Mechanisms of resveratrol-induced platelet apoptosis

Kuan H. Lin[1], George Hsiao[2], Chwen M. Shih[3], Duen S. Chou[2], and Joen R. Sheu[1,2]*

[1]Graduate Institute of Medical Sciences, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan; [2]Department of Pharmacology, Taipei Medical University, Taipei 110, Taiwan; and [3]Department of Biochemistry, Taipei Medical University, Taipei 110, Taiwan

Aims Apoptotic events have recently been found to occur in platelets, which are anuclear. Resveratrol is present in red wine and has various biological activities, including inhibition of platelet aggregation. Although considerable evidence is available as to the induction of tumour cell apoptosis by resveratrol, resveratrol's effects on platelet apoptosis have not yet been investigated. In the present study, we demonstrate that resveratrol also markedly stimulates apoptosis in washed human platelets.

Methods and results Resveratrol (5–25 μM) completely inhibited platelet aggregation stimulated by collagen. Furthermore, resveratrol time- and concentration-dependently stimulated dissipation of the mitochondrial membrane potential ($\Delta\Psi$m), activation of caspases-9, -3, and -8, gelsolin and actin cleavage, Bid cleavage into truncated Bid, Bax translocation, cytochrome *c* release, and phosphatidylserine exposure but not P-selectin expression in washed human platelets. The presence of z-IETD-fmk, a caspase-8 inhibitor, markedly reversed tBid formation and caspase activation and partially reversed the dissipation of platelet $\Delta\Psi$m stimulated by resveratrol. In addition, resveratrol also directly evoked dissipation of $\Delta\Psi$m and release of cytochrome *c* from isolated mitochondria. Furthermore, resveratrol shortened platelet survival or enhanced platelet clearance in an *in vivo* study.

Conclusion This study demonstrates for the first time that resveratrol simultaneously inhibits platelet aggregation and stimulates platelet apoptosis. Stimulation of platelet apoptosis by resveratrol may represent the increased therapeutic potential for patients suffering from thrombotic conditions or thrombocytosis to promote platelet destruction and thus prevent pathological clotting. Furthermore, this study also provides a novel conception that rigorous surveillance of platelet numbers may be important during resveratrol treatment in the clinic.

1. Introduction

Platelets, anuclear blood cells, are derived from megakaryocytes, which are small and discoid in shape with dimensions of approximately 2.0~4.0 μm, and they have a lifespan of 7~10 days.[1] It is well known that platelets play important roles in haemostatic processes. As a consequence of vascular injury, blood platelets will tether, adhere, aggregate, and finally form platelet plugs in injured vessel walls in order to arrest bleeding from blood vessels.[2]

Apoptosis or programmed cell death, which usually occurs in nucleated cells, is a form of cellular suicide that is essential for development and tissue homeostasis of all metazoan organisms. Profound alteration of the cellular architecture characterizes apoptotic cell death, including cell shrinkage, membrane blebbing, DNA fragmentation, chromatin condensation, and phosphatidylserine (PS) exposure.[3] Its deregulation in humans can lead to diseases such as cancer, immune disorders, and neurodegenerative diseases.[4,5] Recently, apoptotic events were also found in anuclear platelets.

For example, apoptosis was induced in platelets stored at 37°C or when platelets were subjected to chemical compounds and experimental stress.[6–8] In those circumstances, several features of apoptosis in platelets were found, including PS exposure, mitochondrial dysfunction, strong expression of proapoptotic Bax and Bak, caspase-3 activation, and apoptotic body formation. The function of the apoptotic machinery remains unclear in human platelets; several studies have reported that apoptosis-like events, at least in part, are involved in platelet activation.[9,10] On the other hand, Zhang *et al.*[11] showed that the induction of platelet apoptosis enhances its clearance *in vivo* by the reticuloendothelial system. Mason *et al.*[12] also reported that the inactivation of pro-survival Bcl-x$_L$ reduced platelet half-life and caused thrombocytopenia, suggesting that platelet apoptosis is also related to its lifespan.

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene), a polyphenolic natural product, is a phytoalexin widely present in red wine and other constituents of the human diet (e.g. peanuts, grapes, and mulberries), and has been demonstrated to have various biological activites, including inhibition of platelet aggregation and anticancer, antimutagenic, antifungal, antiinflammatory,

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* Corresponding author. Tel: +886 27361661 3199; fax: +886 2 27390450.
E-mail address: sheujr@tmu.edu.tw

and antioxidant properties.[13,14] Because of the very important role of platelets in the development of acute thrombosis, coronary heart disease (CHD), and atherosclerosis, several studies have investigated the effects of resveratrol on platelet functions. As described previously,[15] we found that resveratrol inhibited collagen-induced platelet activation at lower concentrations (0.15~0.25 μM). In the present study, we further demonstrate that resveratrol markedly stimulated platelet apoptosis at a higher concentration (5 μM). Therefore, we systematically examined the cellular events associated with resveratrol-induced platelet apoptosis in the present study, and utilized the findings to characterize the apoptotic mechanisms involved in this influence.

2. Methods

2.1 Chemicals and reagents

Resveratrol, collagen (type I, bovine Achilles's tendon), apyrase, heparin, phenylmethylsulfonyl fluoride, β-mercaptoethanol, bovine serum albumin (BSA), and an anti-gelsolin monoclonal antibody (mAb) were purchased from Sigma (St Louis, MO, USA). Active caspase-9 and -3 polyclonal antibodies (pAbs), anti-cytochrome *c* (Cyt. *c*) mAb, z-DEVD-fmk, z-IETD-fmk, annexin V-FITC, and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine chloride (JC-1) were purchased from BioVision (Mountain View, CA, USA). An anti-cleaved caspase-8 mAb, anti-Bid, and anti-Bax pAb were purchased from Cell Signaling (Beverly, MA, USA). The anti-α-tubulin mAb was purchased from NeoMarkers (Fremont, CA, USA). The Hybond-P PVDF membrane, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulin G (IgG), sheep anti-mouse IgG, and the enhanced chemiluminescence (ECL) western blotting detection reagent and analysis system were purchased from Amersham (Buckinghamshire, UK). The mitochondria isolation kit was purchased from Pierce (Rockford, IL, USA). The anti-COX-1 mAb was purchased from MitoSciences (Eugene, OR, USA). The anti-human P-selectin (CD62P)-fluorescein mAb was purchased from R&D Systems (Minneapolis, MN, USA). The anti-human fractin (cleaved actin) pAb was purchased from BD Pharmingen (San Diego, CA, USA). The anti-CD95 (ZB4) mAb was purchased from GeneTex (Irvine, CA, USA). The anti-TNF-R1 (H398) mAb was purchased from Serotec (Oxford, UK). 5-Chloromethylfluorescein diacetate (CMFDA) was purchased from Molecular Probes (Eugene, OR, USA). Resveratrol (Mw. 228.24) was dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C until use.

2.2 Preparation of human platelet suspensions

Human platelet suspensions were prepared as described previously.[15] This study was approved by the Institutional Review Board of Taipei Medical University and conformed to the principles outlined in the *Helsinki Declaration*, and human volunteers gave informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine during the preceding 2 weeks, and was mixed with acid/citrate/glucose (9:1, vol/vol). After centrifugation, the supernatant (platelet-rich plasma) was supplemented with prostaglandin E$_1$ (0.5 μM) and heparin (6.4 IU/mL). Washed platelets were finally suspended in Tyrode's solution containing BSA (3.5 mg/mL). The final concentration of Ca^{+2} in Tyrode's solution was 1 mM.

A turbidimetric method was applied to measure platelet aggregation,[15] using a Lumi-Aggregometer (Payton, Scarborough, Ontario, Canada). Platelet suspensions (0.4 mL) were pre-incubated with resveratrol (5~25 μM) or an isovolumetric solvent control (0.06% DMSO) for 3 min before the addition of collagen. The reaction was allowed to proceed for 6 min, and the extent of aggregation was expressed in light-transmission units.

2.3 Quantitative immunoblotting

The method of western blotting was performed as described previously.[15] Platelets (8×10^8/mL) were pre-incubated with or without z-DEVD-fmk (5 μM) or z-IETD-fmk (100 μM) for 10 min, followed by the addition of resveratrol (5, 10, and 25 μM) at the indicated times (0, 10, 30, and 60 min) in a Lumi-Aggregometer. The reaction was stopped with 10 mM EDTA. Proteins were extracted with lysis buffer for 30 min. Lysates were centrifuged, and the supernatant (80 μg protein) was subjected to SDS–PAGE, and electrophoretically transferred onto Hybond-P PVDF membranes by semidry transfer (Bio-Rad, Hercules, CA, USA). Membranes were blocked with TBST (10 mM Tris-base, 100 mM NaCl, and 0.01% Tween 20) containing 5% BSA for 1 h and then probed with primary antibodies (diluted 1:1000 in TBST) specific for anti-active caspase-9 or -3, anti-gelsolin, anti-cleaved caspase-8, anti-Bid, anti-Bax, anti-cleaved actin, and anti-Cyt. *c* for 2 h. Membranes were washed and then incubated with HRP-linked anti-mouse IgG or anti-rabbit IgG (diluted 1:3000 in TBST) for 1 h. The immunoreactive band with peroxidase activity was detected using film exposure with ECL detection reagents (ECL System, Amersham). The bar graph depicts the ratios of quantitative results obtained by scanning reactive bands and quantifying the optical densities using Bio-1D version 99 image software (Vilber Lourmat, France).

2.4 Assay of the dissipation of the mitochondrial membrane potential (ΔΨm)

The ΔΨm was determined with JC-1 according to a method described previously.[16] In brief, platelet suspensions (1×10^6/mL) were incubated with various concentrations of resveratrol (1~25 μM) in the absence or presence of z-IETD-fmk (50 and 100 μM). After 60 min, platelet suspensions were combined with JC-1 (2 μg/mL, dissolved in DMSO) for 20 min. The final volume was brought to 1 mL for immediate analysis by Coulter Epics XL flow cytometry (Beckman Coulter, Miami, FL, USA). JC-1 is a lipophilic cationic fluorescence dye and is capable of selectively entering mitochondria. A shift in fluorescence from red (JC-1 aggregates, FL3) to green (JC-1 monomers, FL1) is detected as a reduction of ΔΨm, which implies JC-1 dissociation from mitochondria into the cytosol. Signal platelets were gated from the forward scatter-orthogonal side scatter dot plot. FL-1 and FL-3 were measured in the logarithmic mode using voltages of 600 and 700 V, respectively.

2.5 Isolation of the mitochondrial fraction in washed platelets

The mitochondrial fraction of platelets was isolated using a mitochondria isolation kit (Pierce).[17] Briefly, washed platelets (1.2×10^9/mL) were incubated with resveratrol (5, 10, and 25 μM) at the indicated times (0, 10, 30, and 60 min) in a Lumi-Aggregometer. Platelets were then suspended in mitochondrion isolation buffer A for 2 min, buffer B for 5 min, and buffer C for another 5 min followed by centrifugation. The supernatant was centrifuged at 12 000 *g* for 20 min. The pellet containing the mitochondria was washed with buffer C, incubated in lysis buffer for 30 min, and centrifuged at 12 000 *g* for 5 min. The supernatant was analysed by immunoblotting.

2.6 Detection of ΔΨm and Cyt. *c* release in isolated mitochondria from platelets

As described earlier, platelet mitochondria were isolated by a mitochondria isolation kit. The platelet mitochondrial suspension was incubated with resveratrol (25 μM) at the indicated times (0, 10, 30, and 60 min). After the reaction, JC-1 was added to detect ΔΨm by flow cytometry. In addition, Cyt. *c* release was determined by immunoblotting as described earlier.

2.7 Measurement of phosphatidylserine exposure and P-selectin expression in washed platelets

The method of Shcherbina and Remold-O'Donnell[18] was modified. In brief, washed platelets (1×10^6/mL) were pre-incubated with or without resveratrol (25 μM) at the indicated times (0~9 h). After the reaction, annexin V-FITC (1 μL/1×10^7 cells) or anti-human P--selectin-fluorescein (1 μg/mL) was added to label PS or P-selectin for 20 min, respectively. Data acquisition and analysis were performed by Coulter Epics XL flow cytometry.

2.8 Identification of isolated mitochondrial fraction by transmission electron microscopy (TEM)

Mitochondrial pellets were fixed with 2% paraformaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) for 2 h. After rinsing, samples were postfixed with 1% osmium tetroxide for 1 h. After dehydration in a graded series of ethanol, samples were embedded in Epon 812 using standard procedures. Ultrathin sections were mounted on nickel grids, and double-stained with uranyl acetate and lead citrate, and then examined under a Hitachi H-600 electron microscope (Hitachi, Tokyo, Japan).

2.9 Measurement of free radicals by electron spin resonance spectrometry

The electron spin resonance spectrometry (ESR) method used a Bruker EMX ESR spectrometer as described previously.[19] In brief, platelet suspensions (3.6×10^8/mL) were pre-incubated with resveratrol (0.25 and 25 μM), H_2O_2 (300 μM), or solvent control (0.06% DMSO) for 3 min. The reaction was allowed to proceed for 5 min, followed by the addition of DMPO (100 μM) for the ESR study.

2.10 Determination of platelet recovery and survival by 5-chloromethylfluorescein diacetate-labelled platelets in a rat model

A modified method of Baker et al.[20] was performed. In brief, platelet suspensions were incubated with Tyrode's solution, resveratrol (25 μM), or 0.06% DMSO and then labelled with CellTracker Green CMFDA (2.5 μM). Unincorporated dye was removed by centrifugation and platelets were re-suspended in Tyrode's solution. 5-Chloromethylfluorescein diacetate-labelled platelets were injected into rats via the femoral vein. For survival determination, blood samples were collected at 10 min and 24 h. The percentage of CMFDA positive platelets was determined by flow cytometry. A total of 50 000 events were collected in each sample. 5-Chloromethylfluorescein diacetate positive platelets measured at 10 min were set as 100%.

2.11 Statistical analysis

The experimental results are expressed as the means $\pm$ SEM and are accompanied by the number of observations. The experiments were assessed by analysis of variance. If this analysis indicated a significant difference among group means, then each group was compared using the Newman–Keuls method. A P-value of <0.05 was considered statistically significant.

3. Results

3.1 Effects of resveratrol on platelet aggregation, $\Delta\Psi$m change, caspase activation, gelsolin, and actin cleavage in washed platelets

Resveratrol (5~25 μM) completely inhibited platelet aggregation stimulated by collagen (1 μg/mL) in washed platelets (*Figure 1A*). The lipophilic cation, JC-1, was used to detect the dissipation of $\Delta\Psi$m by flow cytometry. As shown in *Figure 1B*, resveratrol (1~25 μM) concentration-dependently evoked dissipation of platelet $\Delta\Psi$m, which achieved maximal efficacy at 25 μM (approximately 70%). On the other hand, resveratrol (5~25 μM) markedly promoted both caspase-9 (37 kDa) and -3 (17 kDa) activations (*Figure 1C* and *D*). Resveratrol (5~25 μM) also concentration-dependently stimulated gelsolin cleavage (47/45 kDa), a substrate for caspase-3.[21] The presence of z-DEVD-fmk (5 μM), a caspase-3 inhibitor,[22] obviously reversed resveratrol (25 μM)-induced gelsolin cleavage (*Figure 1E*). Furthermore, resveratrol (5~25 μM) was also found to stimulate actin cleavage (32 kDa), which was reversed in the presence of z-DEVD-fmk (5 μM) (*Figure 1F*).

3.2 Effect of resveratrol on the extrinsic apoptotic pathway: activation of caspase-8 and Bid in washed platelets

As shown in *Figure 2A*, the cleaved fragment of caspase-8 (active caspase-8, 10 kDa) was time-dependently observed 10 min after resveratrol (25 μM) treatment, which achieved maximal efficacy at 60 min. Moreover, concentration-dependent induction of active caspase-8 formation was also observed 30 min after resveratrol (5~25 μM) treatment (*Figure 2B*).

In addition, resveratrol (25 μM) significantly triggered Bid, the downstream signal of active caspase-8, which was cleaved into tBid (truncated Bid, 15 kDa) within 10~30 min, followed by the degradation of tBid within a 60 min period (*Figure 2C*). This degradation of tBid may be mediated by ubiquitin.[23] Concentration-dependent induction of tBid formation stimulated by resveratrol (5~25 μM) for 30 min was also observed (*Figure 2D*). The presence of z-IETD-fmk (100 μM), a caspase-8 inhibitor,[24] markedly diminished resveratrol-induced tBid formation (*Figure 2D*). On the other hand, resveratrol-induced caspase-8 activation did not be reversed in the presence of both antagonistic anti-Fas receptor (ZB4, 500 ng/mL) and anti-TNF-R1 (H398, 10 μg/mL) mAbs (*Figure 2E*).

3.3 Effects of resveratrol on Bax translocation and Cyt. *c* release in washed platelets

In this study, all samples were separated with a mitochondria isolation kit into cytosolic and mitochondrial extracts after the reaction. Time- and concentration-dependent induction of Bax translocation and Cyt. *c* release stimulated by resveratrol were determined. Resveratrol time (0~60 min)- and concentration (5~25 μM)-dependently promoted Bax translocation from the cytosol into mitochondria and Cyt. *c* release from mitochondria into the cytosol (*Figure 3A* and *B*). In this study, platelet mitochondria were isolated using mitochondria isolation kit. According to the manufacturer's instruction, the protocol presented prepares a mitochondrial fraction that may include slight contaminants of plasma membrane debris. However, a high purity of mitochondrial preparations in this study has also been confirmed by TEM (*Figure 3C*).

3.4 Essential role of caspase-8 in downstream activation of caspase-9 and -3 in washed platelets

As shown in *Figure 4A* and *B*, resveratrol (25 μM) markedly triggered both caspase-9 and -3 activations after 60 min of
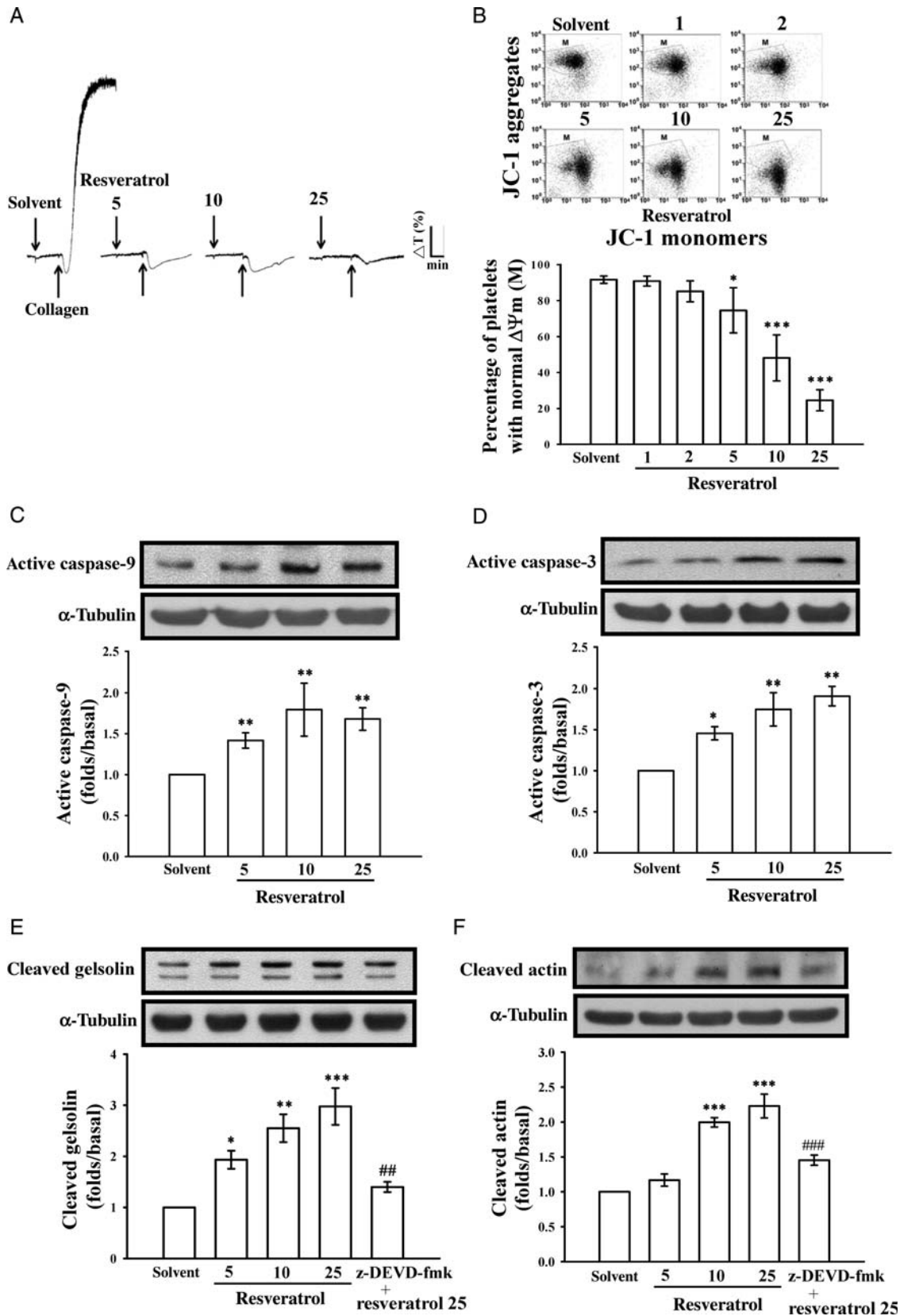
A

B

C

D

E

F

Figure 1 Effects of resveratrol on (*A*) platelet aggregation, (*B*) the mitochondrial membrane potential (ΔΨm), (*C* and *D*) caspase-9 and -3 activations, (*E*) gelsolin, and (*F*) actin cleavage in washed platelets. (A) Washed platelets were pre-incubated with resveratrol (5~25 μM), followed by the addition of collagen (1 μg/mL) to trigger platelet aggregation. (B) Platelets were incubated with resveratrol (1~25 μM), followed by the addition of JC-1 for flow cytometric analysis. (C–F) Platelets were incubated with resveratrol (5~25 μM) in the absence or presence of z-DEVD-fmk (5 μM). Active caspase-9 (37 kDa), active caspase-3 (17 kDa), cleaved gelsolin (47/45 kDa), and actin (32 kDa) were assessed by immunoblotting. Profiles (A) are representative examples of four similar experiments. Data (B–F) are presented as the means ± SEM (*n* = 4). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the solvent control; ##$P < 0.01$ and ###$P < 0.001$ compared with the resveratrol (25 μM)-treated group.
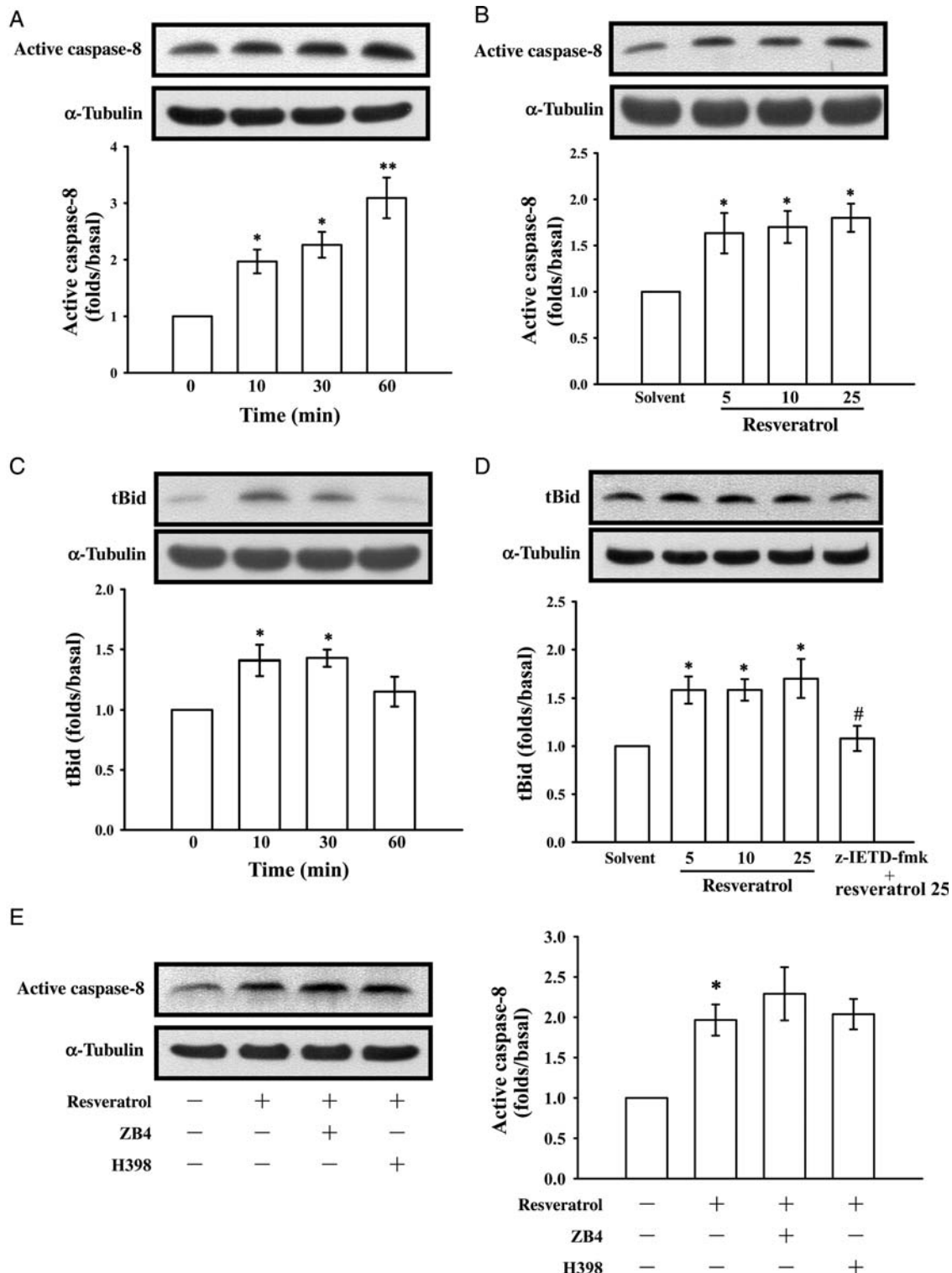
Figure 2 Time- and concentration-dependent induction of caspase-8 activation and Bid cleavage by resveratrol in washed platelets. Platelets (8×10^8/mL) were incubated with various resveratrol concentrations ($5{\sim}25\ \mu$M) for the indicated times ($0{\sim}60$ min) in the absence or presence of z-IETD-fmk (100 μM), antagonistic anti-Fas receptor (ZB4, 500 ng/mL), and anti-TNF-R1 (H398, 10 μg/mL) monoclonal antibodies. (*A*, *B*, and *E*) Active caspase-8 (10 kDa) and (*C* and *D*) tBid (15 kDa) were assessed by immunoblotting. Data are presented as the means $\pm$ SEM ($n = 4$). *$P < 0.05$ and **$P < 0.01$ compared with the solvent control; #$P < 0.05$ compared with the resveratrol (25 μM)-treated group.

treatment. The presence of z-IETD-fmk (100 μM), a caspase-8 inhibitor, obviously reversed the resveratrol-induced activations of both caspase-9 and -3 (*Figure 4A* and *B*), indicating that both caspase-9 and -3 are downstream signals of active caspase-8 in human platelets.

3.5 Effects of resveratrol on the intrinsic apoptotic pathway in both washed platelets and isolated mitochondria

Figure 5A shows that resveratrol (25 μM)-evoked dissipation of platelet $\Delta\Psi$m for 60 min of treatment was partially reversed in
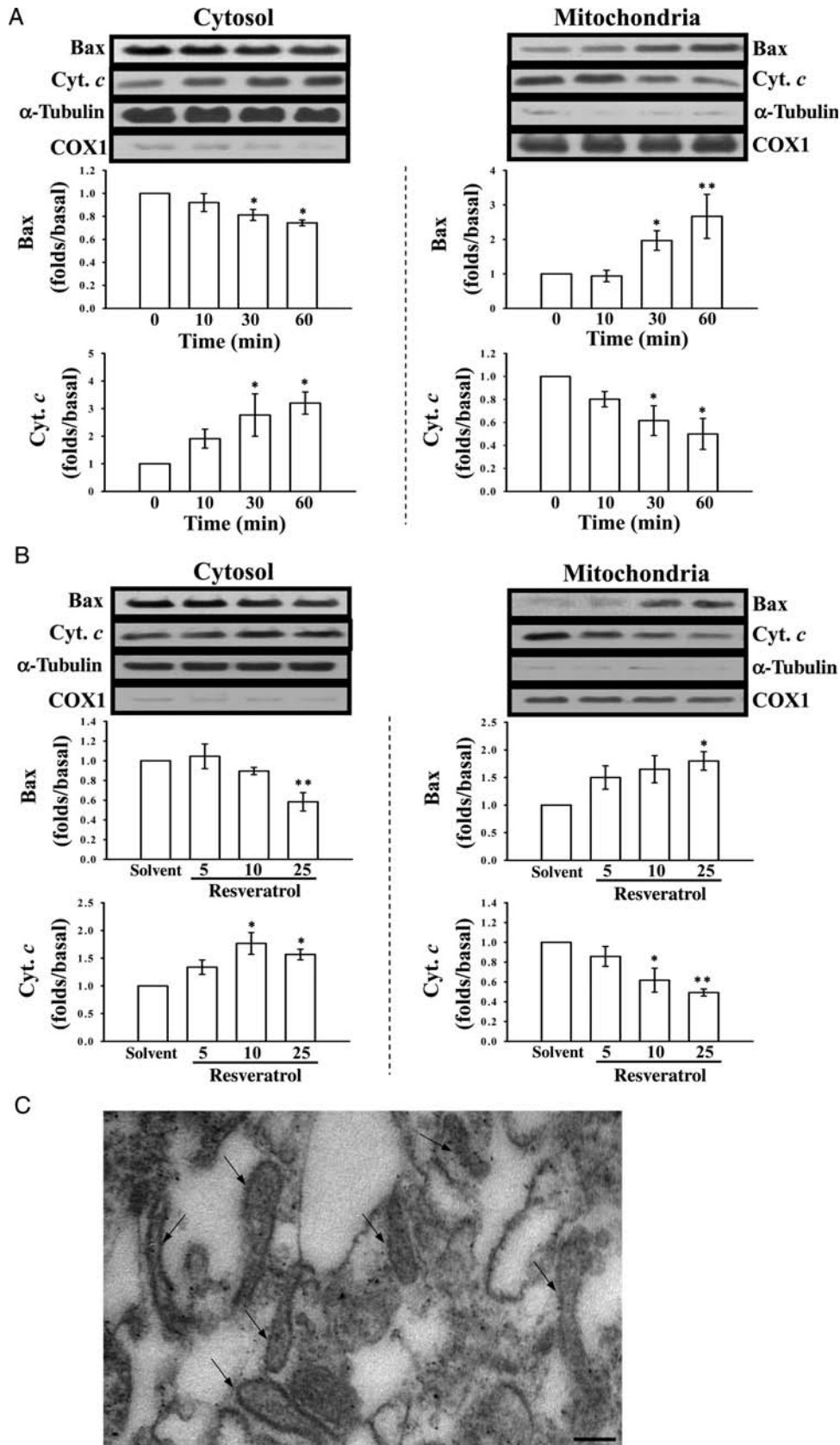
Figure 3 Time- and concentration-dependent induction of Bax translocation and cytochrome *c* (Cyt. *c*) release in cytosol and mitochondrial fractions. Platelets (1.2 × 10^9/mL) were incubated with (*A*) resveratrol (25 μM) for 0∼60 min or (*B*) resveratrol (5∼25 μM) for 60 min. After the reaction, cytosol and mitochondrial fractions were isolated and analysed by immunoblotting with antibodies specific for Bax (20 kDa) and Cyt. *c* (12 kDa), respectively. Cytochrome *c* oxidase subunit 1 (COX1) (for the mitochondrial fraction) and α-tubulin (for the cytosolic fraction) were used as the respective internal controls. (*C*) Isolated mitochondrial fraction from platelets was confirmed by transmission electron microscopy. Arrows indicate intact mitochondria. Bar = 100 nm. Data are presented as the means ± SEM (*n* = 4). *$P < 0.05$ and **$P < 0.01$ compared with the solvent control.

the presence of z-IETD-fmk (100 μM) by approximately 20%. On the other hand, resveratrol (25 μM) also directly evoked dissipation of $\Delta\Psi$m (*Figure 5B*) and triggered Cyt. *c* release from isolated mitochondria (*Figure 5C*) within a 10~60 min period of treatment compared with the solvent control. These results indicate that resveratrol can directly target mitochondria and activate apoptosis through the intrinsic platelet apoptotic pathway.

3.6 Effects of resveratrol on phosphatidylserine exposure, P-selectin expression, and hydroxyl radical formation in washed platelets, and on platelet recovery and survival in a rat model

In this study, annexin V-FITC was used to detect PS exposure, a death knell,[25] on the outer leaflet of plasma membranes by flow cytometry. As shown in *Figure 6A*, resveratrol (25 μM) time-dependently (1~9 h) induced PS exposure in washed platelets, which reached a maximal response at 9 h after treatment. On the other hand, an anti-human P-selectin-fluorescein mAb was also used to detect P-selectin expression, a marker of platelet activation.[26] *Figure 6B* shows that the resveratrol (25 μM) treatment did not significantly induce P-selectin expression for up to 9 h of incubation compared with thrombin (0.1 U/mL) treatment (positive control). These results indicate that resveratrol triggers platelet apoptosis; however, it did not induce platelet activation in the same circumstances.

A typical ESR signal of hydroxyl radical (OH$^{\bullet}$) formation induced by H_2O_2 (300 μM) in platelets was compared with solvent control (0.06% DMSO) (*Figure 6Ca and b*); pre-treated with resveratrol (0.25 and 25 μM) did not significantly induce hydroxyl radical formation (*Figure 6Cc and d*). The antioxidant, catalase (1000 U/mL), markedly suppressed hydroxyl radical formation by about 85% stimulated by H_2O_2 (data not shown). This result indicates that resveratrol does not possess pro-oxidant activity in platelets even at higher concentration (25 μM).

To evaluate the effect of resveratrol in platelet recovery and survival, the percentage of circulating CMFDA-labelled platelets in rats was analysed after 24 h by flow cytometry. A significant reduction of platelet recovery was observed in resveratrol (25 μM)-treated rats when compared with solvent control group (0.06% DMSO, 61.4 $\pm$ 4.4 vs. 46.8 $\pm$ 3.4%, $n = 6$, $P < 0.05$) (*Figure 6D*).

4. Discussion

Results obtained from the present study demonstrate for the first time that resveratrol inhibits platelet aggregation stimulated by collagen; on the other hand, it also stimulates platelet apoptosis at the same concentration. Apoptosis occurs through two main pathways: the extrinsic (cytoplasmic) and intrinsic (mitochondrial) apoptotic pathways. In the extrinsic apoptotic pathway, cytokine ligands [tumour necrosis factor-alpha (TNF-α), Fas ligand (FasL), or TNF-related apoptosis-inducing ligand (TRAIL)] engage to membrane-bound death receptors (DRs; TNF-α, FasL, or the TRAIL receptor), which can recruit the death-inducing signalling complex (DISC) and activates caspase-8. In the cytosol, Cyt. *c*, apoptosis protease activating factor-1 (Apaf-1), and procaspase-9 further form apoptosomes which can execute self-activation of caspase-9 and
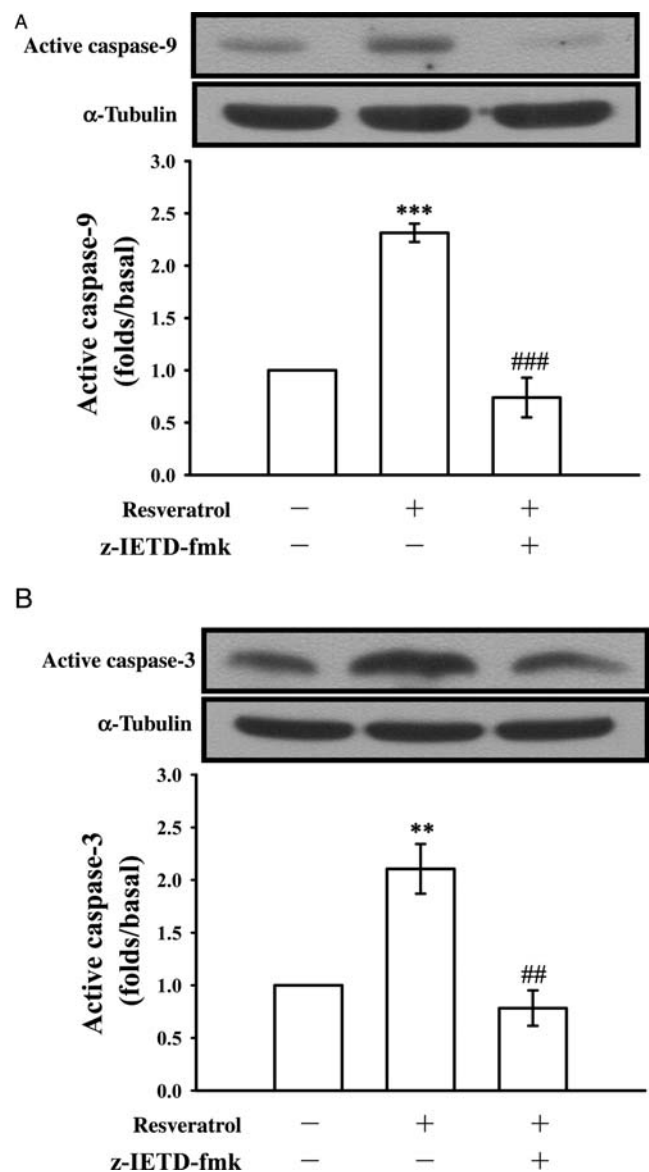

Figure 4 Effects of z-IETD-fmk on resveratrol-induced caspase-9 and -3 activations in washed platelets. Platelets (8×10^8/mL) were incubated with resveratrol (25 μM) for 60 min in the absence or presence of z-IETD-fmk (100 μM). After the reaction, samples were analysed by immunoblotting with antibodies specific for active caspase-9 and -3, respectively. Data are presented as the means $\pm$ SEM ($n = 4$). **$P < 0.01$ and ***$P < 0.001$ compared with the solvent control; ##$P < 0.01$ and ###$P < 0.001$ compared with the resveratrol (25 μM)-treated group.

subsequently activates caspase-3.[27,28] The intrinsic apoptotic pathway can be initiated by a variety of stress stimuli including DNA damage, UV radiation, activation of tumour suppressor genes, and most chemotherapeutic agents, which leads to the release of Cyt. *c* and subsequent activation of caspase-9 and -3.[27,28]

Resveratrol, a naturally occurring compound found in grapes and red wine, possesses chemotherapeutic potential through its ability to trigger apoptosis in several cancer types.[13,29] In this study, we demonstrate resveratrol also triggers platelet apoptosis by such mechanisms as caspase-8 cleavage and tBid formation. Moreover, Bax translocation to mitochondria, dissipation of $\Delta\Psi$m, and Cyt. *c* release into the cytosol were also observed with resveratrol treatment. In addition, resveratrol significantly stimulated
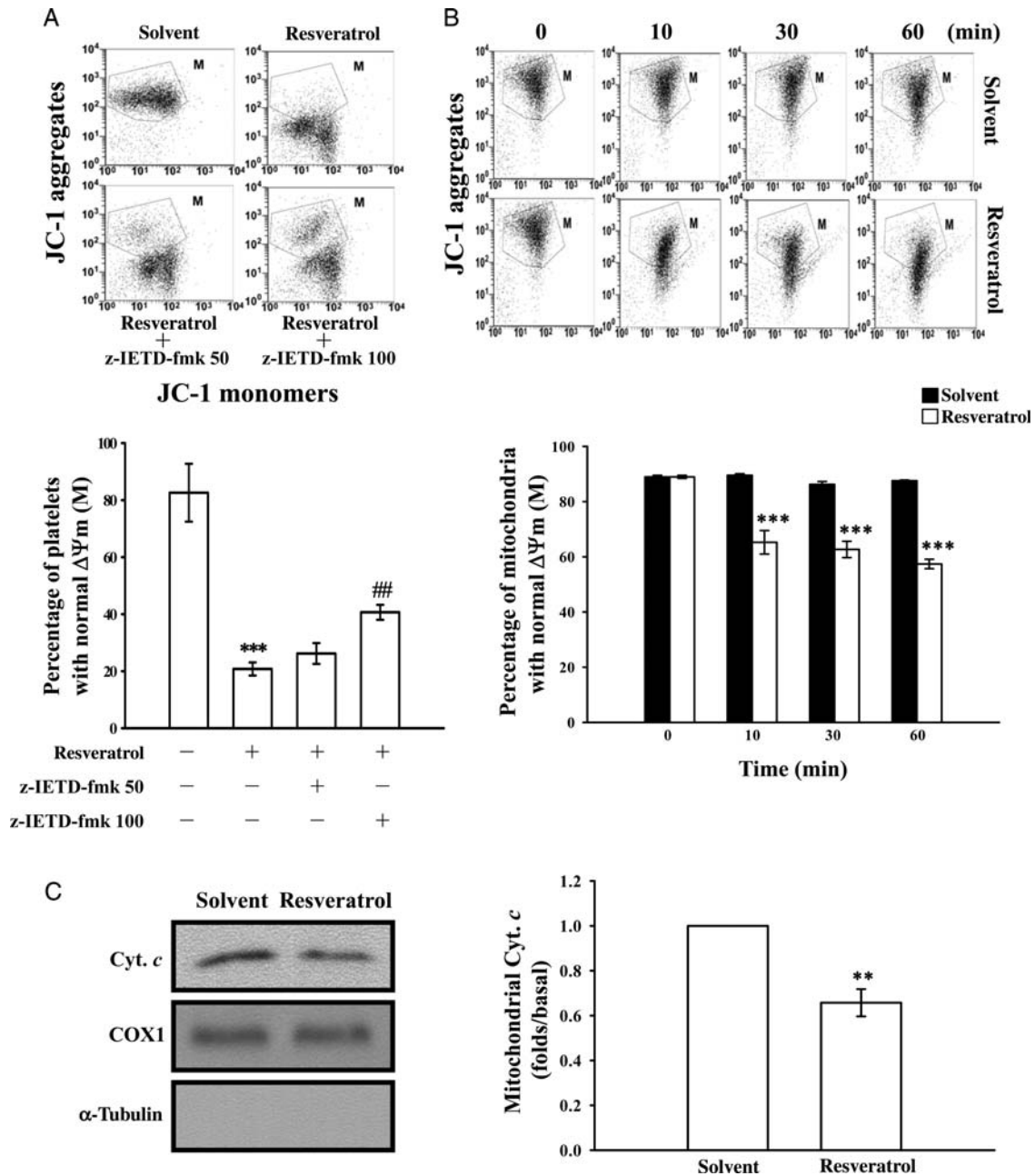
Figure 5 Effects of resveratrol on the mitochondrial membrane potential ($\Delta\Psi$m) and cytochrome *c* (Cyt. *c*) release in whole cells or isolated mitochondria. (*A*) Human platelets (1×10^6/mL) or (*B*) isolated mitochondria were incubated with resveratrol (25 μM) for the indicated times (0~60 min) with or without z-IETD-fmk (50 or 100 μM), followed by the addition of JC-1. After 20 min, samples were immediately analysed by flow cytometry. (*C*) Isolated mitochondria were incubated with resveratrol (25 μM) for 60 min. After the reaction, samples were analysed by immunoblotting with an antibody specific for Cyt. *c* (12 kDa). COX1 (for the mitochondrial fraction) and α-tubulin (for the cytosolic fraction) were used as the respective internal controls. Data are presented as the means $\pm$ SEM ($n = 4$). **$P < 0.01$ and ***$P < 0.001$ compared with the solvent control; ##$P < 0.01$ compared with the resveratrol (25 μM)-treated group.

both caspase-9 and -3 activations which were inhibited in the presence of a caspase-8 inhibitor. These results indicate that both caspase-9 and -3 are downstream regulators of caspase-8 (*Figure 7*). Gelsolin, a substrate of caspase-3 in multiple cell types, causes cells to round up, detach from the plate, and undergo nuclear fragmentation.[21] Cleaved gelsolin generated in cells during apoptosis may preferentially sever actin filaments (*Figure 7*).[21] Thus, cleaved gelsolin and actin filaments may be physiological effectors of morphological change during apoptosis.[21] Resveratrol markedly caused actin and gelsolin cleavage which was reversed by the caspase-3 inhibitor, suggesting that platelets may

undergo apoptosis upon resveratrol treatment, and resveratrol-induced caspase-3 activation is functional. Based on these data, resveratrol-induced platelet apoptosis, at least in part, is involved in the extrinsic apoptotic pathway (*Figure 7*).

On the other hand, we found that the caspase-8 inhibitor only partially reversed resveratrol-evoked dissipation of the $\Delta\Psi$m (*Figure 5A*), indicating that besides the extrinsic apoptotic pathway, other mechanisms may participate in mitochondrial depolarization. In the isolated mitochondrion preparation (cell-free system), resveratrol directly evoked the dissipation of $\Delta\Psi$m and Cyt. *c* release. This study is
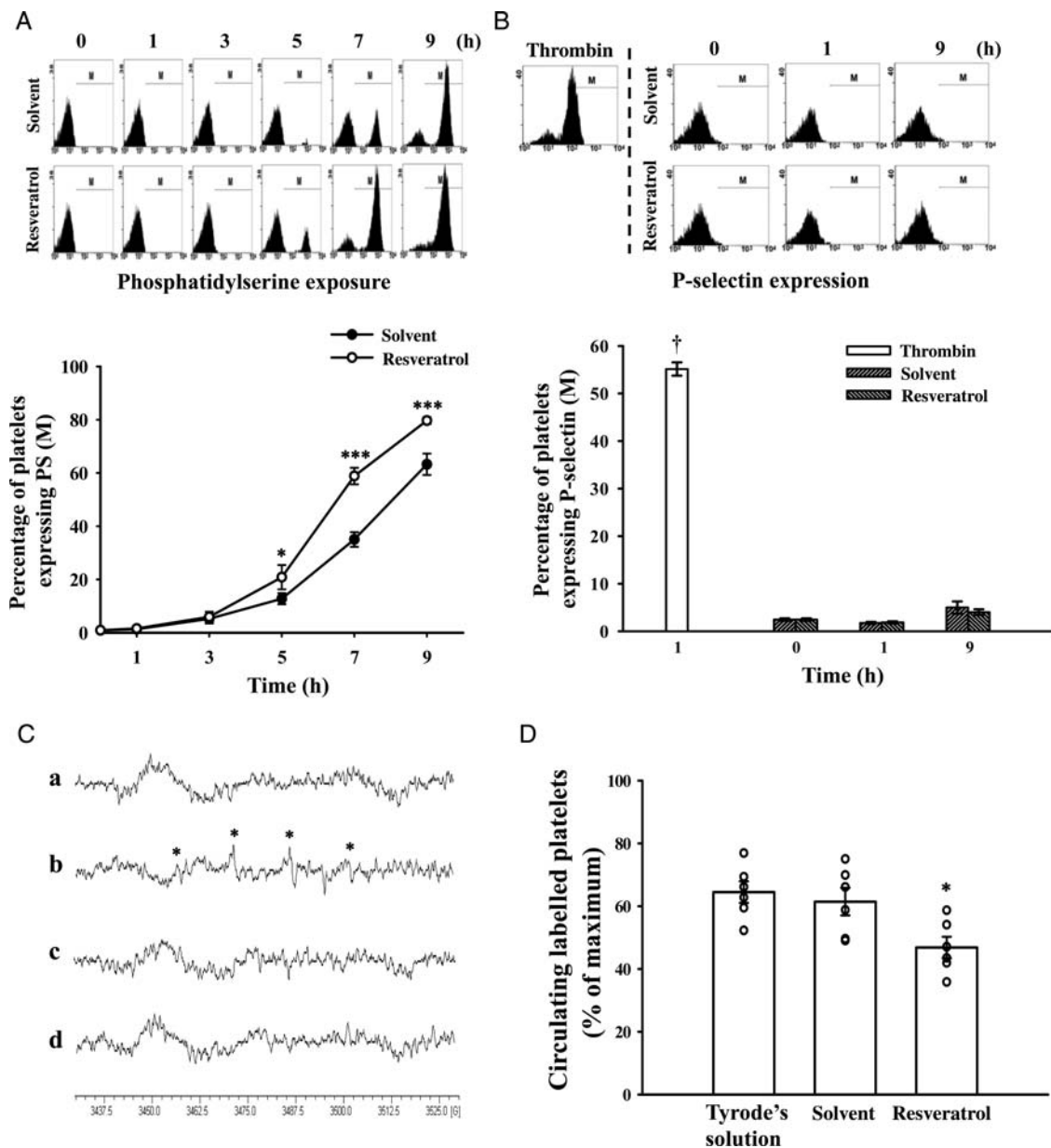
Figure 6 Effects of resveratrol on phosphatidylserine (PS) exposure, P-selectin expression, and hydroxyl radical formation in washed platelets, and on *in vivo* platelet recovery and survival. Platelets were incubated with resveratrol (25 μM) for 0~9 h, followed by the addition of (*A*) an annexin V-FITC or (*B*) an anti-human P-selectin-fluorescein monoclonal antibody. After 20 min, both PS exposure and P-selectin expression were assessed by flow cytometry. Thrombin (0.1 U/mL) was used as the positive control of platelet activation (*B*). (*C*) For the hydroxyl radical (OH·) formation, platelets were incubated with (a) 0.06% DMSO, (b) H_2O_2 (300 μM), and (c and d) resveratrol (0.25 and 25 μM) for the ESR experiments. (*D*) For the *in vivo* study, recovery of CMFDA-labelled platelets in the circulation of rat was determined as described in 'Methods'. The maximum percent of circulating labelled platelets in each animal, as determined at 10 min after injection, was designated as 100%. Bar represents the mean value. Each different symbol represents the platelet recovery (%) of the individual rat. Data are presented as the means ± SEM (A–C, $n = 4$; D, $n = 6$). *$P < 0.05$ and ***$P < 0.001$ compared with the solvent control; †$P < 0.001$ compared with the solvent-treated group at 0 h.

also consistent with a previous report by Sareen *et al.*[30] They found that mitochondria were the primary target of resveratrol-induced apoptosis in human retinoblastoma cells. Therefore, resveratrol-induced platelet apoptosis may also be mediated by the intrinsic apoptotic pathway.

In a study of tumour cells, resveratrol did not enhance DR expression, but could induce apoptosis through redistribution of the Fas receptor or DR4 and DR5 into lipid rafts, together with Fas-associated death domain and procaspase-8.[29] This redistribution was associated with the formation of a DISC and sensitized cells to DR stimulation.[29] Platelets, however, do not express the DRs such as the Fas

receptor, DR4, or DR5 on their plasma membranes.[31] Moreover, Piguet *et al.*[32] demonstrated that TNF could act on platelets directly and stimulates caspase activation via the TNF-R1, but not TNF-R2. In the present study, both antagonistic anti-Fas receptor and anti-TNF-R1 mAbs did not significantly reverse resveratrol-induced caspase-8 activation, suggesting that these DRs are not involved in resveratrol-induced platelet apoptosis. If other receptors are mediated this reaction, it still remains obscurely and needs to be further investigated.

Recently, several reports have indicated that the mitochondrial permeability transition pore, a key intermediate
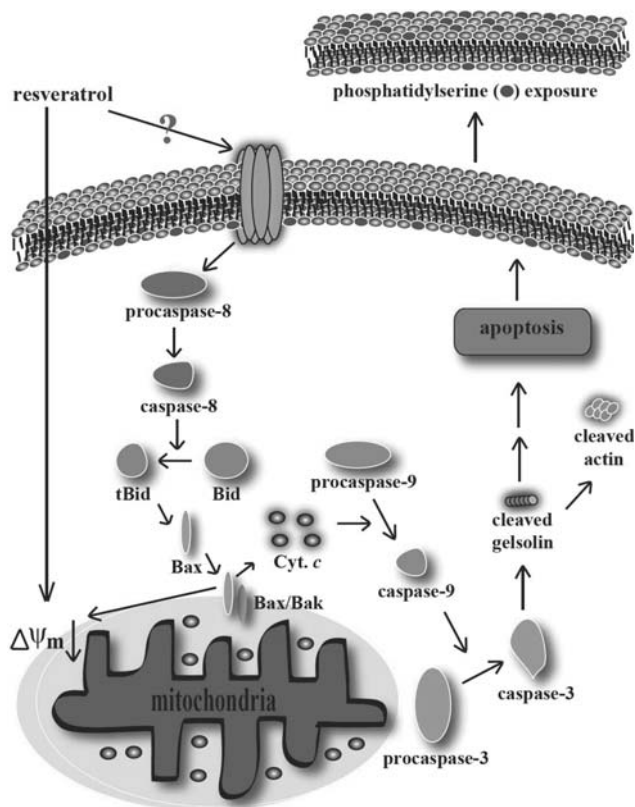
Figure 7 Hypothetical scheme of resveratrol-induced platelet apoptosis. Resveratrol-induced platelet apoptosis may be involved in the extrinsic and intrinsic apoptotic pathways. In the extrinsic pathway, it activates caspase-8 activation, followed by induction of Bax translocation, dissipation of $\Delta\Psi m$, and induction of cytochrome *c* (Cyt. *c*) release. In the intrinsic pathway, resveratrol directly targets mitochondria and evokes dissipation of $\Delta\Psi m$. Both pathways converge to a final common pathway involving the activation of caspase-3, and finally results in phosphatidylserine (PS) exposure and apoptosis.

in apoptosis, and gossypol and methoxy-antimycin, Bax activators, potentiate platelet activation,[9,10] supporting platelet apoptosis being involved in platelet activation. On the other hand, ABT-737, a potent antagonist of the anti-apoptotic Bcl family, leads to activation of key apoptotic processes including Cyt. *c* release, caspase-3 activation, and PS exposure in the absence of platelet activation *in vitro*, and results in enhanced clearance by the reticuloendothelial system *in vivo*.[11] In addition, the reduced recovery of circulating labelled platelets by resveratrol may suggest that it could shorten the platelet survival or enhance the platelet clearance *in vivo*. Mason *et al*.[12] predicted that strategies to promote platelet survival by inhibiting apoptosis may be advantageous in some patients with thrombocytopenia. Conversely, patients suffering from thrombotic conditions or thrombocytosis may well benefit from treatment with ABT-737 to promote platelet apoptosis and thus prevent the sequelae associated with pathological clotting.[12] However, the physiological function of the apoptotic machinery in platelets is not yet clearly understood, and its *in vivo* influence needs to be further investigated.

Platelet activation causes exposure of buried PS on the surface of platelet-derived membranes, and then PS binds to both coagulation factors, X_a and V_a, and regulates their proteolytic and cofactor activities, which leads to assembly of an active prothrombinase.[33] Thus, PS exposure in human

platelet outer membranes is thought to act as a second messenger linking platelet activation to prothrombin activation.[33] Moreover, PS exposure on the outer leaflet of plasma membrane is a surface change common to many apoptotic cells and has several potential biological consequences, one of which is recognition and removal of the apoptotic cell by phagocytes.[34] In this study, platelet activation (P-selectin expression) did not occur during the process of platelet apoptosis stimulated by resveratrol (*Figure 6B*), indicating that resveratrol induces PS exposure without causing platelet activation.

In conclusion, the most important findings of this study demonstrate that resveratrol stimulates platelet apoptosis. This stimulatory apoptotic pathway in human platelets is possibly involved in the following two mechanisms: the extrinsic (cytoplasmic) and intrinsic (mitochondrial) apoptotic pathways (*Figure 7*). Resveratrol causes caspase-8 activation, followed by cleavage of Bid into tBid, which leads to Bax translocation into mitochondria and induction of Cyt. *c* release from mitochondria. Secondarily, resveratrol may directly target mitochondria, and evokes dissipation of $\Delta\Psi m$ and the subsequent induction of Cyt. *c* release. Both pathways converge to a final common pathway involving the activations of caspase-9 and -3, ultimately resulting in platelet apoptosis (*Figure 7*). Stimulation of platelet apoptosis by resveratrol may represent an increased therapeutic potential for patients suffering from thrombotic conditions (i.e. CHD or acute thrombosis) or thrombocytosis, to promote platelet destruction, and thus prevent pathological clotting. On the other hand, our study also provides a novel concept that rigorous surveillance of platelet numbers may be important during resveratrol treatment alone or in combination with other chemotherapies.

Conflict of interest: none declared.

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