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Andrographolide suppresses endothelial cell apoptosis via activation of phosphatidyl inositol-3-kinase/Akt pathway

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Abstract

Andrographolide (Andro), an active component isolated from the Chinese official herbal *Andrographis paniculata*, which has been reported to prevent oxygen radical production and thus prevent inflammatory diseases. In this study, we investigated the molecular mechanisms and signaling pathways by which Andro protects human umbilical vein endothelial cells (HUVECs) from growth factor (GF) deprivation-induced apoptosis. Results demonstrated that HUVECs undergo apoptosis after 18 hr of GF deprivation but that this cell death was suppressed by the addition of Andro in a concentration-dependent manner (1–100 μM). Andro suppresses the mitochondrial pathway of apoptosis by inhibiting release of cytochrome *c* into the cytoplasm and dissipation of mitochondrial potential ($\Delta\psi_m$), as a consequence, prevented caspase-3 and -9 activation. Treatment of endothelial cells with Andro-induced activation of the protein kinase Akt, an anti-apoptotic signal, and phosphorylation of BAD, a down-stream target of Akt. Suppression of Akt activity by wortmannin, by LY-294002 and by using a dominant negative Akt mutant abolished the anti-apoptotic effect of Andro. In contrast, the ERK1/2 activities were not affected by Andro. The ERK1/2 inhibitor, PD98059 failed to antagonize the protective effect of Andro. In conclusion, Andro exerts its anti-apoptotic potential via activation of the Akt-BAD pathway in HUVECs and thus may represent a candidate of therapeutic agent for atherosclerosis.
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Keywords: Andrographolide; Apoptosis; Endothelial cells; Akt; BAD; Mitochondria; Caspase

1. Introduction

Endothelial cells (ECs) play an important role in regulating vascular function and homeostasis [1]. Injury of the vascular endothelium is critical in the pathogenesis of atherosclerosis. Denudation of the endothelium leads to increasing SMCs proliferation, migration, and then increased vascular intimal mass within the atheromatous lesion [2]. On the contrary, re-endothelization can decrease intimal mass. Furthermore, apoptotic endothelial cells could contribute to the destabilization of atherosclerotic plaques and thrombosis [3]. Protection of ECs from apoptosis may represent a fruitful direction for therapy in atherosclerosis.

Several pro-atherogenic factors, including oxidized low-density lipoproteins [4], angiotensin II [5] and oxidative stress [6] can induce the apoptosis of ECs. Through activation of complex intracellular death signals, cells were subsequently led to shrinkage, membrane blebbing, and chromatin condensation, which are characteristics of apoptosis [7]. However, cytokines, hormones and other anti-apoptotic agents such as VEGF [8], oestrogens [9], insulin [10], or antioxidants [11] were observed to protect endothelial cells against apoptosis. The balance of the two opposing signals determines the death or survival of the cells.

Protein kinase Akt is an important regulator of various cellular processes, including glucose metabolism and cell

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Abbreviations: HUVECs, human umbilical vein endothelial cells; Andro, Andrographolide; SMCs, smooth muscle cells; ROS, reactive oxygen species; GF, growth factor; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular regulated kinase; NOS, nitric oxide synthase; NFκB, nuclear factor kappa B.

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survival [12,13]. Activation of receptor tyrosine kinases, G protein-coupled receptors, and stimulation of cells by mechanical force, can lead to the phophorylation and activation of Akt [14–16]. Akt has been identified as a downstream component of survival signaling through PI3K [17]. Akt may be regulated by both phosphorylation and the direct binding of PI3K lipid products to the Akt pleckstrin homology domain. Akt can then phosphorylate substrates such as NFκB [18] and BAD [19]. eNOS also has been identified as an Akt substrate and is activated by Akt-dependent phosphorylation to release NO in endothelial cells [20].

Andro is an active component isolated from the leaves of *Andrographis paniculata* [21], a Chinese herbal medicine used for the treatment of inflammatory disease. It has been reported that Andro has several pharmacological activities, including inhibition of iNOS expression [22], Mac-1 expression, ROS production [23], and that it has protective effects vs. cytotoxicity [24]. However, little is known about the effect of Andro in cell apoptosis or about the molecular mechanism. In the present study, we provide evidence that Andro inhibits serum deprivation-induced HUVEC apoptosis via suppression of the mitochondrial and caspase-dependent death pathway. This effect was mediated by activation of the protein kinase Akt-BAD cascade, but did not affect ERK1/2 activity. Andro may be a candidate therapeutic agent in the treatment of atherosclerosis or vascular disorders.

2. Materials and methods

2.1. Materials

Andrographolide, leupeptin, apotenin, phenylmethylsulfonyl fluoride, SDS, dithiothreitol, and antibody to β-actine were purchased from Sigma-Aldrich. Wortmannin, PD98059, LY-294002, and antibodies to Akt, phospho-Akt, and caspase-3 were from Cell Signaling. Protein assay reagents were purchased from Bio-Rad. N^G-Nitro-arginin, sodium orthovanadate, antibodies to caspase-9 and BAD were from Calbiochem. M199, FBS, endothelial cell growth supplement, heparin, penicillin, RNase, and streptomycin were from Life Technologies. The *R*-phycoerythrin-conjugated anti-rabbit antibody came from Southern Biotech. An enhanced chemiluminescence kit was obtained from Amersham pharmacia biotech. Propidium iodide, $DiOC_6$, was from Molecular probes. The colorimetric assay kit for caspase-3 was from Clontech and caspase-9 was from R&D Systems. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling kit (TUNEL) was from Roche. Anti-c-*myc* antibody, c-*myc*-tagged dominant negative Akt1 (dnAkt) in pUSEamp and control vector pUSEamp were from Upstate Biotechnology. Antibody to phospho-BAD was purchased from Oncogen. Antibody to cytochrome *c* was from BioVision. Antibodies to ERK1/2, phospho-ERK1/2, IκB-α, and the voltage-dependent anion channel (VDAC) were from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Transduction Laboratories. The effectene transfection reagent was purchased from Qiagen. All other chemicals were from Sigma.

2.2. Endothelial cell cultures

HUVECs were isolated from human umbilical cords with collagenase. Cells were maintained in M199 medium supplemented with fetal bovine serum (20%, v/v), endothelial cell growth supplement (0.03 mg/mL), heparin (0.1 mg/mL), 10 units/mL penicillin, and 10 units/mL streptomycin. Growth factor deprivation was induced by changing the medium to M199 without supplements. The cells used in this study were from passages 3 and 4.

2.3. FACS analysis of DNA fragmentation

HUVECs were incubated in serum-free M199 and variant treatments for 18 hr. Floating and adherent cells were collected and fixed with 90% alcohol, and subsequently stained with buffer consisting of 1 mg/mL RNase A and 40 μg/mL propidium iodide by incubation in subdued light for 30 min. Stained cells were then analyzed by flow cytometry. Cells of the hypodiploid DNA group were counted as apoptotic cells.

2.4. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

TUNEL staining was done according to the manufacturer's manual (Roche). Briefly, HUVECs were incubated in serum-free M199 and variant treatments for 18 hr. Floating and adherent cells were collected and fixed with 4% paraformaldehyde in PBS, permeablized with 0.1% Triton X-100, and labeled with TUNEL reaction mixture for 60 min at 37°. Stained cells were then analyzed by flow cytometry.

2.5. Western blot

HUVECs with various treatments were lysed in lysis buffer (1% SDS, 100 mM NaCl, 62.5 mM Tris [pH 7.6], 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL apotenin and 10 mM Na_3VO_4). Cell lysates (30–50 μg per lane) were subjected to SDS–PAGE (10–14%) and transfered to a PVDF membrane (Millipore Corporation). After blockage of non-specific binding sites for 1 hr by 5% non-fat milk in TTBS (0.1% Tween 20 in TBS), the membrane was incubated for 1 hr at room temperature with primary antibody (1:1000). The membrane was then washed three times with TTBS, incubated further with horseradish peroxidase-conjugated secondary antibody (1:2000) at room temperature, and then washed three times

with TTBS. The immunoblot was visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs) and expose to X-ray film. Blots were then subjected to densitometric analysis.

2.6. Assessment of mitochondrial potential ($\Delta\psi_m$)

To evaluate mitochondrial potential, exponentially growing cells (5×10^5 cells/mL) were incubated with GF deprivation M199 for 18 hr. After 15 min incubation, cells were labeled with $DiOC_6$ (40 nM) at 37°. After washing, cells were analyzed by flow cytometry (Becton Dickinson). Control experiments were performed in the growth medium.

2.7. Subcellular fractionation and cytochrome c release

After 18 hr of GF depletion, cells were scraped, washed with ice cold phosphate-buffered saline, and centrifuged; the pellet was then re-suspended in buffer (20 mM Hepes–KOH, pH 7.5, 10 mM KCl, 1.5 $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose, 100 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin and 1 μg/mL aprotinin). Cells were homogenized using a glass Dounce for 20 strokes and centrifuged at 100,000 *g* for 30 min at 4° and the supernatant was collected as the cytosolic fraction. Subsequently, cytochrome *c* and VDAC were analyzed by immunoblotting.

2.8. Caspase-3 and -9 activity assay

Caspase activity was measured using colorimetric assay kits according to the manufacturer of caspase-3 (Clontech, Palo Alto, CA) and caspase-9 (R&D Systems). Briefly, 2×10^6 cells were collected and re-suspended in the lysis buffer and the lysate was centrifuged at 10,000 *g* for 5 min to precipitate cellular debris. The supernatant was then reacted with 5 ng DEVD-*p*-nitroanaline (pNA) or LEHD-pNA, which were the substrates of caspase-3 or -9, at 37° for 2 hr. Absorbance was read at 405 nm wavelength light in a microplate reader (EG&G Berthold, MicroLumat Plus). Control experiments were performed in the present of growth medium.

2.9. Transfection of HUVEC with dominant negative Akt constructs

HUVECs were transfected with 10 μg c-*myc*-tagged dnAkt in pUSEamp (Upstate Biotechnology) and control vector pUSEamp using Effectene (Qiagen). After 24 hr of recovery by culture in complete growth medium, cells transfected with dnAkt or control vector were changed to growth factor deprivation in the presence of Andro (50 μM) for 18 hr. Cells were collected, fixed with 4% paraformaldehyde, permeablized with 0.1% Triton X-100, and labeled with TUNEL stain following the standard procedures (Roche). Separate analysis of c-*myc*-positive cells in both transfected cell populations was performed by fluorescence-activated cell sorter analysis using a primary anti-c-*myc*-antibody (1:100) (Upstate Biotechnology) and a secondary *R*-phycoerythrin-conjugated anti-rabbit antibody (1:100) (Southern Biotech). Apoptosis was calculated as percent c-*myc*-positive apoptotic cells from all c-*myc*-positive cells in the vector control and dnAkt-transfected cell populations.

3. Results

3.1. Andrographolide protects HUVEC from growth factor-deprived apoptosis

ECs undergo apoptosis when deprived of growth factors [25]. To investigate whether Andro protects HUVECs from

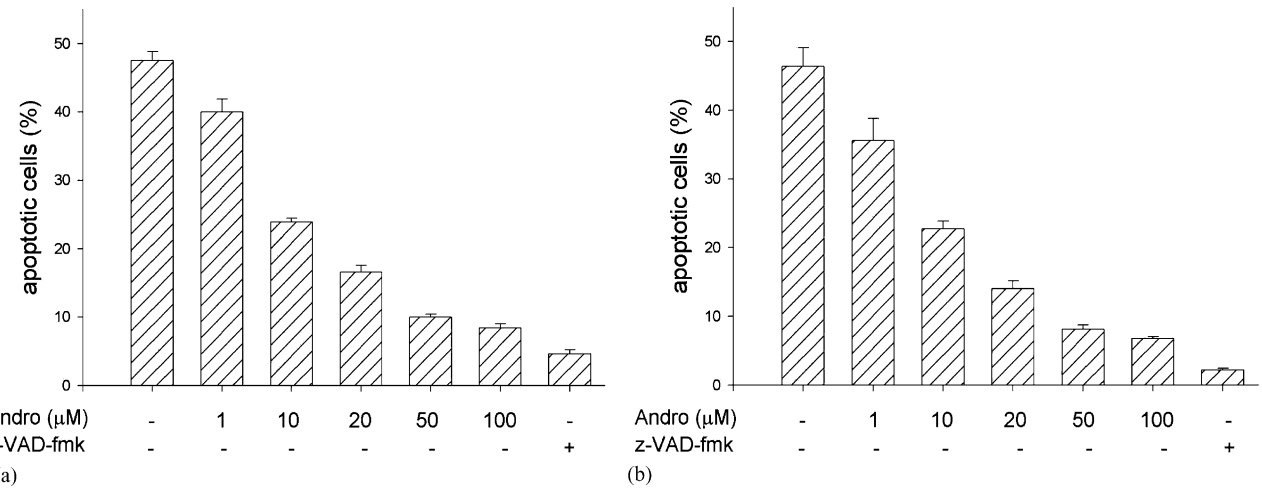

Fig. 1. Andrographolide (Andro) suppresses growth factor (GF) deprivation-induced apoptosis. HUVECs were exposed to GF deprivation M199 with 0–100 μM Andro or 50 μM z-VAD-fmk for 18 hr. The percentage of apoptotic cell death was determined by flow cytometry with (a) TUNEL and (b) propidium iodide (PI). Data represent mean ± SD (N = 5).

serum deprivation-induced cell death in our system, HUVECs were cultured in GF free M199 with Andro (0–100 μM), and DNA fragmentation was quantified using PI or TUNEL stain as a measure of apoptosis. GF deprivation led to a significant increase in the hypodiploid group to ∼46% of control cells grown in the presence of GF. The GF-free induced cell death was abolished by administration of z-VAD-fmk, an inhibitor of caspases. This indicated that the serum deprivation-induced EC death was caspase-dependent apoptosis. The addition of Andro (1–100 μM) also reversed the GF deprivation-induced apoptosis in a concentration-dependent manner (Fig. 1) with a maximal suppression at 50–100 μM, and with a ∼80% inhibition of apoptosis at 100 μM Andro.

3.2. Andrographolide inhibits caspase-3/-9 activity

Activation of the caspase family of cysteine proteases was for the execution of apoptosis. One principal mechanism of caspase activation requires the release of cytochrome *c* from mitochondria, which associates with Apaf-1 to form the complex known as the apoptosome

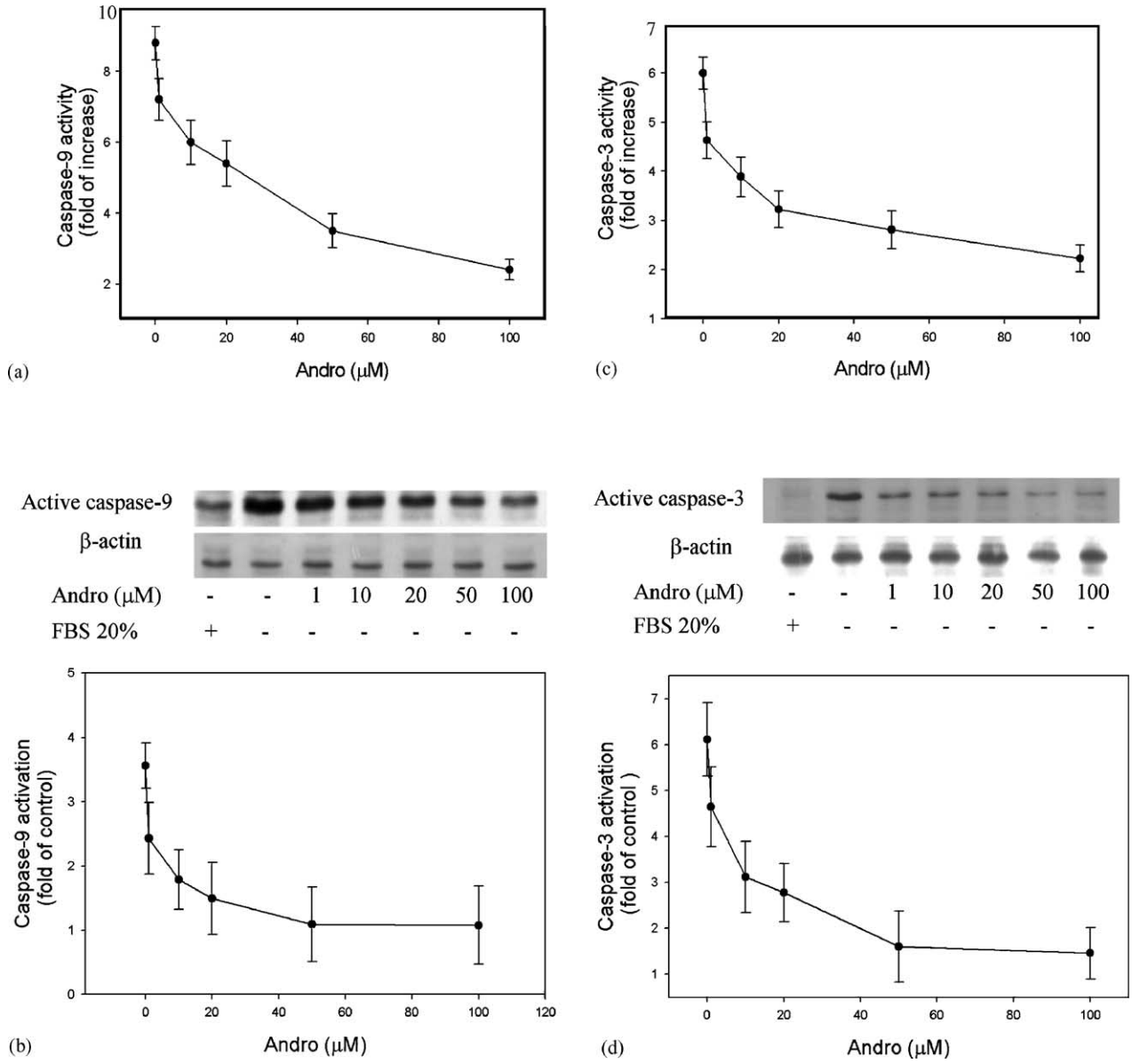

Fig. 2. Andro inhibits GF deprivation-induced caspase-3/-9 activation. HUVECs were exposed to GF deprivation in the absence or presence of Andro (1–100 μM) for 18 hr. (a) Caspase-9 and (c) caspase-3 activity in lysates from Andro-treated and control cells were measured by the amount of chromogenic assay using synthetic substrate LEHD-pNA or DEVD-pNA, respectively, and were expressed as *N*-fold increase over the basal level in control cells. Control experiments were performed in the present of GF. Data represent mean ± SD (N = 5). Lysates from (0–100 μM) Andro-treated and control cells were separated on 14% SDS–PAGE and transferred into PVDF membrane. Protein bands were visualized by Western blot using antibodies for activated caspase-9 (b) and caspase-3 (d), blots were then subjected to densitometric analysis. The quantitative data represent mean ± SD (N = 4).

[26]. The complex then recruits and activates caspase-9, which in turn activates caspases such as caspase-3 [27]. We therefore examined the effect of Andro on the activation of caspase-9 and -3 by colorimetric assay. Andro suppressed the activity of both caspase-9 and -3 after 18 hr of GF withdrawal in a concentration-dependent manner (Fig. 2a and c). We also determined the effect of Andro on the proteolytic processing of pro-caspase-3 and -9. Andro markedly reduced the active form of caspase-3 (23 kDa) and caspase-9 (37 kDa) (Fig. 2b and d).

3.3. Andrographolide suppress the mitochondrial pathway of apoptosis

The mitochondrial death pathway is upstream of caspase activation and can be induced by GF depletion [28]. Dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$) and the release of cytochrome *c* into the cytoplasm are features of common apoptotic pathway or mitochondrial death pathway [29,30]. We therefore examined the effect of Andro on $\Delta\psi_m$ and found that the release of cytochrome *c*. $\Delta\psi_m$ was markedly decreased by 45% compared to the control group after 18 hr of GF deprivation. Addition of Andro exhibited a concentration-dependent inhibition of $\Delta\psi_m$ dissipation, with a maximal suppression at 50–100 μM (Fig. 3a). As expected, the release of cytochrome *c* into the cytoplasm was also inhibited by Andro (Fig. 3b). To exclude contamination of the cytosolic fraction with mitochondria, VDAC was examined, but no contamination was detected.

3.4. Akt is essential for Andro-mediated EC survival

Protein kinase Akt is an important survival factor in endothelial cells. Activation of Akt is known to suppress the mitochondrial death pathway [31] or directly regulate the activity of caspases [10]. To assess the involvement of Akt in the protective effect of Andro, cells were starved for 24 hr in M199 containing 1% FBS and treated with 50 μM Andro for the indicated times. A phosphospecific antibody to serine 473 was used to detect the activation of Akt. The results demonstrated that Akt was activated after 5–15 min administration of 50 μM Andro (Fig. 4a). In addition, BAD, a down-stream target of Akt, was also phosphorylated rapidly (Fig. 4b). To identify whether Akt is responsible for the protective effect of Andro, two structurally unrelated inhibitors of phosphatidylinositol 3-kinase, LY-294002 and wortmannin, were used. Both LY-294002 and wortmannin reversed the inhibitory effect of Andro on cell apoptosis, 50 nM wortmannin and 20 μM LY-294002 almost abrogated the anti-apoptotic effect of Andro (Fig. 5a and b). Moreover, we also transfected ECs with a c-*myc*-tagged dnAkt in pUSE to suppress the activity of endogenous Akt, and then exposed the cells to GF deprivation in the presence of 50 μM Andro. Successful transfection of cells with control vector pUSE or dnAkt was determined by anti-c-*myc* and *R*-phycoerythrin-conjugated anti-rabbit IgG antibodies. The apoptotic cells were identified by TUNEL stain and the percentage was calculated by flow cytometry from the cells which were c-*myc* positive. The results show that the protective effect of Andro (50 μM) was abolished by over-expression of dnAkt (Fig. 5c). These experiments demonstrate that Andro activates the PI3K-Akt pathway, which provides the protective effect.

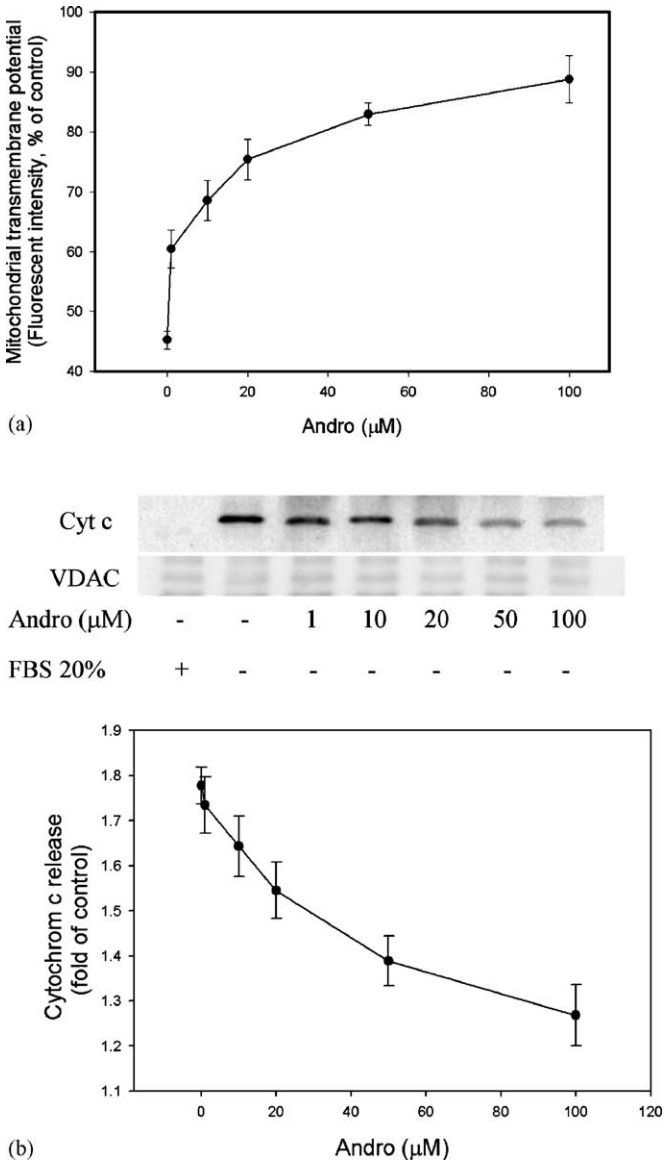

Fig. 3. Andro suppresses GF deprivation-induced mitochondrial apoptosis. HUVECs were exposed to GF deprivation in the absence or presence of Andro (1–100 μM) for 18 hr. (a) Cells were then labeled with $DiOC_6$ (40 nM) at 37° at the end of 15 min incubation and subsequently analyzed by flow cytometry. (b) Lysates from (0–100 μM) Andro-treated and control cells were separated on 14% SDS–PAGE and transferred onto PVDF membrane. Protein bands were visualized by Western blot using antibodies for cytochrome *c*; blots were then subjected to densitometric analysis. Control experiments were performed in the present of GF. Data represent mean ± SD (N = 4).

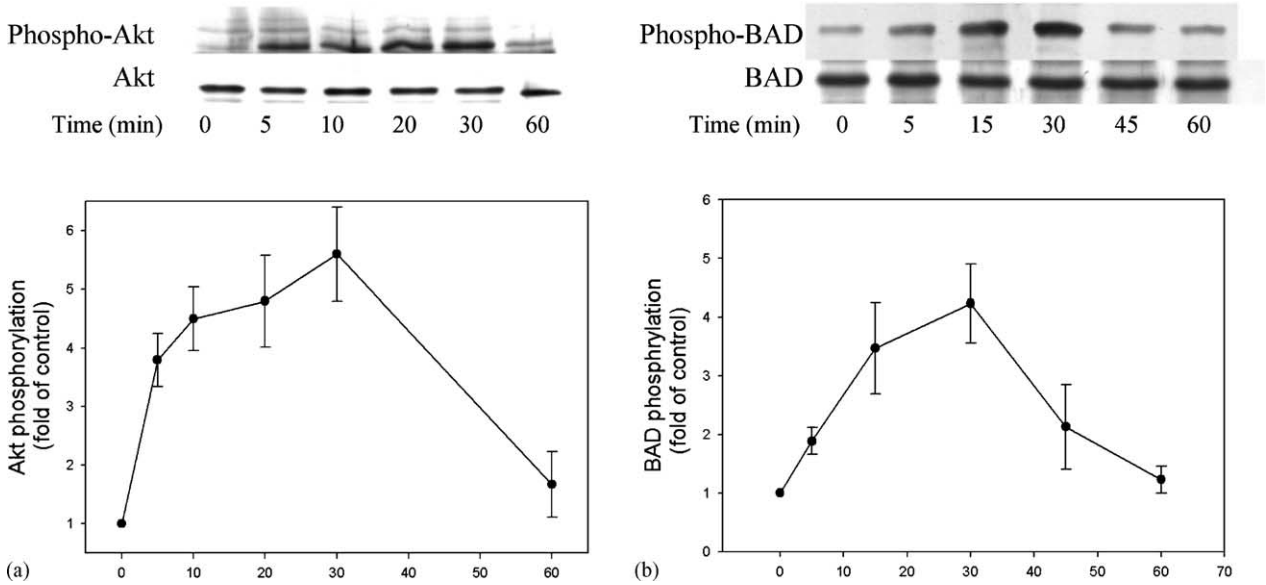

Fig. 4. Andro induces Akt phosphrylation in HUVECs. HUVECs were starved for 24 hr in medium containing 1% FBS and treated with Andro (50 μM) for the indicated times. Cell lysates were subjected to 10% SDS–PAGE and Western blot analysis using antibodies against phosphoserine 473 of Akt (a) or phosphoserine 136 of BAD (b). Equal protein loading was performed with Akt and BAD antibodies. Blots were then subjected to densitometric analysis. Quantitative data represent mean ± SD (N = 4).

3.5. ERK1/2, NOS and NFκB were not involved in Andro-mediated EC survival

ERK1/2 activity has been reported to rescue certain types of cells from apoptosis. We therefore analyzed the involvement of this kinase activity in the protective effect of Andro. As shown in Fig. 6a, ERK1/2 did not activate after administration of Andro as compared to control group. The ERK inhibitor, PD98059 (20 μM) did not reverse the protective effect of Andro (Fig. 6c). This indicated that ERK1/2 activity was not the mediator of Andro.

Another survival factor, NFκB, has been reported to be regulated by Akt [18]. IκB degradation, one characteristic of NFκB activation, was not seen after administration of Andro (Fig 6b). It therefore appears that NFκB also was not involved in the protective effect of Andro.

Recent studies provide evidence that Akt stimulates endothelial nitric oxide synthase (eNOS) and thereby activates the enzyme [16]. However, the NOS inhibitor N^G-Nitro-L-arginine (2.5 mM) did not reduce the anti-apoptotic effect of Andro (Fig. 6d). Furthermore, the nitrate concentration was not altered after administration of 50 μM Andro (data not show), suggesting that the anti-apoptotic effect of Andro is independent of the generation of nitric oxide.

4. Discussion

Endothelial cells play a key role in the maintenance of vascular homeostasis, including regulation of the permeability of plasma lipoproteins, adhesion of leukocytes, and release of growth factors and vascular regulators [32]. Once ECs are impaired, the pathogenesis of atherosclerosis is induced [33]. In this study, we first demonstrated that Andro, a Chinese herbal remedy, promotes endothelial cell survival and protects against apoptosis. Andro suppresses the mitochondrial pathway of apoptosis by inhibiting cytochrome *c* release and $\Delta\psi_m$ dissipation as well as the subsequent activation of the caspase-9/-3 cascades. We identified the protein kinase Akt, a survival factor in a number of experimental systems, as the mediator upstream of the mitochondrium. Similar to growth factors such as vascular endothelial growth factor [8] and oestrogens [9], Andro activates Akt via the PI3K-dependent pathway to protect against apoptosis in HUVECs. Additional anti-atherogenesis activity also has been reported, i.e. anti-oxidative and inhibition of Mac-1 expression [23]. This compound thus may be a promising therapeutic agent against atherosclerosis or other vascular disorders.

Andro has been reported to protect hepatocytes from carbon tetrachloride-induced cytotoxicity *in vivo* [24]. Although the type of cell death was not identified in that report, previous evidence has suggested that apoptosis may play an important role in carbon tetrachloride-induced cell death [34]. Our results suggest that the anti-apoptotic effect of Andro may be contributing to the protective effect *in vivo*, at least in part.

Mitochondrial release of cytochrome *c* is a crucial pro-apoptotic signal in many forms of apoptosis. Once the cytochrome *c* is released into the cytosol, it associates with Apaf-1 and procaspase-9 to form the apoptosome [26]. The

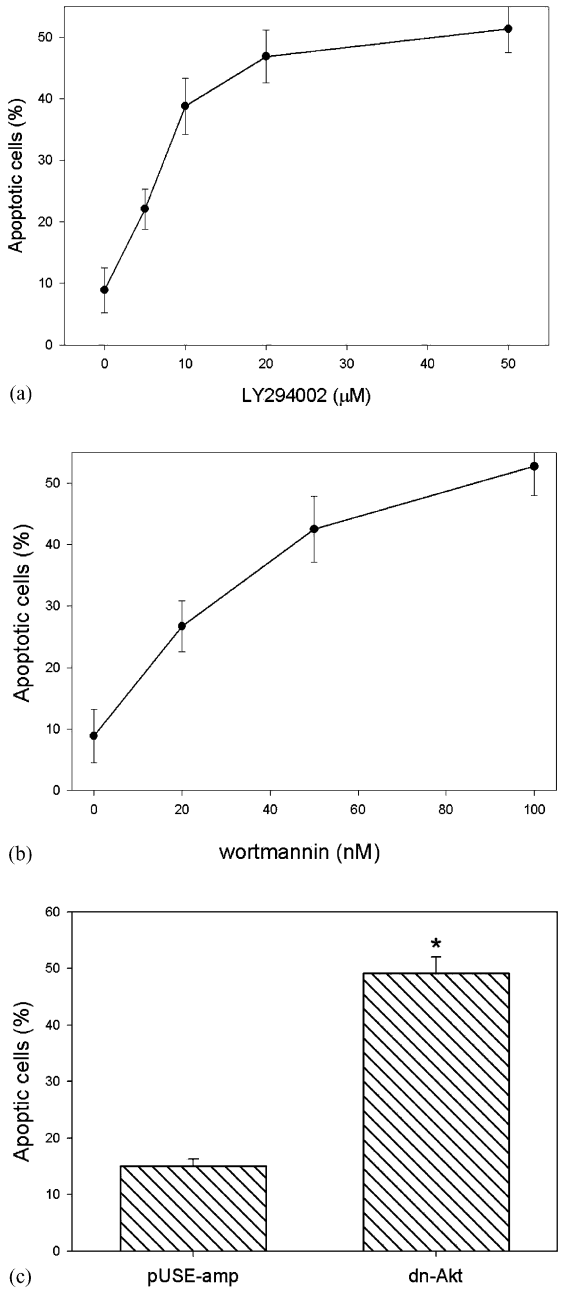

Fig. 5. Akt mediates the survival signal induced by Andro. HUVECs were pre-incubated for 30 min with (a) LY-294002 (0–50 μM) or (b) wortmannin (0–100 nM) and then exposed to GF deprivation in the presence of 50 μM Andro for 18 hr. The percentage of apoptotic cells was determined by flow cytometry with PI. (c) HUVECs were transfected with the expression vector pUSEamp encoding a c-*myc*-tagged dominant negative form of Akt (dnAkt) or with control plasmid. Cells were allowed to recover for 24 hr in complete medium and then exposed to GF deprivation in the presence of 50 μM Andro for 18 hr. Cells were assessed by flow cytometry with anti-c-*myc* antibodies and TUNEL stain. The percentage of apoptotic cells (TUNEL positive) was calculated by dividing the number of c-*myc*-positive floaters by the total number of c-*myc*-positive cells. Data represent mean ± SD (N = 5).

result is activation of caspase-9, which then processes and activates other caspases to orchestrate the biochemical execution of cells. We showed that Andro induces phosphorylation of the pro-apoptotic protein BAD, an important substrate of Akt [19]. This phosphorylation is known to promote the association of BAD with 14-3-3 and its dissociation from BCL-X_L, which is then free to suppress mitochondrial apoptosis. We demonstrated that Andro protects HUVECs from cytochrome *c* release to the cytosol and dissipation of the mitochondrial potential ($\Delta\psi_m$). Although we have not provided evidence for a linkage between BAD phosphorylation and the mitochondrial pathway in our results, we speculate that the anti-apoptotic effect of Andro is associated with the phosphorylation of BAD.

Akt activity is known to rescue HUVEC from apoptosis [35,36]. Our results strongly indicated that Andro activates the protein kinase Akt. Furthermore, the PI3K inhibitors, wortmannin, LY-294002, and transfection of dominant negative Akt all abolished the protective effect of Andro (Fig. 5). This indicates that Akt activation was the necessary survival signal of Andro in HUVECs. In addition, activation of Akt has been reported to regulate the mitochondrial death pathway [31] and caspase-9 activity [10]. It seems that the Andro-mediated inhibition of the mitochondrial pathway and caspase-3/-9 activity was through activation of the PI3K-Akt pathway. Many cytokines and growth hormones have been reported to activate protein kinase Akt [8,9,35,36]. Most of these factors act on their receptor and subsequently activate Akt. Interestingly, the process of Andro-mediated Akt activation is rapid (Fig. 4). It appears that the Akt activation by Andro is transcription-independent and it may act on a receptor or on PI3K directly. However, to understand the detailed molecular action and therapeutic effect *in vivo* will require further research.

ROS have been assumed to play a key role in cell apoptosis. Previous research has demonstrated that Andro has anti-oxidative activity [23] and this effect may contribute to the anti-apoptotic action. However, hydrogen peroxide also has been reported to be an activator of Akt [37,38]. It thus seems that the anti-oxidative activity was not responsible for the Akt activation. In addition, our results demonstrate that the Akt activity was essential for the protective effect of Andro. We suggest that the anti-oxidative potential of Andro is not involved in, or plays only a minor role in protection of the cell from apoptosis.

In our results, the down stream targets of Akt, NOS [20] and NFκB, were not activated by Andro. This may be due to the absence of other required conditions for activation of NOS or NFκB, or perhaps another action of Andro that inhibits these two factors. We are still investigating these relationships.

To summarize, we have demonstrated the anti-apoptotic effect of Andro and its molecular mechanism. Andro protects HUVECs from GF deprivation-induced apoptosis via enhancement of PI3K-Akt activity. This molecular action suggests that Andro may prove to be useful as a research tool or in clinical applications.

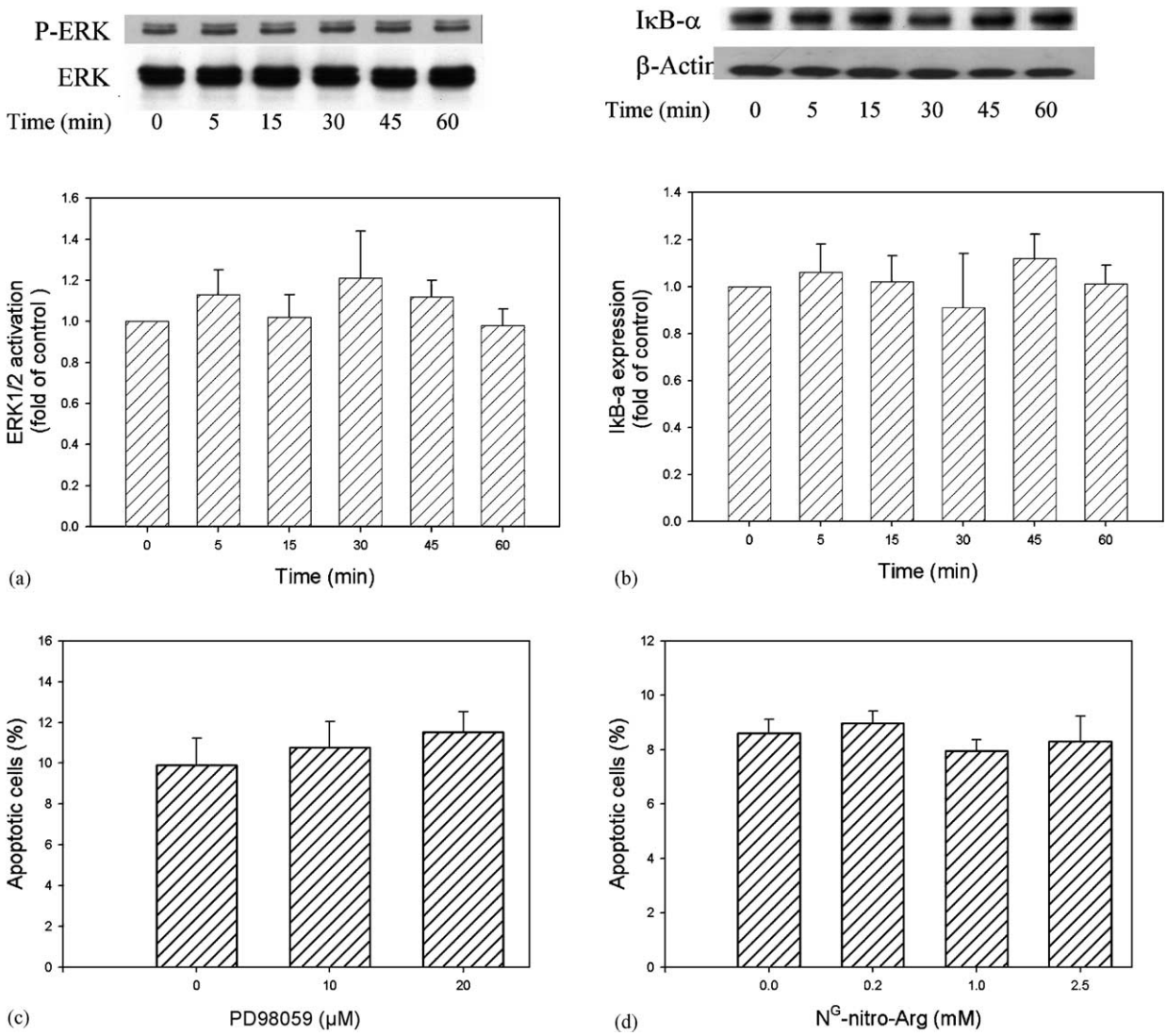

Fig. 6. Effect of Andro on ERK1/2, NOS and NFκB in HUVECs. HUVECs were starved for 18 hr in medium containing 1% FBS and treated with Andro (50 μM) for indicated times. Cell lysates were subjected to 10% SDS–PAGE and Western blot analysis using antibodies against phospho-ERK1/2 (a) or IκB-α (b). Equal protein loading was performed with ERK1/2 and β-actin antibodies. Blots were then subjected to densitometric analysis. Quantitative data represent mean ± SD (N = 4). HUVECs were pre-incubated for 30 min with (c) PD98059 (0–20 μM) or (d) N^G-nitro-arginine (0–2.5 mM) and then exposed to GF deprivation in the presence of 50 μM Andro for 18 hr. The percentage of apoptotic cells was determined by flow cytometry with PI. Quantitative data represent mean ± SD (N = 5).

Acknowledgments

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