inhibit VDAC activity awaits further investigation.

It has been demonstrated that hopeaphenol has various effects, namely, anti-inflammatory, anti-tumor, and anti-microbial effects (Huang et al., 2001; Mishima et al., 2003; Zgoda-Pols et al., 2002). All these effects are useful for medical care. Recently, we demonstrated that the prevention of apoptosis by cytochrome c release from mitochondria induced by large amount of NO produced by iNOS in the reperfusion therapy after acute myocardial infarction is useful for the care of acute myocardial infarction (Seya et al., 2007). In this study, we show that hopeaphenol inhibits Ca^{2+} - and NO donor-induced cytochrome c release and apoptosis in the primary cultured cardiomyocytes induced by vitisin A. These results show that hopeaphenol may be useful for the care of acute myocardial infarction and prevention of secondary myocardial infarction. It is expected that further clinical study using hopeaphenol on myocardial infarction will be needed.

In conclusion, resveratrol tetramers have at least two opposite effects on cardiomyocyte apoptosis. Vitisin A and vitisin B, having (+)- ϵ -viniferin unit, markedly increased the mitochondrial swelling and cytochrome c release from mitochondria and induced cardiomyocyte apoptosis. On the other hand, hopeaphenol and vitisifuran A inhibited Ca^{2+} - and NO donor-induced cytochrome c release from cardiac mitochondria, and cardiomyocyte apoptosis induced by vitisin A. These results show one way that resveratrol tetramers having ampelopsin B structure are useful for clinical care, for example the prevention of acute myocardial infarction.

JPET #143172

Acknowledgements

We thank Ms Izumi Miki in this laboratory, Hirosaki University Graduate School of Medicine, for various technical assistances. We also thank Drs Soroku Yagihashi, Ryuichi Wada and Shin-Ichiro Yamagishi, Department of Pathology, Hirosaki University Graduate School of Medicine for their appropriate advices about our research.

JPET #143172

References

- Abdalla M, Mowafy EL and White RE (1999) Resveratrol inhibits MAPK activity and nuclear translocation in coronary artery smooth muscle: reversal of endothelin-1 stimulatory effects. *FEBS Lett* :63-67.
- Andriambeloson E, Kleschyov AL, Muller B, Beretz A, Stoclet JC and Andriantsitohaina R (1997) Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *Br J Pharmacol* **120**:1053-1058.
- Bavaresco L, Fregoni C, Cantu E and Trevisan M (1999) Stilbene compounds: from the grapevine to wine. *Drugs Exptl Clin Res* **XXV**; 57-63.
- Beavis AD and Davatol-Hag H. (1996) The mitochondrial inner membrane anion channel is inhibited by DIDS. *J Bioenerg Biomembr* **28**:207-214.
- Bernardes CF, Meyer-Fernandes JR, Basseres DS, Castilho RF and Varcési AE (1994) Ca²⁺-dependent permeabilization of the inner mitochondrial membrane by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) *Biochim Biophys Acta* **1188**:93-100.
- Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-254.
- Clement M-V, Hirpara JL, Chawdhury S-H and Pervaiz S (1999) Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. *Am Soc Hemat* **92**:996-1002.
- Dercks W and Creasy LL (1989) The significance of stilbene phytoalexins in the plasmopara viticola-grapevine interaction. *Physiol Mol Plant Pathol* **34**:189-202.
- Eppenberger-Eberhardt M, Flamme I, Kurer V and Eppenberger HM (1990) Reexpression of α -smooth muscle actin isoform in cultured adult rat cardiomyocytes. *Dev Biol*

JPET #143172

139:269–278.

Frangogiannis NG, Smith CW and Entman ML (2002) The inflammatory response in myocardial infarction. *Cardiovasc Res* **53**:31-47.

Gavrieli Y, Sherman Y and Ben-Sasson SA (1992) Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* **119**:493-501.

Hain R, Reif HJ, Krause E, Langebartels R, Kindl H, Vornam B, Wiese W, Schmelzer E, Schreier P, Stocker R and Stenzel K (1993) Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature* **361**:153-156.

Huang C, Ma W-Y, Goranson A and Dong Z (1998) Resveratrol suppresses cell transformation and induced apoptosis through a p53-dependent pathway. *Carcinogenesis* **20**:237-242.

Huang K-S, Lin M and Cheng G-F (2001) Anti-inflammatory tetramers of resveratrol from the roots of *Vitis amurensis* and the conformations of the seven-membered ring in some oligostilbenes. *Phytochemistry* **58**:357-362.

Imamura G, Bertelli AA, Bertelli A, Otani H, Maulik N and Das DK (2002) Pharmacological preconditioning with resveratrol: an insight with iNOS knockout mice. *Am J Physiol: Heart Circ Physiol* **282**:H1996-H2003.

Ito J, Gabaru K, Shimamura T, Niwa M, Takaya Y and Oshima Y (1998) Absolute configurations of some oligostilbenes from *Vitis vinifera* ‘Kyohou’. *Tetrahedron* **54**:6651-6660.

Ito J, Takaya Y, Oshima Y and Niwa M (1999) New oligostilbenes having a benzofuran from *Vitis vinifera* ‘Kyohou’. *Tetrahedron* **55**:2529-2544.

Kaga S, Zhan L, Altaf E and Maulik N (2006) Glycogen synthase kinase-3beta/beta-catenin promotes angiogenic and anti-apoptotic signaling through the induction of VEGF, Bcl-2 and survivin expression in rat ischemic preconditioned myocardium. *J Mol Cell Cardiol*

JPET #143172

40:138-147.

Kirichok Y, Krapivinsky G and Clapham DE (2004) The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* **427**:360-364.

Liswidowati F, Melchior F, Hohmann F, Schwer B and Kindl H (1991) Induction of stilbene synthase by *Botrytis cinerea*, in cultured grapevine cells. *Planta* **183**:307-314.

Mishima S, Matsumoto K, Futamura Y, Araki Y, Ito T, Tanaka T, Iinuma M, Nozawa Y and Akao Y (2003) Antitumor effect of stilbenoids from *Vateria indica* against allografted sarcoma S-180 in animal model. *J Exp Ther Onco.* **3**:283-88.

Moens AL, Claeys MJ, Timmermans JP and Vrints CJ (2005) Myocardial ischemia/reperfusion-injury, a clinical view on a complex pathophysiological process. *Int J Cardiol* **100**:179-190.

Okimasu E, Akiyama J, Shiraishi N and Utsumi K (1979) The mechanism of inhibition on the endogenous respiration of Ehrlich ascites tumor cells by the cyanine dye diS-C3-(5). *Physiol Chem Phys* **11**:425-433.

Olivetti G, Quaini F, Sala R, Lagrasta C, Corradi D, Bonacina E, Gambert SR, Cigola E and Anversa P (1996) Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart. *J Mol Cell Cardiol* **28**:2005-2016.

Oshima Y, Kamijou A, Moritani H, Namao K-I and Ohizumi Y (1993) Vitisin A and cis-vitisin A, strongly hepatotoxic plant oligostilbenes from *Vitis coignetiae* (Vitaceae). *J Org Chem* **58**:850-853.

Oshima Y, Kamijou A, Ohizumi Y, Niwa M, Ito J, Hisamichi K and Takeshita M (1995a) Novel oligostilbenes from *Vitis coignetiae*. *Tetrahedron* **51**:11979-11986.

Oshima Y, Namao K, Kamijou A, Matsuoka S, Nakano M, Terao K and Ohizumi Y (1995b) Powerful hepatoprotective and hepatotoxic plant oligostilbenes, isolated from the

JPET #143172

- Oriental medicinal plant *Vitis coignetiae* (Vitaceae). *Experientia* **51**:63-66.
- Seya K, Furukawa K-I, Taniguchi S, Kodzuka G, Oshima Y, Niwa M and Motomura S (2003) Endothelium-dependent vasodilatory effect of vitisin C, a novel plant oligostilbene from *Vitis* Plants (Vitaceae) in rabbit aorta. *Clin Sci* **105**:73-79.
- Seya K, Motomura S, and Furukawa K-I (2007) Cardiac mitochondrial cGMP stimulates cytochrome *c* release. *Clin Sci* **112**:113-121.
- Smith AL (1967) Preparation, properties, and conditions for assay of mitochondria: slaughterhouse material, small-scale. *Methods Enzymol* **10**:81-86.
- Vermes I, Haanen C, Steffens-Nakken H and Reutelingsperger CA (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Meth* **184**:39-51.
- Wu JM, Wang ZR, Hsieh TC, Bruder JL, Zou JG and Huang YZ (2001) Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine. *Int J Mol Med* **8**:3-17.
- Yamamoto T, Terauchi S, Tachikawa A, Yamashita K, Kataoka M, Terada H and Shinohara Y (2005) Two critical factors affecting the release of mitochondrial cytochrome C as revealed by studies using N,N'-dicyclohexylcarbodiimide as an atypical inducer of permeability transition. *J Bioener Biomem* **37**:299-306.
- Zgoda-Pols JR, Freyer AJ, Killmer LB and Porter JR (2002) Antimicrobial resveratrol tetramers from the stem bark of *Vatica oblongifolia* sp. *J Nat Prod* **65**:1554-1559.

JPET #143172

Footnotes

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan to Dr. Ken-Ichi Furukawa. Financial support to Dr. Ken-Ichi Furukawa from Hirosaki University Educational Improvement Promotional Aid is also acknowledged.

Address for print requests: Dr. Ken-Ichi Furukawa, Department of Pharmacology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. E-mail: furukawa@cc.hirosaki-u.ac.jp

JPET #143172

Legends for Figures

Figure 1 Structure of resveratrol (A), (+)- ϵ -viniferin (B), ampelopsin B (C), vitisin A (D), hopeaphenol (E), vitisin B (F) and vitisifuran A (G).

Vitisin A (D) is composed of two resveratrol dimers, (+)- ϵ -viniferin (B) and ampelopsin B (C). Hopeaphenol is formed by two ampelopsin B units (C). Vitisin B (F) is partially formed by an (+)- ϵ -viniferin unit. Vitisifuran A (G) contains an ampelopsin B unit.

Figure 2 Effects of vitisin A on swelling (A) and membrane potential (B) of cardiac mitochondria

(A) Mitochondrial swelling was monitored as an absorbance change of a mitochondrial suspension (0.05 mg of protein/ml) at 540 nm in a high-potassium buffer. (Left panel) Mitochondria were pre-incubated in the absence (control; trace a) or presence of 30 μM Ca^{2+} (positive control; trace b) or 30 μM Ca^{2+} + 1 μM cyclosporin A (trace c). (Center panel) Mitochondria were pre-incubated in the absence (control; trace a) or presence of 10-300 nM vitisin A (trace b: 10 nM, trace c: 30 nM, trace d: 100 nM, trace e: 300 nM). (Right panel) Mitochondria were pre-incubated in the absence (control; trace a) or presence of 300 nM vitisin A (trace b), 300 nM vitisin A + 0.2 mM EGTA (trace c), 300 nM vitisin A + 1 μM Ru360 (trace d), or 300 nM vitisin A + 1 μM cyclosporin A (trace e).

(B) Mitochondrial membrane potential was monitored using the cyanine dye diS-C3(5) in a high-potassium buffer. (Left panel) Mitochondria were pre-incubated in the absence (control; trace a) or presence of 30 μM Ca^{2+} (positive control; trace b) or 30 μM Ca^{2+} + 1 μM cyclosporin A (trace c). (Center panel) Mitochondria were pre-incubated in the absence (control; trace a) or presence of 30-300 nM vitisin A (trace b: 30 nM, trace c: 100 nM, trace d: 300 nM). (Right panel) Mitochondria were pre-incubated in the absence (control; trace a) or presence of 300 nM vitisin A (trace b), 300 nM vitisin A + 0.2 mM EGTA (trace c), 300 nM

JPET #143172

vitisin A + 1 μ M Ru360 (trace d), or 300 nM vitisin A + 1 μ M cyclosporin A (trace e).

Figure 3 Effects of vitisin A on cytochrome c release from cardiac mitochondria

(A) Isolated cardiac mitochondria were incubated with various concentrations of vitisin A (0-300 nM) in a high-potassium buffer for 10 min at 30 °C. Cytochrome c release from mitochondria was determined by Western blot. Band intensity was normalized to the mean value obtained from the control. Lane 1, control (Cont.); lane 2, 10 nM vitisin A; lane 3, 30 nM vitisin A; lane 4, 100 nM vitisin A; lane 5, 300 nM vitisin A; lane 6, cytochrome c (0.2 μ g). Values are the mean \pm S.E.M. of five experiments. *p <0.05 and **p <0.01 compared with control.

(B) Cytochrome c release was induced by vitisin A in the presence of cyclosporin A, Ru360 or DIDS. Lane 1, control (Cont.); lane 2, 300 nM vitisin A; lane 3, 300 nM vitisin A + 1 μ M cyclosporin A; lane 4, 300 nM vitisin A + 1 μ M Ru360; lane 5, 300 nM vitisin A + 0.1 mM DIDS; lane 6, cytochrome c (0.2 μ g). Values are the mean \pm S.E.M. of five experiments. *p <0.05 compared with 300 nM vitisin A. #p < 0.01 compared with control.

Figure 4 Vitisin A-induced apoptosis in primary cultured adult rat cardiomyocytes

(A) Left upper panel shows representative images of annexin V-FITC/propidium iodide staining of cardiomyocytes incubated for 4 h under the following conditions: (a) control; (b) in the presence of 300 nM vitisin A; (c) in the presence of vitisin A + 1 μ M Ru360. The left lower panel shows the phase-contrast views of each cardiomyocyte. The right graph shows the alternation of vitisin A-induced cardiomyocyte apoptosis in the absence (control) or presence of 300 nM vitisin A, 300 nM vitisin A + 1 μ M cyclosporin A, or 300 nM vitisin A + 1 μ M Ru360. The values for the apoptotic cells were calculated by comparing the rates of the apoptotic cells relative to the total number of cells. Values are the mean \pm S.E.M. of seven

JPET #143172

(vitisin A), three (vitisin A + cyclosporin A), or four (vitisin A + Ru360) experiments. * $p < 0.01$ compared with control. # $p < 0.05$ compared with the presence of 300 nM vitisin A alone. (B) Vitisin A-induced apoptosis of adult rat cardiomyocytes was estimated by TUNEL method. Bar graph show the rate of apoptotic cells after 1 day of culture in the various concentrations of vitisin A (0 to 300 nM), 300 nM vitisin A + 1 μ M cyclosporin A, and 300 nM vitisin A + 1 μ M Ru360. The value of apoptotic cells was calculated as comparison of the rate of apoptotic cells to the total cells. Values are mean \pm SEM of five (vitisin A), four (vitisin A + cyclosporin A), or three (vitisin A + Ru360) experiments. Significant differences: * $p < 0.05$ compared with control. # $p < 0.05$ compared with the presence of 300 nM vitisin A alone.

Figure 5 Effects of hopeaphenol on swelling (A) and membrane potential (B) of cardiac mitochondria

(A) Absorbance of a mitochondrial suspension (0.05 mg of protein/ml) was measured at 540 nm in a high-potassium buffer. (Left graph) Mitochondria were pre-incubated in the absence (control; trace a) or presence of 30 μ M Ca^{2+} (positive control; trace b), 1 μ M hopeaphenol (trace c), 3 μ M hopeaphenol (trace d), or 10 μ M hopeaphenol (trace e). (Right graph) Mitochondria were pre-incubated in the absence (control; trace a) or presence of 30 μ M Ca^{2+} (trace b), 30 μ M Ca^{2+} + 1 μ M hopeaphenol (trace c), 30 μ M Ca^{2+} + 3 μ M hopeaphenol (trace d), or 30 μ M Ca^{2+} + 10 μ M hopeaphenol (trace e). (B) Mitochondrial membrane potential was measured using the cyanine dye diS-C3(5) in a high-potassium buffer. Mitochondria (0.1 mg of protein/ml) were pre-incubated in the absence (control; trace a) or presence of 30 μ M Ca^{2+} (trace b), 30 μ M Ca^{2+} + 10 μ M hopeaphenol (trace c), 10 μ M hopeaphenol (trace d).

Figure 6 Effects of hopeaphenol on Ca^{2+} -induced cytochrome c release from cardiac

JPET #143172

mitochondria and cardiomyocyte apoptosis

(A) Isolated cardiac mitochondria were incubated with 30 μM Ca^{2+} and/or hopeaphenol in a high-potassium buffer for 10 min at 30 °C. Cytochrome c release from mitochondria was determined by Western blot. Band intensity was normalized to the mean value obtained from the control. Lane 1, control (Cont.); lane 2, 30 μM Ca^{2+} ; lane 3, 30 μM Ca^{2+} + 1 μM cyclosporin A; lane 4, 30 μM Ca^{2+} + 3 μM hopeaphenol; lane 5, 30 μM Ca^{2+} + 10 μM hopeaphenol; lane 6, cytochrome c (0.2 μg). Values are the mean \pm S.E.M. of four experiments. * $p < 0.01$ compared with control. # $p < 0.05$ and ## $p < 0.01$ compared with 30 μM Ca^{2+} . (B) Isolated cardiac mitochondria were incubated with 1 mM SNAP and/or hopeaphenol in a high-potassium buffer for 10 min at 30 °C. Cytochrome c release from mitochondria was determined by Western blot. Lane 1, control (Cont.); lane 2, 1 mM SNAP; lane 3, 1 mM SNAP + 10 μM hopeaphenol. (C) The changes in the rate of apoptotic cells by incubating for 4 h under the following conditions in the absence (control; closed bar) or presence of 300 nM vitisin A (grey bar), or 300 nM vitisin A + 1 μM hopeaphenol (open bars). The values for the apoptotic cells were calculated by comparing the rates of the apoptotic cells relative to the total number of cells. Values are the mean \pm S.E.M. of three experiments. * $P < 0.01$ compared with control. # $P < 0.05$ compared with the presence of 300 nM vitisin A alone.

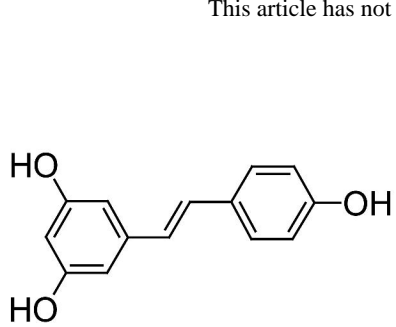
Figure 7 Effects of vitisin B and vitisifuran A on cardiac mitochondria

(A and B) Absorbance of a mitochondrial suspension (0.05 mg of protein/ml) was measured at 540 nm in a high-potassium buffer. (A) Mitochondria were pre-incubated in the absence (control; trace a) or presence of 30 μM Ca^{2+} (trace b) or 1-10 μM vitisin B (trace c: 1 μM , trace d: 3 μM , trace e: 10 μM). (B) Mitochondria were pre-incubated in the absence (control; trace a) or presence of 30 μM Ca^{2+} (trace b), 10 μM vitisifuran A (trace c), or 30 μM Ca^{2+} + 10

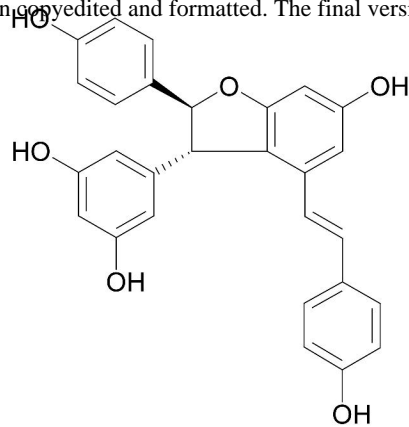
JPET #143172

μM vitisifuran A (trace d).

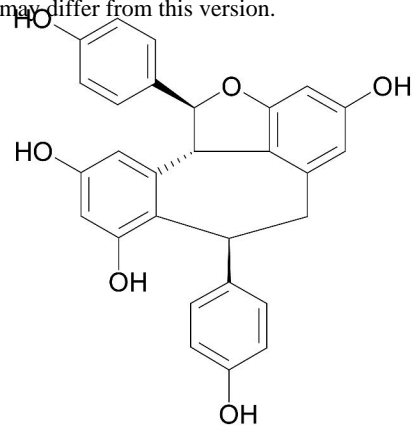
(C and D) Isolated cardiac mitochondria were incubated with $30 \mu\text{M Ca}^{2+}$, vitisifuran A, or vitisin B in a high-potassium buffer for 10 min at 30°C . Cytochrome c release from mitochondria was determined by Western blot. (C) Lane 1, control (Cont.); lane 2, $30 \mu\text{M Ca}^{2+}$; lane 3, $10 \mu\text{M}$ vitisin B; lane 4, $10 \mu\text{M}$ vitisifuran A; lane 5, cytochrome c ($0.2 \mu\text{g}$). (D) Lane 1, control (Cont.); lane 2, $30 \mu\text{M Ca}^{2+}$; lane 3, $30 \mu\text{M Ca}^{2+} + 10 \mu\text{M}$ vitisifuran A; lane 4, $10 \mu\text{M}$ vitisifuran A.



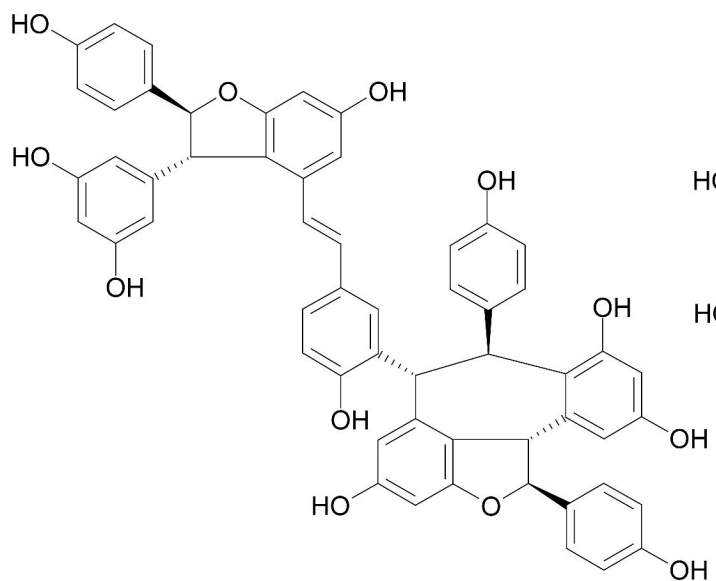
A Resveratrol



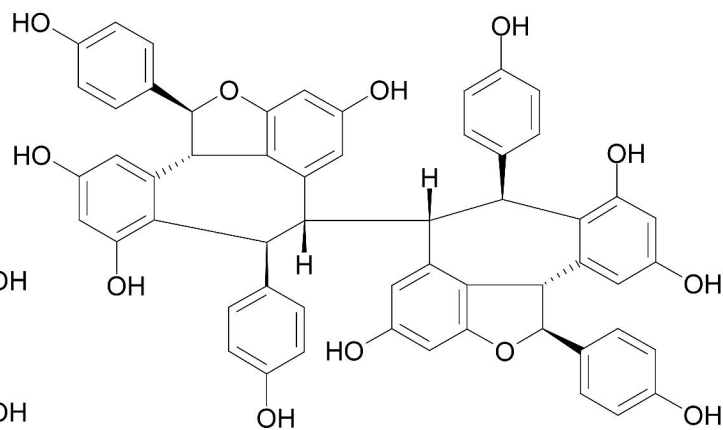
B (+)-ε-Viniferin



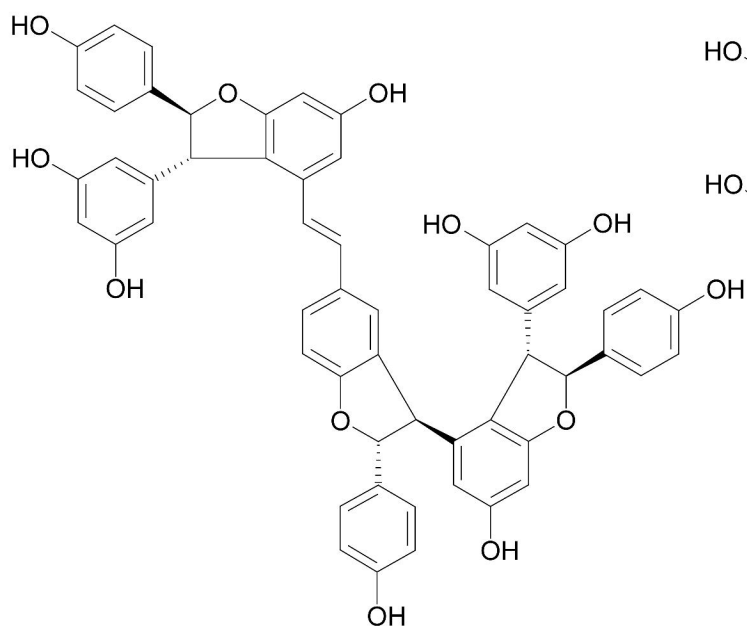
C Ampelopsin B



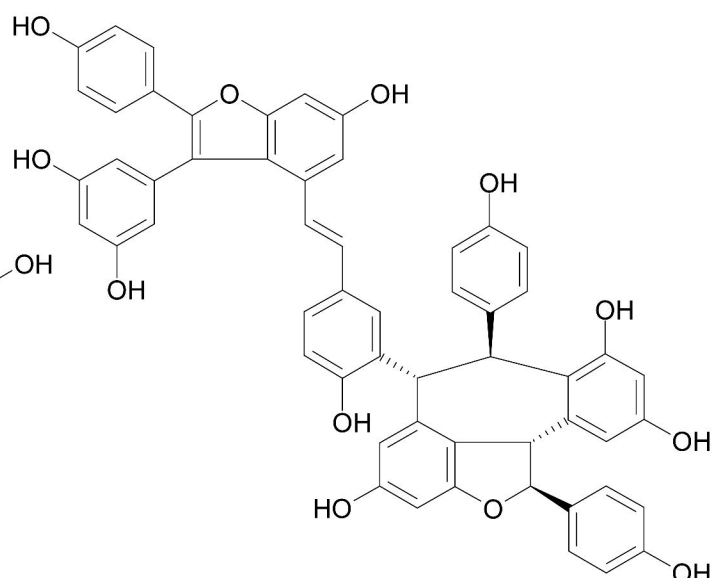
D Vitisin A



E Hopeaphenol



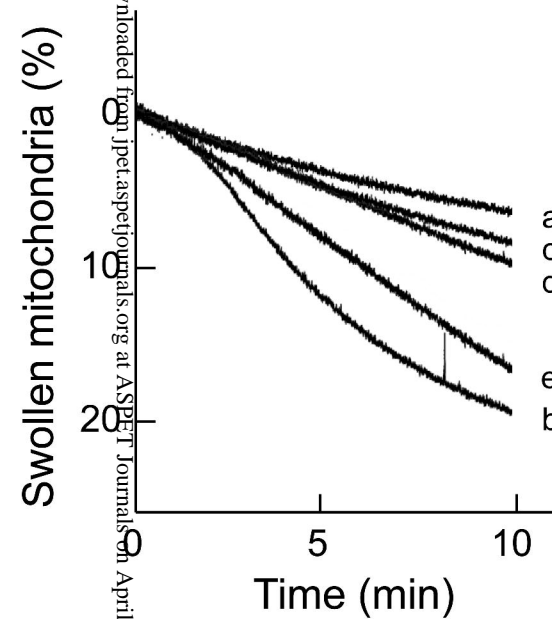
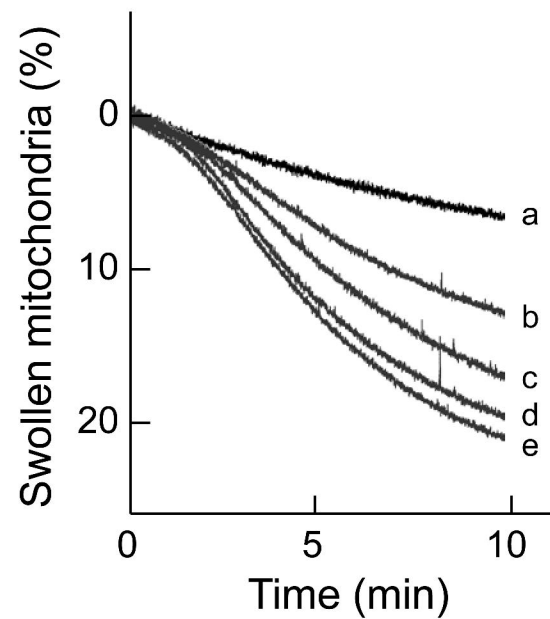
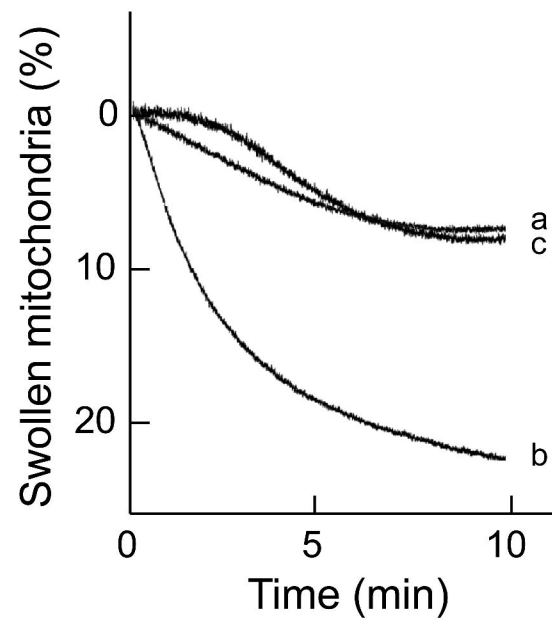
F Vitisin B



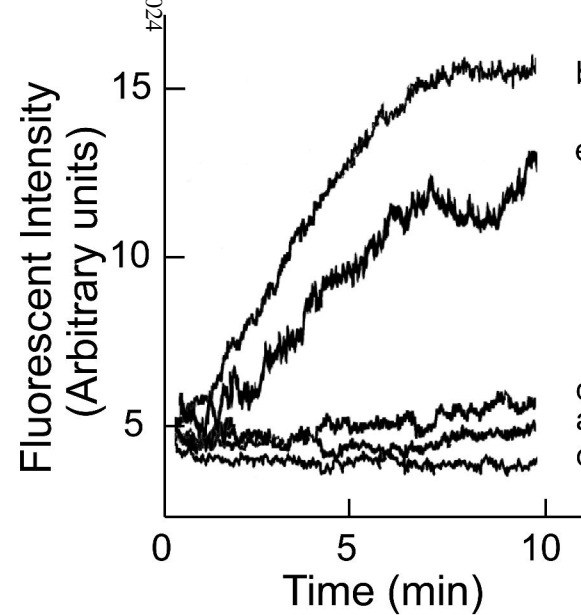
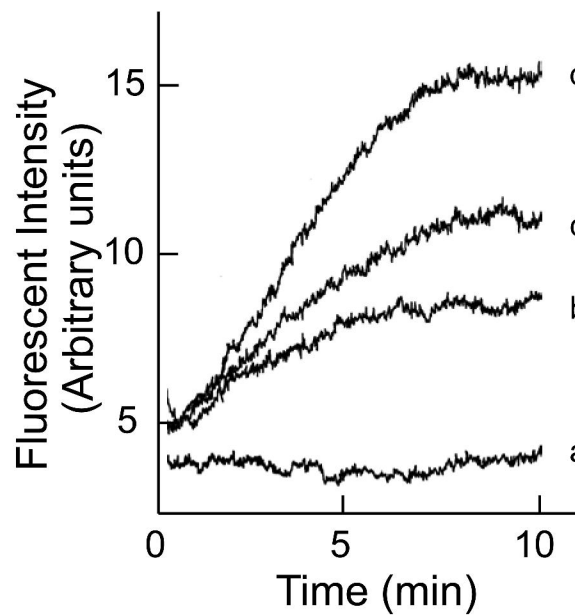
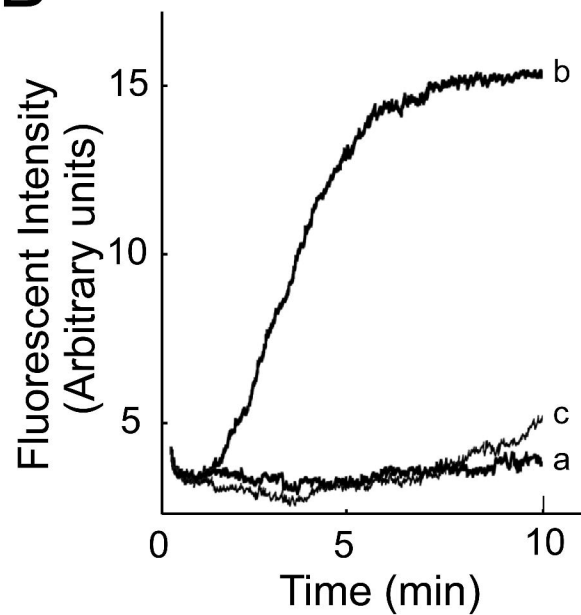
G Vitisifuran A

Figure 2

A



B



Downloaded from jpeel.aspetjournal.org at ASPET Journals on April 19, 2024

Figure 3

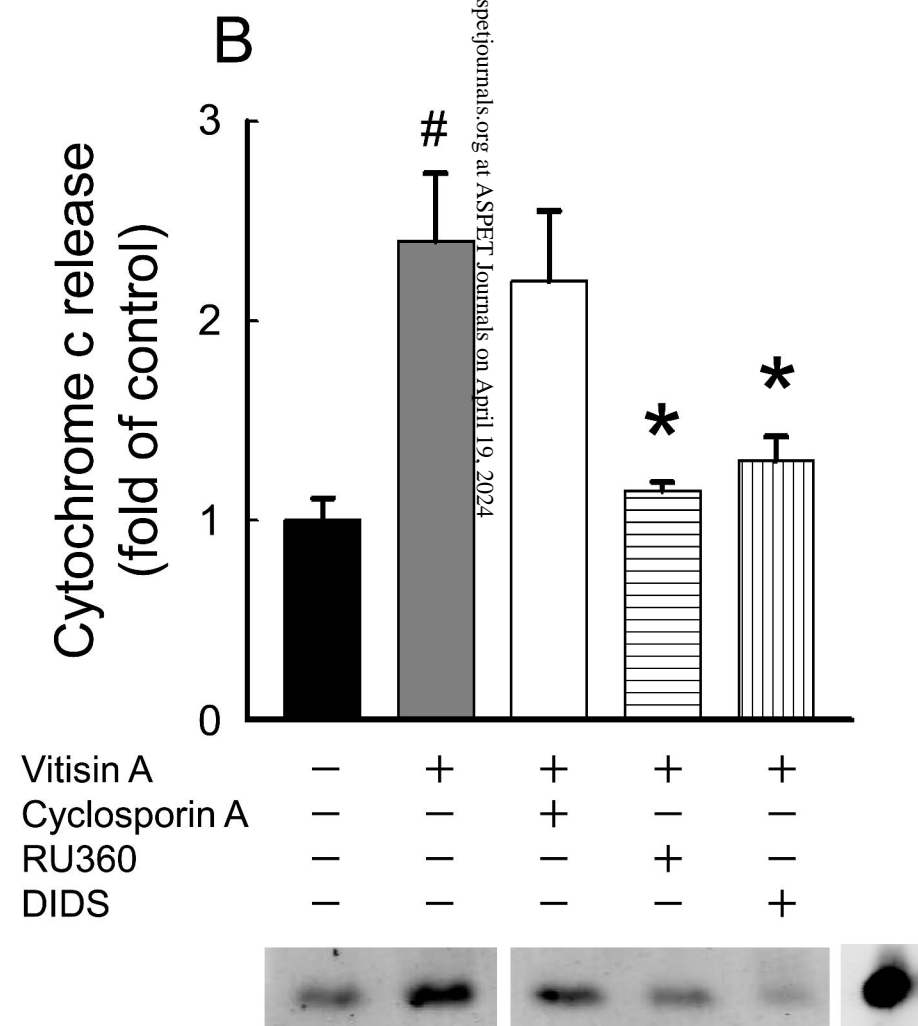
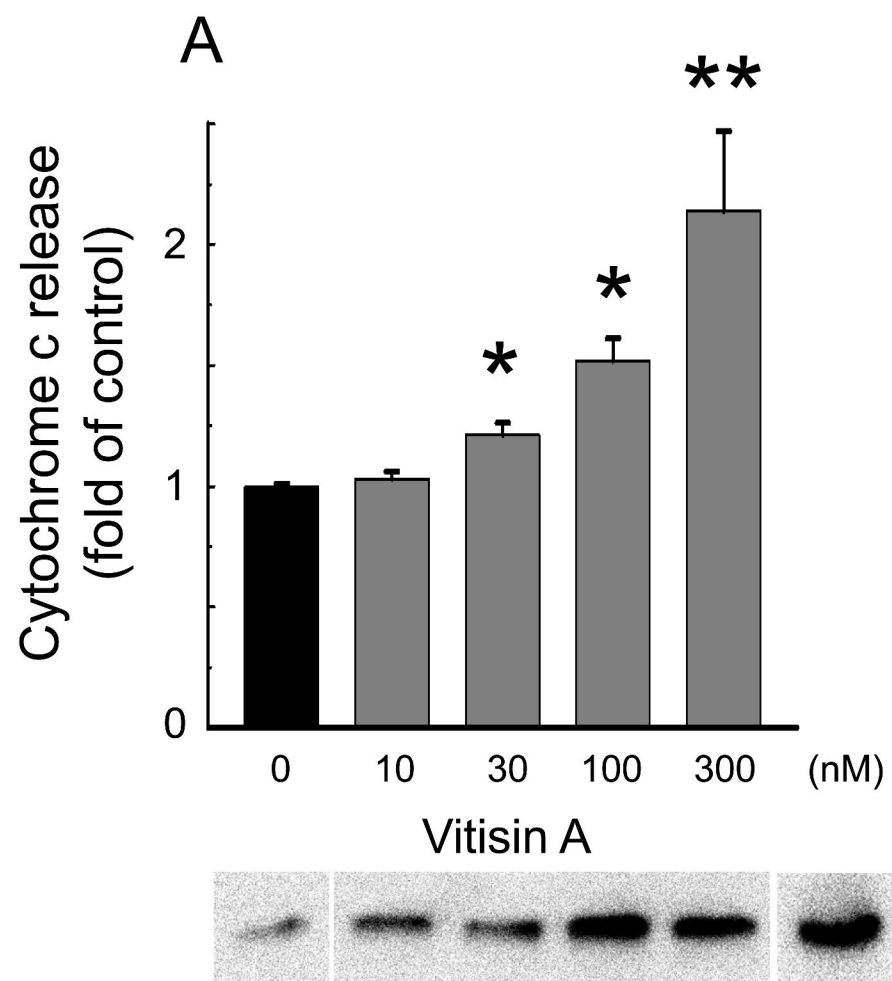
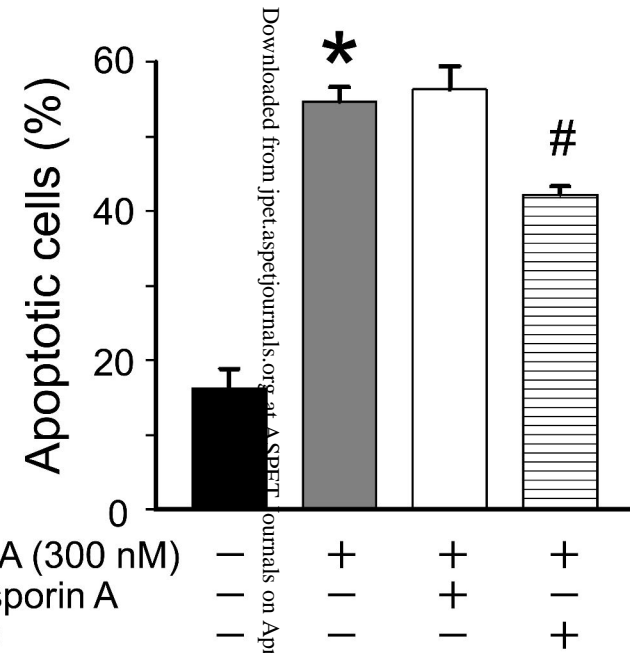
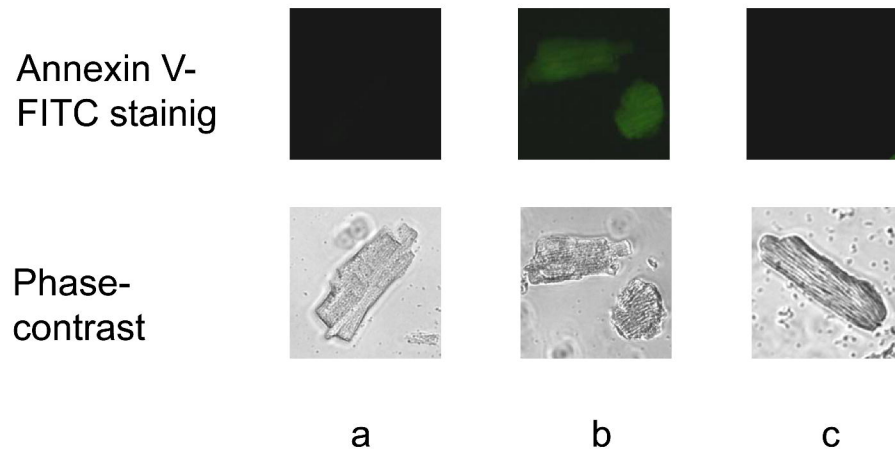
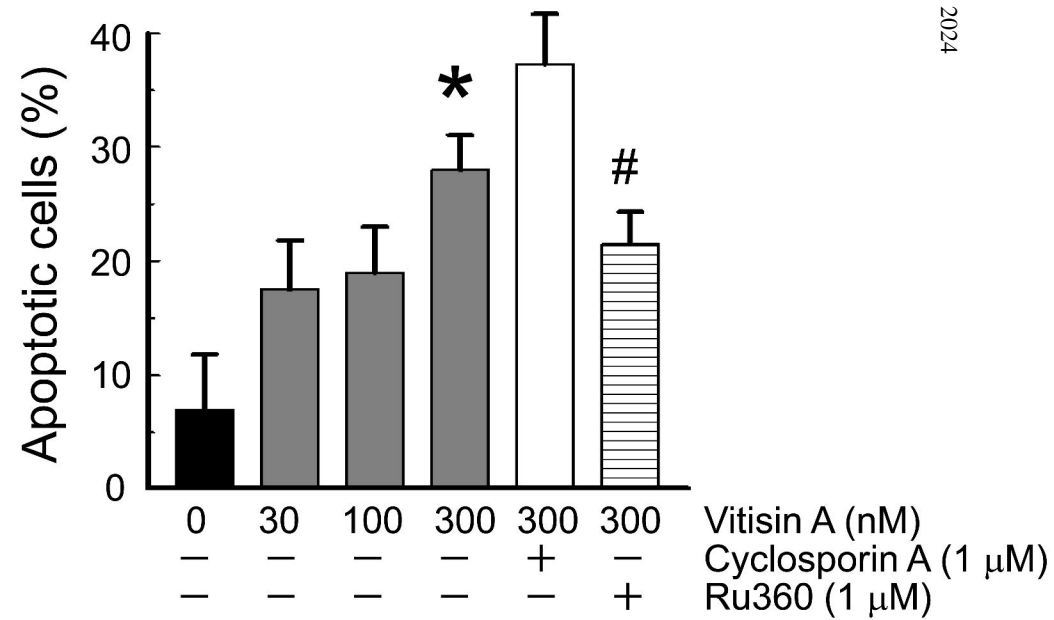


Figure 4

A

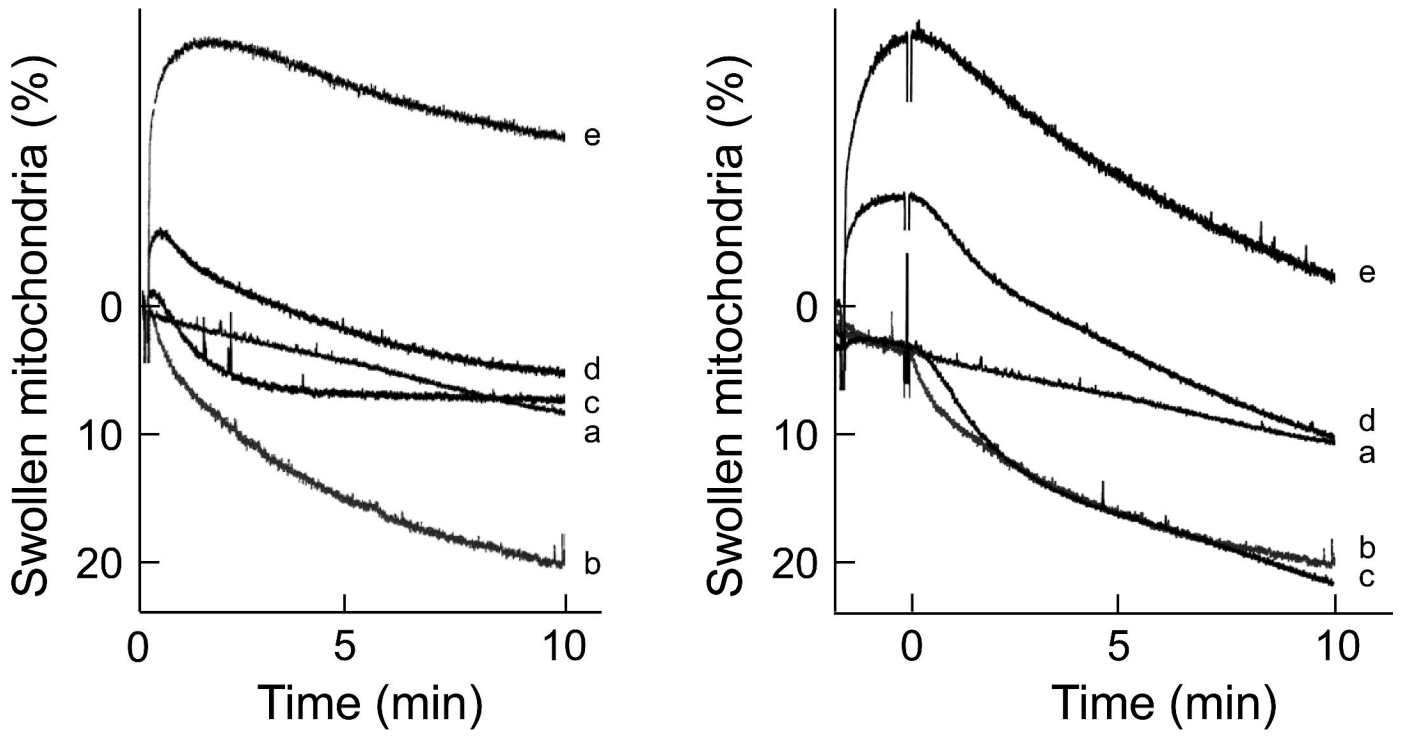


B

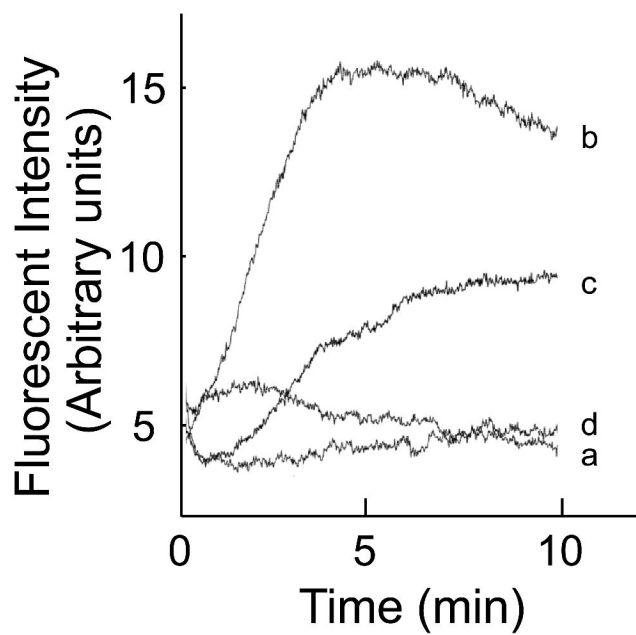


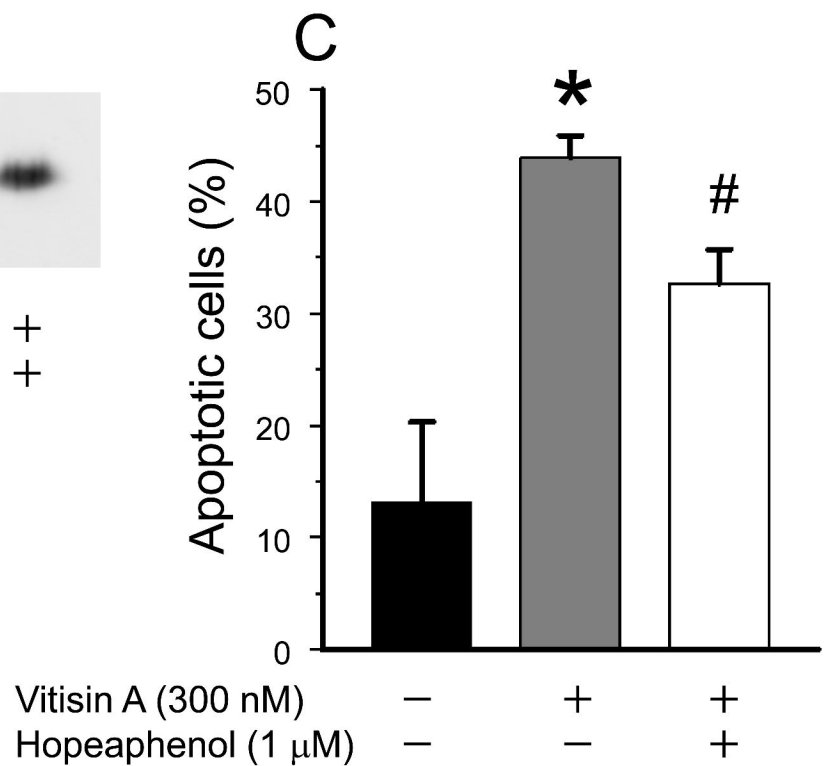
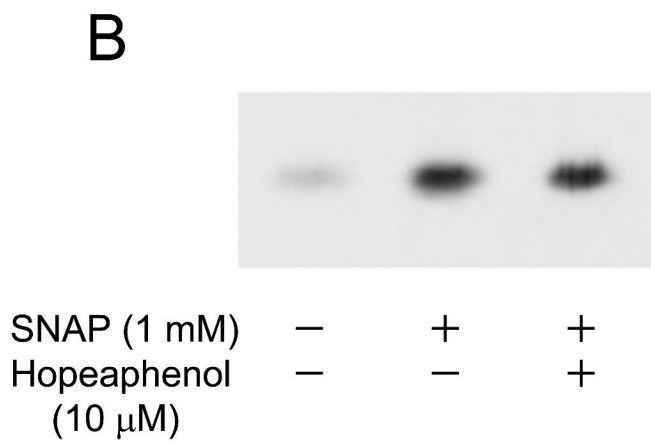
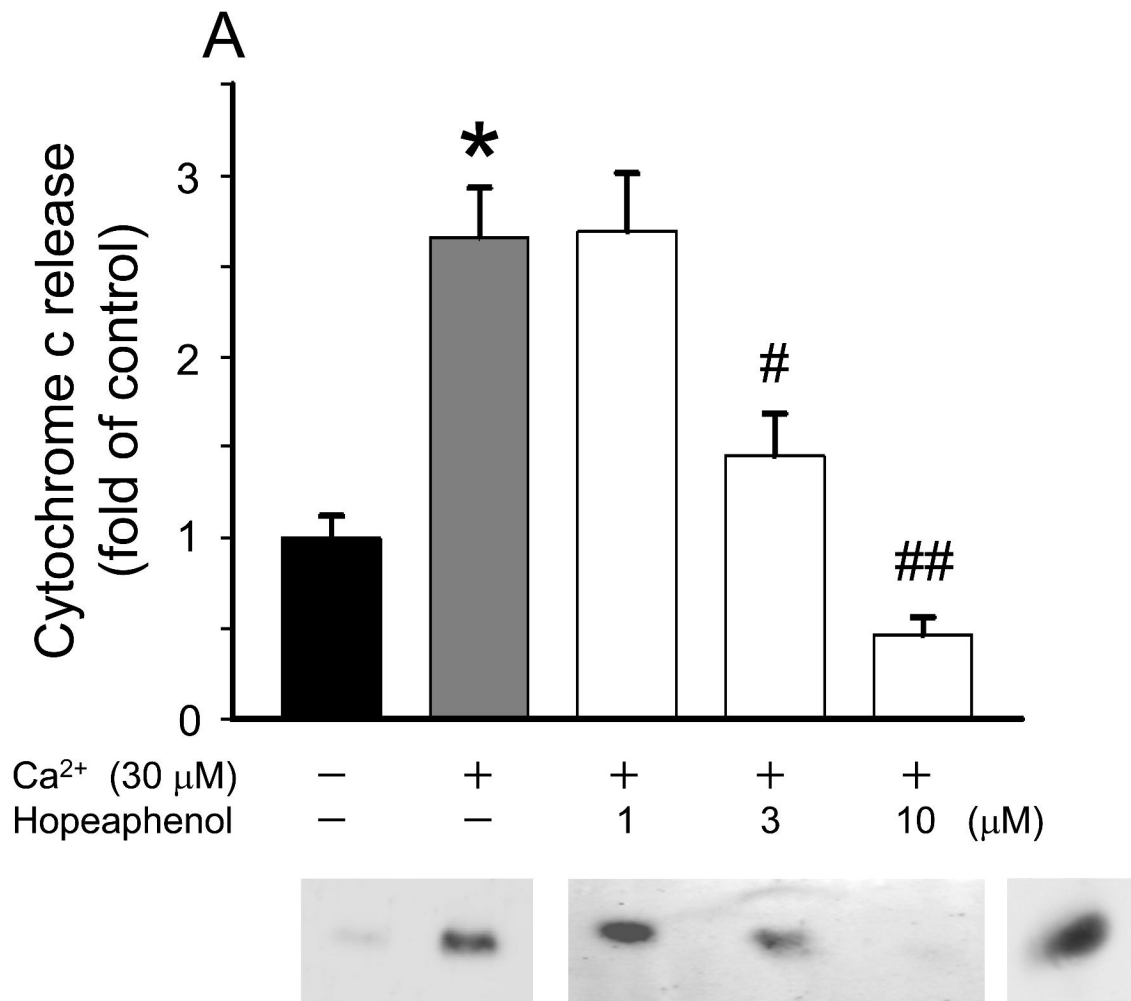
Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

A



B





JPET Fast Forward. Published on October 16, 2008 as DOI: 10.1124/jpet.108.143172
 This article has not been copyedited and formatted. The final version may differ from this version.

