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Saponin monomer 13 of dwarf lilyturf tuber (DT-13) protects serum withdrawal-induced apoptosis through PI3K/Akt in HUVEC

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ABSTRACT

Dwarf lilyturf tuber is widely used in clinics to prevent cardiovascular diseases. DT-13, the saponin monomer 13 of dwarf lilyturf tuber, shows protective activities in anti-thrombosis, anti-inflammation, and cardioprotective. However, little is known about the cellular function of DT-13 in cardiovascular system. Vascular endothelial cells (EC) are important to maintain the integrity of the vasculature throughout entire body. Dysregulation of EC may lead to pathophysiological processes of numerous cardiovascular diseases. We thus tested the function of DT-13 in EC. In the present study, we are the first to report that DT-13 has anti-apoptosis activity on human umbilical vein endothelial cells (HUVEC), potentially through down regulation of cleaved caspase-3 and cleaved PARP expression. DT-13 also increased mitochondrial membrane potential. To explore the potential mechanism, we investigated the effect of DT-13 on Akt and MAPK pathways and found that DT-13 was involved in Akt signaling confirmed by using PI3 K/Akt inhibitor LY294002. Thus, DT-13 could improve survival of EC and therefore be a potential clinical use in the treatment of cardiovascular diseases.

1. Introduction

Vascular endothelial cells (EC) cover the inner surface of blood vessels and serve as a crucial barrier between tissues and circulation [1]. Health EC are critical in maintaining vascular integrity and participating in the immune responses. They secrete a variety of substances and protect vessels from activation of clotting and proinflammatory factors [2]. An imbalance in repair and injury of EC results in endothelial dysfunction, which is associated with many vascular complications [3,4].

Apoptosis is a natural physiological process which uses programmed cell death to control cell number and maintain homeostasis and development of tissues in organisms. Dysregulation of apoptosis in EC may destroy the vascular barrier integrity and leads to pathophysiological processes which are associated with numerous cardiovascular diseases such as thrombosis, diabetes, hypertension, atherosclerosis and pathological angiogenesis [5].

The saponin monomer 13 of dwarf lilyturf tuber (DT-13) is a major saponin monomer isolated from *Liriope muscari*. Our group and others have shown multiple protective activities of DT-13 in cardiovascular systems, including inhibition of thrombus induced by inferior vena cava (IVC) ligation [6], inhibition of lymphocyte adhesion to extracellular matrix [7] and reducing L-type calcium currents during hypoxia in adult rat ventricular myocytes [8,9]. It has also been reported that DT-13 can improve immunological liver injury by selectively inducing liver-infiltrating lymphocytes dysfunction [10]. However, despite the widely effects of DT-13 to prevent cardiovascular diseases, little is known about the cellular function of DT-13 in EC. Thus, the aim of this study was to investigate the effects of DT-13 on EC to further extend our understanding its pharmacologic functions and to provide experimental evidence for its potential clinical use in the treatment of cardiovascular diseases.

In the present study, we demonstrate for the first time that DT-13 prevents human umbilical vein endothelial cells (HUVEC) from serum withdrawal induced apoptosis by modulating Akt signaling, caspase-3 and Poly ADP-Ribose Polymerase (PARP) cleavage, indicating that DT-13 could be a potential agent in improving endothelial function.

2. Materials and methods

2.1. Materials

HUVECs were obtained from the tissue culture core of Yale Vascular Biology and Therapeutic program. DT-13 (purity > 98%) was

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separated from Liriopes Radix by the authors and dissolved in dimethyl sulfoxide (DMSO). The stock solution (100 mM) was stored at −20 °C and was diluted to 1, 2, and 5 μM, before treatment. Propidium iodide (PI) was purchased from Life Technologies Corporation (NY, USA). JC-1 assay kit was from Molecular Probes, Inc (OR, USA). Primary antibodies against caspase-3, cleaved PARP, phosphorylated Akt, Akt, phosphorylated p38, p38, phosphorylated extracellular signal-regulated protein kinases 1/2 (p-ERK), ERK, phosphorylated c-Jun N-terminal kinases (p-JNK) and JNK were purchased from Cell Signaling Technology, Inc (MA, USA). PI3 K inhibitor LY294002 was obtained from Cell Signaling Technology, Inc (MA, USA).

2.2. Cell culture and treatment

HUVEC were cultured in EBM-2 (Lonza, Singapore) supplemented with 10% fetal bovine serum (FBS) and EGM-2 bullet kit in a humidified 37 °C incubator with 5% CO_2 atmosphere. Low passage HUVEC (<6 passages) were used in this study. To examine the effect of DT-13 on EC apoptosis, HUVEC were treated with serum free media with DT-13 (1, 2, 5 μM) or its vehicle control (0.01% DMSO) for 24 h.

2.3. PI staining of cells to assess DNA content

The DNA cell cycle assay was done by flow cytometry analysis. Both floating cells and attached cells were collected in the appropriate manner and then fixed in cold 70% ethanol for 30 min at 4 °C. Cells were washed in cold PBS after fixation and spun down at 850 g. RNase treatment was done before adding PI to ensure only DNA, not RNA, was stained. Cells were stained by 200 μL PI (50 μg/mL) and then measured by flow cytometer. DNA content was analyzed, and the sub-G1 population was obtained as an indicator of apoptosis.

2.4. Membrane potential assay

Mitochondrial membrane potential (MMP) was assessed to indicate mitochondrial function. HUVEC were treated with serum free medium for 24 h with different concentrations of DT-13 (1, 2, 5 μM) or its vehicle control (0.01% DMSO). JC-1 labeling was done according to the manufacturer's directions. Cells were analyzed on a flow cytometry.

2.5. Western blot analysis

After treatments, cultured HUVEC were washed with cold PBS and lysed in a buffer containing 50 mM Tris–HCl, pH 7.4, 1% NP-40, 2% SDS, 0.1% deoxycholic acid, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM sodium pyrophosphate, 5 mM sodium fluoride, 1 mM Na_3VO_4 and protease inhibitor (Roche 11836145001). Proteins were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Blots were blocked with 5% BSA and 0.1% Tween in Tris-buffered saline (TBS-T) for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C. After incubation, membranes were washed with TBS-T and incubated with fluorescent secondary antibodies at room temperature for 1 h followed by Odyssey infrared imaging system detection (Li-Cor, Lincoln, NE).

2.6. Statistical analysis

All the experiments were repeated at least three times. The results are presented as mean ± SEM. Data were analyzed using a student *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. DT-13 attenuates serum withdrawal-induced apoptosis in HUVEC

To evaluate the effect of DT-13 on serum withdrawal-induced apoptosis, DNA content of HUVEC was measured by flow cytometry. HUVEC were stained by PI and the Sub-G1 population, which is associated with apoptotic cells, was counted [11]. As shown in Fig. 1A, serum withdrawal significantly elevated percentage of cells with hypo-diploid DNA content compared to normal control cells (4.29% and 24.85%, respectively). HUVEC treated with different concentrations of DT-13 (1, 2, or 5 μM) reduced the percentage of apoptotic cells (18.9%, 12.16%, and 6.64%, respectively) in a dose dependent manner.

3.2. DT-13 inhibits serum withdrawal-induced caspase-3 and PARP cleavage

At the protein level, apoptosis is characterized by the sequential activation of caspase cascades. Caspase-3 is a key protease of cell apoptosis [12,13]. Therefore, we next investigated the effect of DT-13 on caspase-3 in HUVEC. Serum withdrawal increased caspase-3 cleavage, characterized by the increased density of 17- and 19-kDa protein bands (Fig. 1B). In contrast, DT-13 treatment dose dependently decreases the amount of cleaved caspase-3. At 5 μM, DT-13 significantly decreased cleaved caspase-3 compared to vehicle treated cells. PARP, as a target of caspase-3, has been used extensively as a marker of apoptosis [14,15]. In accordance with caspase-3 results, increased PARP cleavage was detected in HUVEC in serum free media compared to those in normal complete media. DT-13 inhibited the increase in cleaved PARP and indicated a significant anti-apoptotic effect at 5 μM (Fig. 1B). These findings suggest that apoptosis is induced in HUVEC after serum withdrawal for 24 h, and DT-13 dose dependently protects HUVEC from apoptosis.

3.3. DT-13 attenuates serum withdrawal-induced mitochondrial dysfunction

The mitochondrion is an important organelle for the production of cellular energy and has key role in programmed cell death. The maintenance of membrane potential is essential for mitochondrial integrity and bioenergetic functions [16]. We then assessed the MMP using the JC-1 probe and flow cytometry analyses. As shown in Fig. 2, HUVEC in serum free condition exhibit a higher incidence of cells with decreased membrane potential from 7.11% (normal control with complete media) to 22% (serum free). DT-13 treatment results in a decline of the percentage of JC-1 aggregate cells in a dose dependent manner with an effect reaching statistical significance at 5 μM concentration.

3.4. DT-13 up-regulates Akt phosphorylation in serum free treated HUVEC

To gain further insight into the potential underlying mechanisms of DT-13 on EC apoptosis, we investigated the effect of DT-13 on Akt activation. Akt phosphorylation decreases 5-fold after 24 h starvation in vehicle treatment cells compared to normal control cells. On the other hand, DT-13 shows a dose dependent effect on increasing Akt phosphorylation, indicating that DT-13 may be involved in Akt pathway (Fig. 3A).

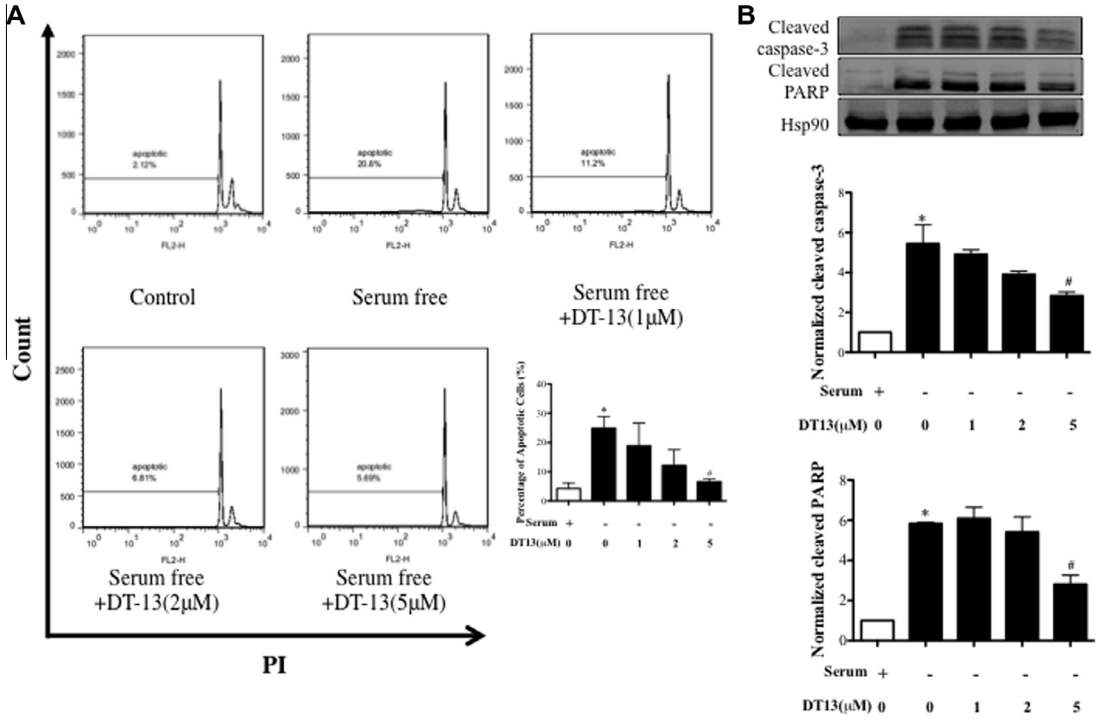

Fig. 1. DT-13 attenuates serum withdrawal-induced apoptosis in HUVEC. (A) DNA content was detected by flow cytometer after PI staining. Cells were treated by 24 h serum-free starvation with or without DT-13. (B) Cleaved PARP and cleaved caspase-3 were detected by Western blot after 24 h serum-free starvation with or without DT-13. $^{*,\#}P < 0.05$ based on *t*-test.

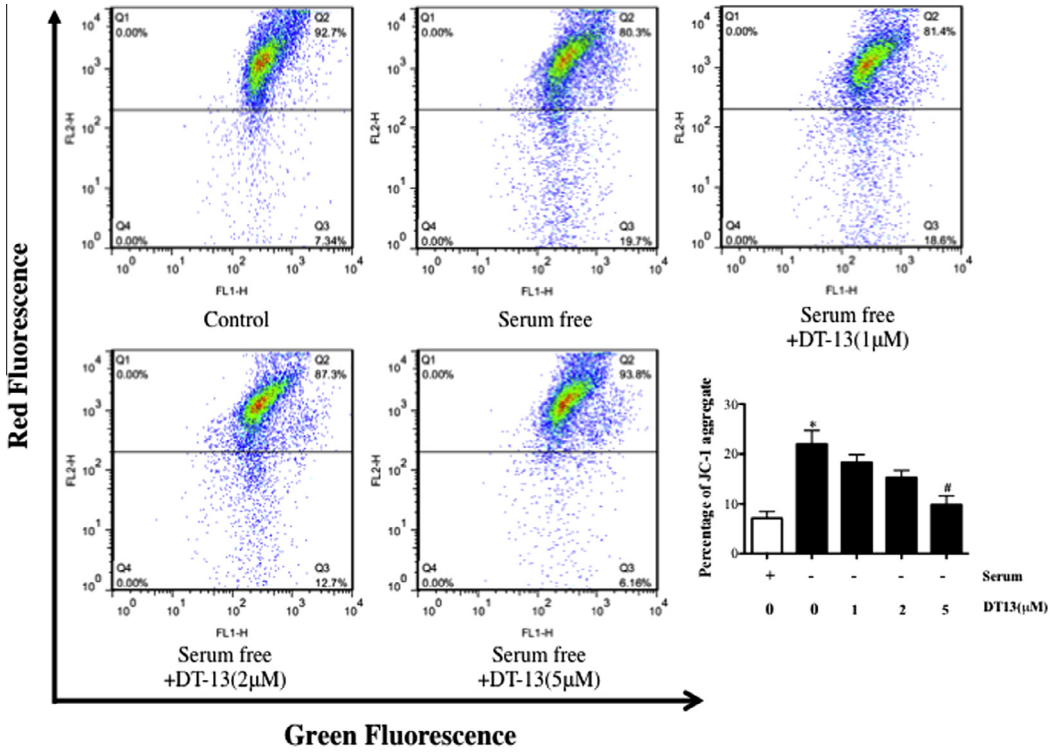

Fig. 2. DT-13 protects HUVEC from serum withdrawal-induced mitochondrial dysfunction. Mitochondrial membrane potential was detected by flow cytometer after JC-1 staining. Cells were treated 24 h serum-free starvation with or without DT-13 followed by JC-1 staining. $^{*,\#}P < 0.05$ based on *t*-test.

3.5. DT-13 has no effect on serum withdrawal-induced MAPK activation

The MAPK pathways are also known to play roles in cell survival. Thus, we examined phosphorylation of p38, ERK, and JNK to study the effect of DT-13 on the MAPK pathway. Serum withdrawal markedly enhances phosphorylation of p38 (Fig. 3B) and JNK (Fig. 3D) and slightly increases ERK phosphorylation (Fig. 3C). DT-13 did not affect phosphorylation of p38, JNK, nor ERK compared with vehicle. Although we see an increase in ERK

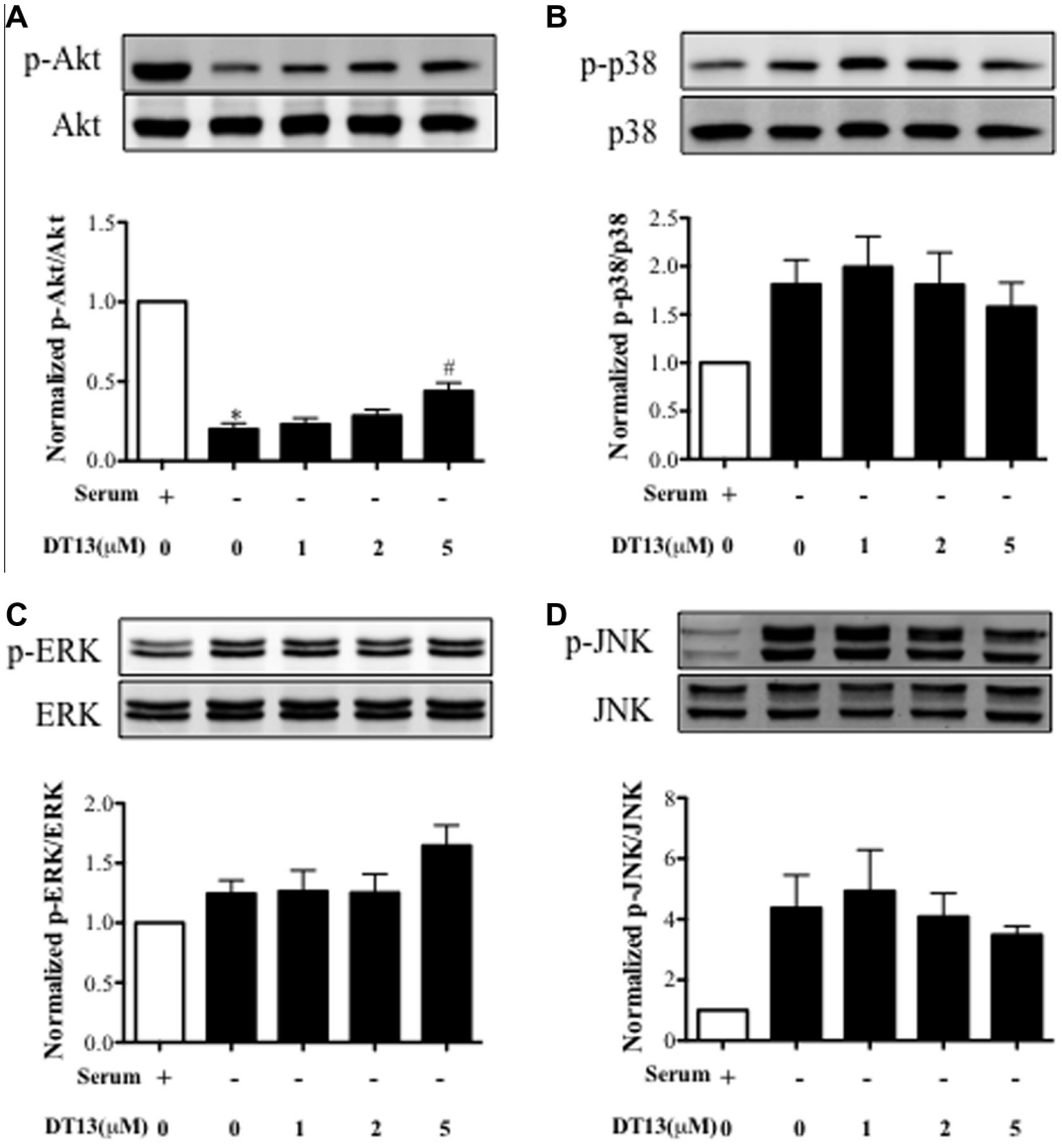

Fig. 3. DT-13 increases Akt phosphorylation after 24 h serum-free starvation. (A) p-Akt and Akt, (B) p-p38 and p38, (C) p-ERK and ERK, and (D) p-JNK and JNK were detected by Western blot after 24 h serum-free starvation with different concentrations of DT-13 or without DT-13. $^{*,\#}P < 0.05$ based on *t*-test.

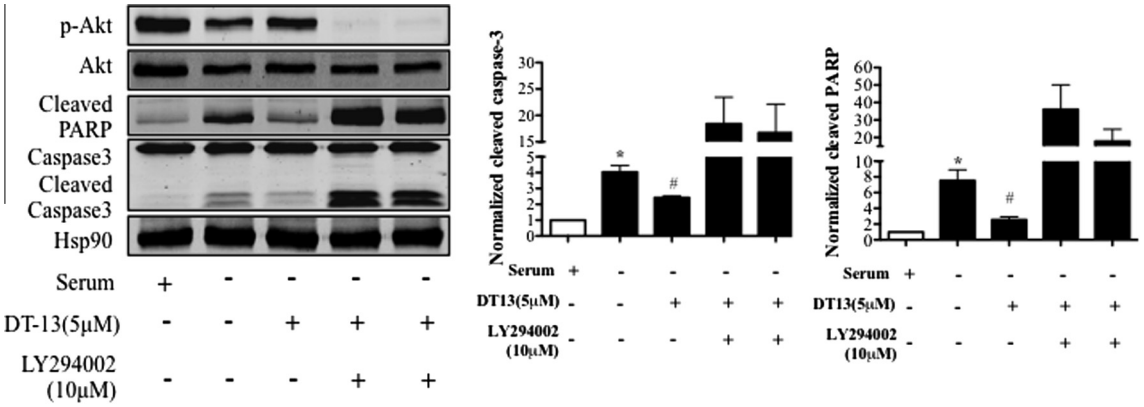

Fig. 4. The anti-apoptotic effect of DT-13 is PI3 K/Akt dependent. Cleaved PARP and cleaved caspase-3 were detected by Western blot after 24 h serum-free treatment. HUVEC were pretreated with PI3 K inhibitor LY294002 (10 μM) for 30 min followed by 24 h serum-free starvation with or without DT-13. $^{*,\#}P < 0.05$ based on *t*-test.

phosphorylation when cells are treated with 5 μM DT-13, this increase is not significant. These results suggest the effects of DT-13 are MAPK independent.

3.6. Anti-apoptotic effect of DT-13 was eliminated by PI3K inhibitor

To confirm DT-13 is PI3K/Akt-dependent, we inhibited Akt phosphorylation by using PI3K inhibitor LY294002 and examined apoptosis of cells indicated by cleavage of caspase-3 and PARP. After PI3K inhibitor treatment, the phosphorylation of Akt is blocked and levels of cleavage caspase-3 and PARP increase (Fig. 4). Both HUVEC with or without 5 μM of DT-13 treatment show similar amounts of cleaved caspase-3, indicating the protective effect of DT-13 on apoptosis might be Akt specific or dependent. Interestingly, cleaved PARP shows a decreasing, though not significant, trend in cells treated with PI3K inhibitor and DT-13 compared with cells treated with PI3K inhibitor alone.

4. Discussion

Dwarf lilyturf tuber is a traditional Chinese medicine widely used in clinics [17]. As one of saponin monomers of dwarf lilyturf tuber, DT-13 has potent cardiovascular protective effects. In the previous studies on the cardiovascular system, DT-13 reduced L-type calcium currents and exhibited anti-angiogenic activity [8,18]. However, the effect of DT-13 on apoptosis of vascular endothelial cells was unknown. Here, we report that DT-13 decreases serum withdrawal-induced apoptosis in HUVEC through down regulation of cleaved caspase-3 and cleaved expression, and increasing mitochondrial membrane potential. Furthermore, the anti-apoptotic effect of DT-13 is Akt-dependent. These results suggest that DT-13 could potentially improve survival of endothelial cells and correct endothelium dysfunction.

Apoptosis is characterized by marked morphological changes, including cytoplasm shrinkage, chromatin condensation, plasma membrane blebs, DNA fragmentation and apoptotic body formation [19,20]. DNA content measurement reveals the cell cycle phase of the non-apoptotic cells and can be applied to investigate specificity of apoptosis indicated by the sub-G1 population [21,22].

Serum deprivation causes cell apoptosis [23]. As shown in the cell cycle analysis (Fig. 1A), the sub-G1 population, or apoptotic DNA, increased dramatically after serum withdrawal. The percentage of apoptotic cells decreased in a dose dependent manner while DT-13 was present, indicating DT-13 can prevent apoptosis in HUVEC and help to maintain vascular integrity.

Apoptosis is mainly regulated by the intrinsic mitochondrial pathway such as Bcl-2 family related signals and the extrinsic death receptor pathway such as MAPK and Akt signals [24]. The loss of MMP is an early stage of apoptosis. In our study, we use JC-1 to measure MMP levels (Fig. 2). The flow cytometry analysis shows that serum free treated cells exhibit lower MMP levels (Fig. 2). DT-13 decreases the percentage of cells with green fluorescence in a dose-dependent manner, indicating DT-13 prevents cells from losing MMP induced by serum withdrawal. Cytochrome C release is accompanied with decreased MMP and leads to activate the caspase cascade. In parallel with the DNA content and MMP data, DT-13 decreases serum withdrawal-induced cleaved caspase-3 and cleaved PARP expression, which are viewed as key executioners of apoptosis (Fig. 1B). Thus, in our system, DT-13 shows function in increasing MMP and decreasing caspase-3 and PARP cleavage after 24 h serum starvation, indicating that DT-13 is involved in mitochondrial apoptotic pathways.

The PI3K/Akt and MAPK signaling pathways are known to be involved in apoptosis of endothelial cells. Akt activation serves as a pro-survival signal, which promotes cell survival and inhibits apoptosis [25,26]. It is generally accepted that ERK activation is essential for cell survival, whereas activation of JNK and p38 is thought to play an important role in cell death by controlling the activities of downstream transcription factors. [27,28]. Thus, we studied Akt and MAPK phosphorylation in our system. All of the MAPK are activate while Akt is inactive during serum deprivation. DT-13 markedly increased Akt phosphorylation (Fig. 3A) but without effect on the MAPK pathway (Fig. 3B, C, and D). In order to confirm the effect of DT-13 on Akt, we treated cells with PI3K inhibitor, LY294002, which blocks Akt phosphorylation without changing Akt protein expression. As shown in Fig. 4, DT-13 does not protect against serum withdrawal-induced apoptosis while the Akt phosphorylation is blocked, indicating DT-13 is Akt-dependent. Interestingly, we found a decreasing, though not significant, trend in cells treated with PI3K inhibitor and DT-13 compared with cells treated with PI3K inhibitor alone. This data indicates DT-13 may be involved another pathway except Akt to prevent cells from apoptosis. In previous studies of the anti-tumor effects of DT-13, DT-13 inhibited the phosphorylation of p38 in MDA-MB-435 cells and suppressed the hypoxia induced p-ERK and p-Akt in human breast cancer cells [18,29]. The different signaling pathways affected by DT-13 between previous studies and our study could be due to different cell types.

It can be concluded that DT-13 exerts remarkable protective effects on serum withdrawal-induced HUVEC apoptosis. Collectively, DT-13 blocks mitochondria-dependent apoptosis including the change of mitochondrial membrane potential and cleaving of caspase-3 and PARP protein. DT-13 also participates in the Akt signaling pathway by increasing Akt phosphorylation, which may also improve mitochondrial function by regulating Bcl-2 protein family. Considering the anti-apoptotic activity of DT-13 in HUVEC, our findings support the potential application of DT-13 for treatment of apoptosis related cardiovascular diseases.

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