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FERM domain promotes resveratrol-induced apoptosis in endothelial cells via inhibition of NO production

Hye-Rim Lee [a,1], Jongmin Kim [b,1], Jinsun Park [a], Sunyoung Ahn [a], Eunsil Jeong [a], Heonyong Park [a,*]

[a] Department of Molecular Biology & Institute of Nanosensor and Biotechnology, Dankook Univiersity, 126, Jukjeon-dong, Suji-gu, Yongin-si, Gyeonggi-do 448-701, Republic of Korea
[b] Department of Life Systems, Sookmyung Women's University, 52, Hyochangwon-gil, Yongsan-gu, Seoul 140-742, Republic of Korea

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ABSTRACT

Focal adhesion kinase (FAK) consists of an N-terminal band 4.1; ezrin, radixin, moesin (FERM) domain; tyrosine kinase domain; and C-terminal FA targeting domain. Here we show that ectopically expressed FERM is largely located in the cytosolic fraction under quiescent conditions. We further found that this ectopically expressed FERM domain aggravates endothelial cell apoptosis triggered by 100 μM resveratrol, whereas FERM had no effect on apoptosis induced by TNF-α. We determined that resveratrol at low doses (<20 μM) promotes phosphorylation (S1177) of eNOS via an AMPK-dependent pathway. The presence of the FERM domain blocked this resveratrol-stimulated eNOS phosphorylation and NO production. Thus, the pro-apoptotic activity of cytosolic FERM domain is at least partially mediated by down-regulation of NO, a critical cell survival factor. Consistently, we found that the apoptosis induced by cytosolic FERM in the presence of resveratrol was reversed by an NO donor, SNAP. In conclusion, FERM located in the cytosolic fraction plays a pivotal role in aggravating cell apoptosis through diminishing NO production.

1. Introduction

Endothelial cells comprise the innermost layer of blood vessels, providing a lining between the bloodstream and the vessel wall. Endothelia are structurally and functionally maintained through cell–cell junctions and cell-to-extracellular matrix (ECM) adhesions. Focal adhesions (FAs) are points of contact between the basal membrane of endothelial cells and the ECM [1]. The FA complex consists of various proteins; including integrins, talin, paxillin, vinculin, XIAP, and FAK; providing anchorage to the cytoskeleton and ECM. Recruitment of FAK into FAs is crucial for maintenance of FA and endothelial cell survival [1].

FAK consists of an N-terminal band 4.1; ezrin, radixin, moesin (FERM) domain; tyrosine kinase (Kinase) domain; and C-terminal FA targeting (FAT) domain [2]. The FAT domain plays an important role in the recruitment of FAK to FAs through interaction with paxillin/talin [3,4]. FAK-mediated signal transduction at the FA site is facilitated by recruitment of other FAK-activating molecules, including Src kinase, subsequently triggering phosphorylation of Tyr-576/Tyr-577 in the Kinase domain [5]. It is established that phosphorylation at Tyr-576/Tyr-577 is essential for FAK-activated cell signaling, including activation of MAP kinases. The FERM domain contains nuclear export signal sequences (NES) and nuclear target sequences (NTS), triggering nuclear localization of FAK under certain conditions, such as treatment with staurosporine [1,6]. It is also well-accepted that nuclear FAK interacts with p53 through the FERM domain, controlling cell apoptosis [7,8].

Full-length FAK is cleaved by apoptotic stimulants, such as growth factor deprivation or staurosporine [6,9]. Previously it was shown that FAK cleavage occurs in part by caspase-3, caspase-7, and caspase-6, alone or in combination [9]. Immunoblotting using an antibody against the N-terminus of FAK revealed that a cleaved fragment (~48 kD) contains most of the FERM domain. To date, little is known as to a role of the FERM domain produced by proteolysis under apoptotic stimulation in endothelial cells. Although it remains to be determined, it is highly possible that XIAP regulates FERM domain cleavage from FAK. Indeed, XIAP interacts with FAK in FAs, thereby controlling cell responses [10,5], and inhibits the caspases known to cleave FAK [11].

Resveratrol is a naturally occurring phytoalexin found in grapes. It was reported that at high doses (100 μM) resveratrol acts as an

Abbreviations: XIAP, X-chromosome linked inhibitor of apoptosis protein; FA, focal adhesion; FAK, focal adhesion kinase; FERM domain, band 4.1, ezrin, radixin, moesin domain; FAT, focal adhesion targeting; FRNK, FAK-related non-kinase; BAEC, bovine aortic endothelial cell; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; AMPK, AMP-activated protein kinase; FA, focal adhesion; ECM, extracellular matrix; CAM, cell adhesion molecule; siRNA, small interfering RNA; NES, nuclear export signal sequence; NTS, nuclear target sequence; SNAP, S-Nitroso-N-acetyl-DL-penicillamine.

* Corresponding author. Fax: +82 31 8021 7201.
E-mail address: heonyong@dankook.ac.kr (H. Park).

[1] These authors contributed equally to this work.

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apoptogenic agent, activating caspase-3 in BAECs [12], whereas at low doses (~nM) resveratrol activates eNOS by enhancing phosphorylation at S1177 [13], consequently producing NO. The endothelial level of NO is a critical factor in determining cellular fate. Previous reports revealed that resveratrol at ~nM level activates eNOS through an estrogen receptor α/caveolin-1/Src pathway [13] or an AMP-activated protein kinase (AMPK)-dependent pathway [14]. However, the detailed mechanisms by which resveratrol at high doses induces apoptosis remain to be determined. In this study, we report for the first time that the FERM domain of FAK down-regulates NO production, thus aggravating apoptosis triggered by resveratrol.

2. Materials and methods

2.1. Cell culture

Bovine aortic endothelial cells (BAECs) obtained from descending thoracic aortas were cultured in DMEM (Wel GENE Inc.) containing 20% fetal bovine serum (FBS, Wel GENE Inc.) and antibiotics (penicillin/streptomycin) at 37 °C with 5% CO_2. Cells from passage 5 to 10 were used.

2.2. Transfection of BAECs with ferm genes

BAECs cultured without antibiotics were grown to 90% confluency. The cells were then transfected with vectors carrying V5-tagged *ferm* gene or empty vector (2 μg/well each) in the presence of 5 μl Lipofectamine 2000 Trasfection Reagent (Invitrogen Life Technologies), as previously described [12].

2.3. Preparation of cell lysates

Cells were washed in ice-cold phosphate buffered saline (PBS), and then placed in lysis buffer A (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM vanadate, 10% glycerol, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) containing a protease inhibitor cocktail (Roche Molecular Biochemicals) or lysis buffer B (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride) containing protease inhibitor cocktail (Sigma). Soluble lysates were fractionated by centrifugation. Total protein amounts in the cell lysates were measured using a Bio-Rad DC assay kit (Bio-Rad) or a SMART BCA protein assay kit (iNtRON). In addition, nuclear/cytosol fractionation was performed using the nuclear/cytosol fractionation kit supplied by BioVision.

2.4. Western blotting

Proteins (25 μg) in the soluble lysates were resolved by SDS–PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), and blotted with antibodies specific to V5 (Invitrogen Life Technologies), FAK (Milipore), p~S1177 eNOS (Cell Signaling), eNOS (Cell Signaling), caspase-3 (Cell Signaling), p53 (Santa Cruz Biotechnology), and actin (Santa Cruz Biotechnology). Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies and developed using the enhanced chemiluminescence detection method (Amersham).

2.5. NO measurement

Cellular NO was quantified by fluorescence spectra of intracellular DAF-2 DA (Calbiochem). Cells were pre-incubated with a HEPES buffer (5 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Glucose, pH7.4) containing 1 μM Ca^{2+} ionophore, A23187 (Sigma), for 20 min. Subsequently, cells were incubated at 37 °C and 5% CO_2 with 10 μM DAF-2 DA for 15 min, and were harvested and lysed by sonication. Supernatants were then obtained by centrifugal fractionation, and were diluted and scanned by a Spectrofluorophotometer (RF 5301PC Shimadzu) at excitation and emission of 495 and 515 nm (slit 10 nm), respectively. NO was calculated via the DAF-2 DA fluorescence intensity [12].

2.6. Cell apoptosis

Confluent BAECs were treated with 20 ng/ml TNF-α or 100 μM resveratrol for 18 h. For FAK cleavage experiments, BAECs were serum-starved for 24–48 h and subsequently treated with 100 μM resveratrol. Apoptotic cells (round, shrunken cells) were then examined under the microscope. For quantification, we counted the number of apoptotic cells in the same visual field. For Hoechst staining, confluent cells were incubated with DMEM containing 100 μM resveratrol or 20 ng/ml TNF-α for 18 h. BAECs were then fixed with Conroy's fixative for 10 min and washed with PBS. The cells were air-dried for 10 min. After air-drying, the cells were stained with Hoechst 33,258 (12.5 μg/ml, Sigma) for 30 min at room temperature. Stained cells were then thoroughly washed with PBS, and the stained nuclei were observed under a fluorescence microscope (Zeiss Autoplan 2).

2.7. Cell proliferation

Cell proliferation was measured using a cell viability assay kit (Daeil Lab Service Co., LTD., Seoul Korea), as previously described [15]. Briefly, confluent FERM-transfected BAECs were serum-starved for 16 h, and then incubated for the indicated period of time. The cells were then thoroughly washed with PBS and incubated with WST-1 reagent. Live cells were measured at 450 nm using an ELISA plate reader (Bio-Rad, Model 550).

2.8. Flow cytometry

BAECs grown to 80% confluency were transfected with the empty or *ferm*-containing vectors. Twenty-four hours after tansfection, serum-starved BAECs were treated with 200 μM SNAP and 100 μM resveratrol for 24 h. Then, BAECs were stained with propidium iodide (PI) and FITC-tagged annexin V, using the ApoScan Annexin V FITC apoptosis detection kit (Biobud Co., LTD., Korea). Stained cells were detected by flow cytometry (Guava easyCyte, Milipore).

3. Results

3.1. Ectopically expressed FERM is largely located in the cytosolic fraction

FAK consists of three functional domains; the FERM domain, kinase domain, and FRNK domain. Since it is known that FAK is cleaved under certain conditions [6,9], we hypothesized that a free FERM domain is produced upon FAK cleavage, and plays an important role in vascular endothelial cells. We first examined the cellular expression of FERM in BAECs transfected with a vector containing the *ferm* gene. As shown in Fig. 1A, FERM was found to be expressed in BAEC by Western blotting using an anti-FERM antibody. Then, we executed an additional experiment to discover the cellular location of ectopically expressed FERM. We found that FERM was largely located in the cytosol under quiescent conditions (Fig. 1B and C), consistent with our previous report [16].

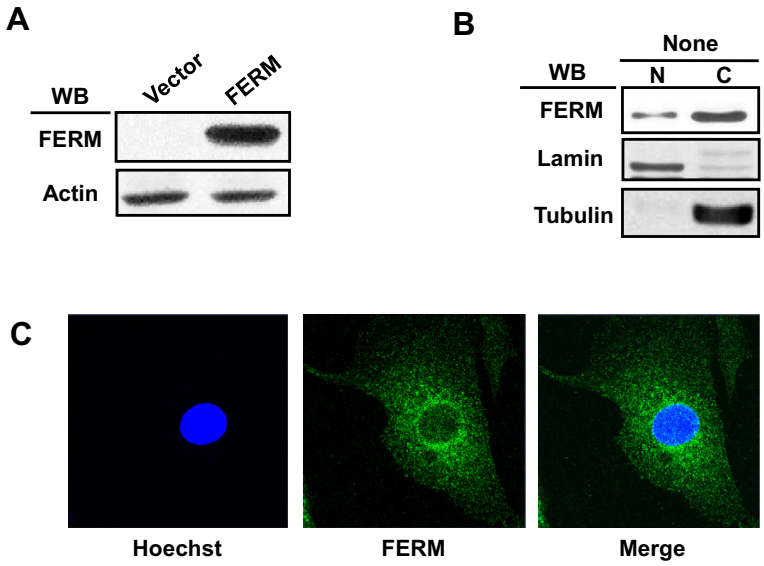

Fig. 1. Ectopically expressed FERM is largely located in the cytsolic fraction. (A) BAECs were transfected with vectors containing a *ferm* gene and FERM was detected by Western blotting. (B) Nuclear (Nu) and cytosol (Cy) fractionations were performed in BAECs transfected with the *ferm* gene. Lamin and Tubulin were used as nuclear and cytosolic markers, respectively. (C) BAECs transfected with V5-tagged *ferm* genes were immunostained with anti-V5 antibodies and also stained with Hoechst 33,258.

3.2. FERM augments cell apoptosis triggered by resveratrol

We then examined whether cell proliferation and apoptosis were affected by FERM overexpression in BAECs. As shown in Fig. 2A, FERM overexpression had no effect on cell proliferation. From apoptotic assays monitoring floating round cells and Hoechst staining, it was revealed that FERM overexpression markedly enhanced the resveratrol-induced apoptosis by ~140%. In contrast, FERM overexpression had no effect on the TNF-α-induced apoptosis (Fig. 2B). These findings suggest that resveratrol and TNF-α differentially regulate apoptosis.

3.3. FERM inhibits eNOS phosphorylation, thereby diminishing NO production

We executed additional experiments to elucidate the mechanisms by which FERM enhances resveratrol-induced apoptosis. As shown in Fig. 3A, FERM overexpression appeared to reduce resveratrol-stimulated phosphorylation of eNOS by ~50% (Fig. 3B), whereas FERM overexpression had no effect on caspase-3 activation and p53 stability. We measured NO levels upon stimulation of cells with resveratrol and TNF-α in order to confirm an inhibitory effect of FERM on eNOS activation. As shown in Fig. 3C, FERM overexpression de-

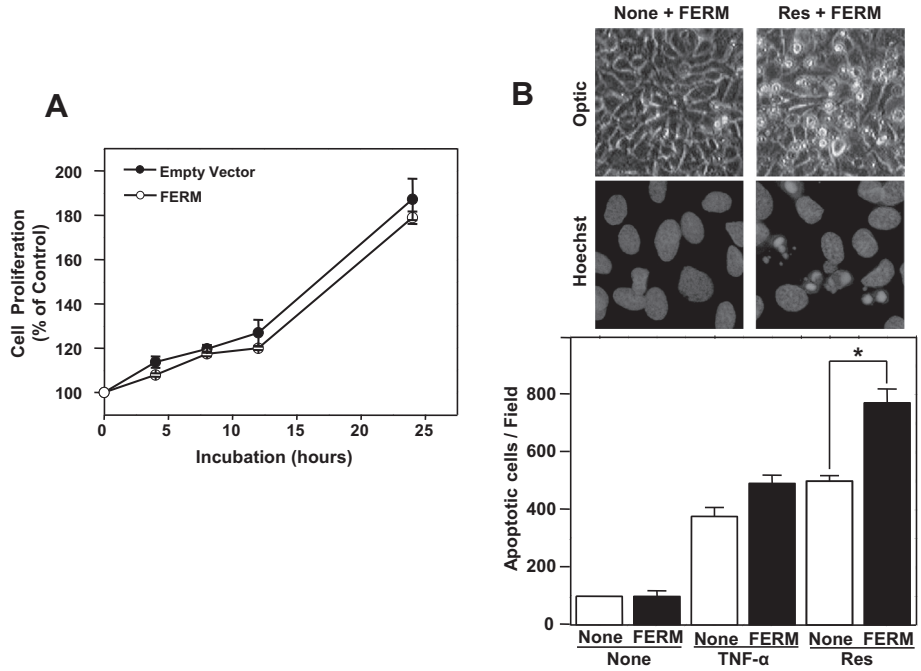

Fig. 2. FERM overexpression promotes resveratrol-triggered apoptosis. Cell proliferation (A) and apoptosis (B) of transfected BAECs were examined. For apoptotic analysis, transfected cells were treated with TNF-α (20 ng/ml) and resveratrol (100 μM). Quantified data were plotted as line graphs (cell proliferation, means ± S.E., $n = 3$) and bar graphs (apoptosis, means ± S.E., $n = 3$). $^{*}P < 0.05$.

creased resveratrol-induced NO production by ~30%. These findings indicate that the ectopically expressed FERM domain inhibits NO-associated cell survival. Conversely, we found that TNF-α had no effect on eNOS phosphorylation and NO production (Fig. 3A and C), consistent with previous reports [17,18]. This result indicates that TNF-α-induced apoptosis is not associated with regulation of NO production. Therefore, it is appropriate that FERM overexpression appeared to have no effect on TNF-α-induced apoptosis.

It is well established that resveratrol at lower doses (50 nM–10 μM) triggers NO production [13,19]. However, our current data reveal that the ectopically expressed FERM domain diminishes NO production induced by 100 μM resveratrol. Therefore, we hypothesized that resveratrol at lower doses enhances NO production via eNOS activation, whereas resveratrol at higher doses (~100 μM) down-regulates NO production through the ectopically expressed FERM. To prove this hypothesis, we examined eNOS phosphorylation at various doses of resveratrol. As shown in Fig. 3C, eNOS phosphorylation was shown to be maximally induced by resveratrol at 20 μM, lower than the apoptotic concentration (100 μM) of FERM/resveratrol. Additionally, we found that Compound C (an AMPK inhibitor) completely ablates resveratrol-induced eNOS phosphorylation, indicating that resveratrol-triggered NO production is crucially mediated by AMPK (Fig. 3D). From these results, resveratrol-induced NO production was principally regulated by an AMPK-dependent pathway due to greater dose-sensitivity and stronger inhibition by Compound C. The inhibitory effect of FERM is likely secondary to the AMPK-dependent pathway in the regulation of resveratrol-induced NO production.

3.4. FERM-induced NO deprivation enhances apoptosis

Based on our previous findings, we hypothesized that NO deprivation aggravates apoptosis triggered by resveratrol in endothelial cells. To test this, we examined whether an NO donor was able to reverse FERM-enhanced apoptosis. Interestingly, based on FACS data (Fig. 4), FERM over-expression in the presence of resveratrol appeared to profoundly induce apoptosis (up to 70%). This apoptogenic effect was reversed by SNAP (an NO donor) treatment, indicating that NO acts as an anti-apoptotic agent under these conditions.

4. Discussion

A variety of proteins are associated with FAK degradation/dissociation from FAs, thus controlling apoptosis. This notion is supported by our previous report that XIAP knockdown induces dissociation of FAK from FAs, subsequently promoting apoptosis [10]. Interestingly, we recently found that BAECs treated with 100 μM resveratrol after long-term starvation showed diminished XIAP expression, markedly increased caspase-3 activity, and cleaved FAK (data not shown). Since XIAP blocks caspase-3 activity [20], XIAP knockdown likely rendered resveratrol-activated cells susceptible to caspase-3-induced FAK degradation. These unpublished data give an insight into the production of FERM under physiological conditions.

FAK is known to be crucially associated with the development of the cardiovascular system, cardiac hypertrophy, and angiogenesis [21]. These vascular functions of FAK are largely explained by a plethora of molecular evidence showing binding of FAK with a variety of molecules, including integrins, Src kinase, p130Cas, and paxillin [22,23]. In addition, molecular studies reveal that three important functional domains of FAK; FERM, kinase, and FRNK domains, have specific molecular and cellular activities. For instance, the FRNK domain is essential for FA recruitment of FAK [16] through an association with other cell adhesion molecules (CAMs)

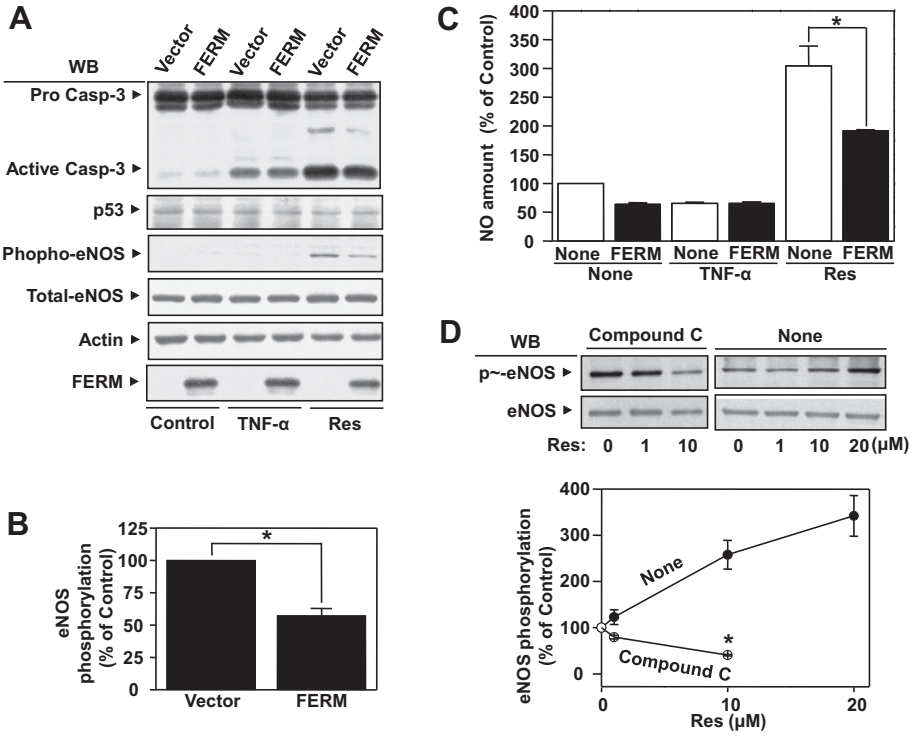

Fig. 3. FERM overexpression inhibits resveratrol-induced eNOS phosphorylation and NO production. (A) BAECs were transfected with a vector containing V5-tagged *ferm* genes and treated with TNF-α (20 ng/ml) and resveratrol (100 μM) for 18 h before Western blotting with indicated antibodies. (B) Western blots for p~eNOS were quantified and plotted as bar graphs (means ± S.E., $n = 3$). $^*P < 0.05$. (C) Transfected cells were treated with 1 μM Ca^{2+} ionophore, A23187, alone or in the presence of TNF-α (20 ng/ml) and resveratrol (100 μM). NO was then measured and levels are plotted as bar graphs (mean ± S.E., $n = 3$). $^*P < 0.05$. (D) BAECs were treated with 20 μM Compound C and indicated concentrations of resveratrol. BEACs treated with 20–100 μM resveratrol in the presence of Compound C were largely degraded, so that the cell lyaste could not be obtained. Immunostaining was performed with anti-p~eNOS and anti-eNOS antibodies. Quantified data were plotted as line graphs (means ± S.E., $n = 3$). $^*P < 0.01$.

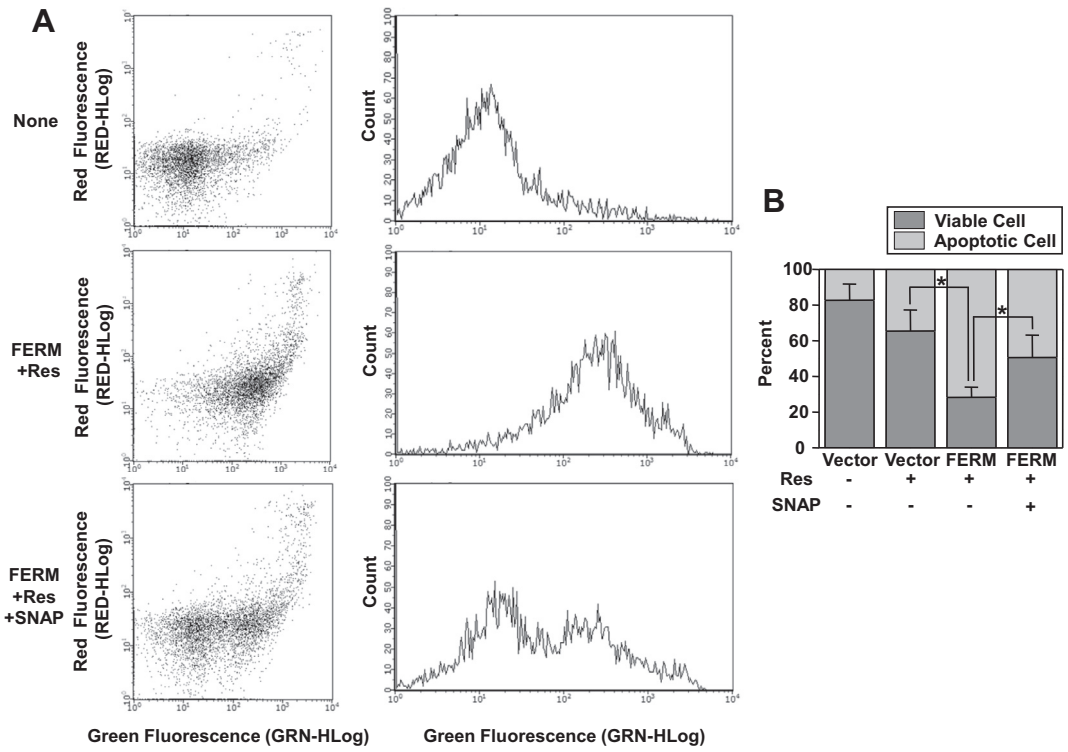

Fig. 4. SNAP reverses FERM overexpression/resveratrol-induced apoptosis. (A) Transfected BAECs were treated with resveratrol (100 μM) or SNAP (200 μM). Then cells were stained with propidium iodide (PI) and FITC-tagged annexin V according to manufacture's protocol. Stained cells were counted using a flow cytometer. (B) Percent apoptotic cells and viable cells were plotted as bar graphs (means ± S.E., $n = 3$). $^{*}P < 0.05$.

and cytoskeletal proteins [24]. The FERM domain interacts with the PH domain of Etk, regulating integrin signaling [25].

A growing body of recent studies has revealed that the FERM domain translocates into the nucleus upon treatment with FAK inhibitors [26], subsequently regulating transcriptional factors, including GATA 4 and p53 [27]. Binding of the nuclear FERM domain with p53 reduces the level of p53, promoting cell survival. The present study showed that the ectopically expressed FERM domain was largely located in the cytosolic fraction, not in the nucleus. Accordingly, our current findings demonstrate for the first time that the cytosolic FERM controls eNOS phosphorylation in endothelial cells, thereby inducing apoptosis. Nevertheless, there is a possibility that the FERM domain or cytosolic FAK translocates into nucleus under certain conditions. Indeed we previously reported that shear stress induces nuclear translocation of FAK in BAECs [10]. The mechanisms by which FAK translocates into nucleus remain to be determined. Taken together, FERM and FAK have differential functions according to their cellular location.

Our present data also pose a query as to the vascular roles of resveratrol. Resveratrol concomitantly regulates the production of two functionally opposite well-known vaso-regulators, i.e., the active form of caspase-3 (an apoptotic molecule) and NO (a cell survival factor). Owing to contradictory outcomes at the cellular level, the overall vascular effects of resveratrol are not easily predicted. However, these contradictory outcomes are associated with different doses of resveratrol. Resveratrol at lower doses (<20 μM) activates eNOS via AMPK, whereas a high dose (100 μM) activates caspase-3, to possibly cleave FAK and decrease NO. The present findings suggest a new notion in the vascular biology arena, that the cleavage of FAK switches the cellular fate from cell survival to apoptosis. This notion is also supported by the inhibitory effect of an NO donor on FERM/resveratrol-induced apoptosis (see Fig. 4).

In conclusion, biphasic endothelial functions of resveratrol are summarized as follows; (1) AMPK-mediated eNOS activation at low dose and (2) caspase-3 activation at high dose. Through these biphasic effects of resveratrol, we found in this study that cytosolic FERM aggravates resveratrol-triggered apoptosis via NO suppression. Given the established anti-apoptotic effects of nuclear FERM, this pro-apoptotic effect of cytosolic FERM suggests that nuclear translocation of FERM is a key factor in determining cellular fate (survival vs. apoptosis).

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