Upregulation of DAPK contributes to homocysteine-induced endothelial apoptosis via the modulation of Bcl2/Bax and activation of caspase 3

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Abstract. Hyperhomocysteinemia is characterized by an abnormally high level of homocysteine (Hcy) in the blood and is associated with cardiovascular diseases such as atherosclerosis. Endothelial dysfunction may lead to the pro-atherogenic effects associated with hyperhomocysteinemia. Endothelial dysfunction induced by Hcy has been previously investigated; however, the underlying molecular mechanism remains to be fully elucidated. The present study investigated whether death-associated protein kinase (DAPK) is involved in Hcy-induced apoptosis in human umbilical vein endothelial cells (HUVECs). It was determined that Hcy treatment upregulated the mRNA and protein expression levels of DAPK in HUVECs. Additionally, it was identified that the knockdown of DAPK using small interfering RNA may attenuate the Hcy-induced apoptosis and dissipation of mitochondrial membrane potential. DAPK inhibition may also reverse the effect of Hcy by the upregulation of B cell leukemia/lymphoma 2 (Bcl2) and poly ADP-ribose polymerase, and the downregulation of Bcl2-associated X protein (Bax) and of caspase 3. In conclusion, the present study demonstrated that DAPK contributed to the Hcy-induced endothelial apoptosis via modulation of Bcl2/Bax expression levels and activation of caspase 3.

Introduction

Hyperhomocysteinemia, defined as elevated levels of homocysteine (Hcy) in blood plasma total is identified as an independent risk factor for the development of atherosclerosis (1-3). Previous studies have determined that the elevation of Hcy levels in the blood plasma may lead to endothelial dysfunction (4,5), which is identified as an early event in the pathogenesis of atherosclerosis (6,7). A previous study reported that the apoptosis of endothelial cells contributed to endothelial dysfunction and destabilization of atherosclerotic plaques and thrombosis (8). Apoptosis or programmed cell death differs from necrosis in that it is an active process of cell suicide. In the vasculature, misdirected control of apoptosis in endothelial cells may lead to pathological conditions including inflammation, clotting and recruitment of smooth muscle cells. A previous study demonstrated that Hcy may activate a mitochondrial pathway, which may lead to reduction of the mitochondrial membrane potential ($\Delta\psi m$) and induce the apoptosis of endothelial cells and result in cardiac dysfunction *in vitro* (9).

Death-associated protein kinase (DAPK), is an established mediator of programmed cell death (10). It has previously been observed to upregulate expression levels in atherosclerotic lesions (11). The upregulation of DAPK was observed to increase cell turnover and arterial wall instability, which increased susceptibility to low-density lipoprotein absorption (12). DAPK may function as a tumor suppressor due to its ability to promote apoptosis and autophagy (13,14), suppress cellular transformation (15) and inhibit metastasis (16,17). Additionally, DAPK may be activated by various stimuli, including tumor necrosis factor (TNF-α), interferon-γ and p53; therefore, acts as a converging point for apoptotic signaling (17-19). DAPK is upstream of the caspases, with the exception of caspase 8, and may induce caspase-independent cell death or autophagy. Previous studies have determined that DAPK contributed to shear stress-induced endothelial apoptosis (20,21). However, the importance of DAPK in Hcy-induced apoptosis in endothelial cells remains to be fully elucidated.

The present study investigated the function of DAPK in Hcy-induced apoptosis in endothelial cells. It was determined that DAPK may contribute to the modulation of the mitochondrial pathway in Hcy-induced apoptosis in human umbilical vein endothelial cells (HUVECs).

Materials and methods

Materials. D,L-Hcy, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and rhodamine 123 (Rh123),

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a mitochondrial-specific fluorescent dye, were purchased from Sigma-Aldrich, Merck Millipore (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Dimethyl sulfoxide (DMSO) was purchased from Shenggong Biology Engineering Technology Service, Ltd. (Shanghai, China). The Bicinchoninic Acid (BCA) protein assay kit and Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit were purchased from KeyGen Biotech Co., Ltd. (Nanjing, China). Polyclonal antibodies against β-actin (cat. no. AA128; 1:1,000) and horseradish peroxidase-conjugated secondary antibodies (goat-anti rabbit; cat. no. A0208; 1:1,000; goat-anti mouse; cat. no. A0216; 1:2,000) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The DAPK antibody (cat. no. ab109382; 1:1,000) was obtained from Abcam (Cambridge, UK). Polyclonal antibodies against B cell leukemia/lymphoma 2 (Bcl2; cat. no. 2876; 1:1,000), Bcl2-associated X protein (Bax; cat. no. 2772; 1:1,000), caspase 3 (cat. no. 9662s; 1:1,000) and polyclonal ADP-ribose polymerase (PARP; cat. no. 9542; 1:1,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The western blotting detection kit was purchased from EMD Millipore (Billerica, MA, USA).

Cell culture. HUVECs were obtained from China Center for Type Culture Collection (Wuhan, China) and maintained in DMEM supplemented with 10% FBS at 37˚C in a humidified atmosphere with 5% CO_2. The medium was changed every 2-3 days. Endothelial cells of passage 4-6 in the actively growing condition were used for the subsequent experiments.

Cell viability assay. Cell viability was evaluated by MTT assays as previously described (22). Briefly, non-transfection HUVECs were seeded in 96-well plates ($1.0x10^4$ cells/well) and were treated with Hcy at concentrations of 0, 0.1, 1, 5, 10 and 15 mM. After 24 h, 500 μg/ml MTT reagent was added and the cells were incubated for an additional 4 h. Subsequently, 150 μl DMSO was added to dissolve the formazan crystals. The absorbance was determined using a microplate reader (Thermo Fisher Scientific, Inc.) at 570 nm.

Cell transfection. DAPK small interfering RNA (siRNA) and control siRNA were obtained from GenePharma Co., Ltd. (Shanghai, China). HUVECs were cultured in 6-well plates at a density of $2.0x10^5$ cells/well for 24 h and then transfected with DAPK siRNA (30 nM) or control siRNA (30 nM) in DMEM medium without FBS using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Hoechst 33342 staining for nuclei fragmentation. HUVECs were treated with 0, 5, 10 mM Hcy for 24 h. The cells were fixed with 4% paraformaldehyde for 30 min at room temperature, then stained with Hoechst 33342 (10 μg/ml) at 37˚C for 20 min, followed by two washes with phosphate-buffered saline (PBS). Subsequently, the cells were resuspended with PBS in order to determine alterations in their nuclear morphology under a fluorescence microscope (1X71; Olympus Corporation, Tokyo, Japan).

Apoptosis analysis by flow cytometry. Flow cytometry was used to detect the apoptotic rate of HUVECs using the Annexin V-FITC/ propidium iodide (PI) staining kit. Following exposure to different concentrations of Hcy (0, 5 and 10 mM), cells were collected and washed with PBS and resuspended in binding buffer containing Annexin V-FITC and PI according to the manufacturer's protocol. Subsequent to staining, cells were analyzed using a flow cytometer.

Flow cytometry determination of $\Delta\psi m$. The alterations in $\Delta\psi m$ were investigated using Rh123 and flow cytometry. Cells were seeded in 6-well plates ($2.0x10^5$ cells/well), transfected with siRNA, which downregulated DAPK expression for 48 h and then treated with 10 mM Hcy for an additional 24 h. Subsequently, cells were incubated with the Rh123 (10 μM) at 37˚C in the dark for 20 min. Following filtration through 200-mesh sieve with pore size of 75 μm, the samples were analyzed using a flow cytometer.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were collected to isolate total RNA and remove genomic DNA using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in a sterile, RNase-free environment. Reverse transcription was performed using RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Inc.) at 37˚C for 1 h. The qPCR reaction was performed using Maxima SYBR Green PCR Master mix (Thermo Fisher Scientific, Inc.). The 25 μl final reaction volume was comprised of 12.5 μl Maxima SYBR Green PCR Master mix, 0.3 μM primer and 1 μl cDNA. The thermocycling conditions were as follows: Initial step of 50˚C for 2 min, followed by a second step at 95˚C for 15 min, then 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. Fold changes in target gene expression between treatments and controls were determined using the $2^{-\Delta\Delta Cq}$ method (23), normalizing to GAPDH RNA expression as an internal reference. All results were repeated in six independent experiments and performed in triplicate each time. The following primers for qPCR were used: DAPK forward, ACA CATTGCCCTTCATCTGG and reverse AGTATTGCCGTG CCTGTCTT; GAPDH forward CGCTCTCTGCTCCTCCTG TTC and reverse ATCCGTTGACTCCGACCTTCAC.

Protein analysis. For protein analysis, cells were collected following each experiment and lysed with radioimmunoprecipitation assay buffer and ultrasound on ice. The supernatant fluids were collected following centrifugation at 13,000 x *g* for 5 min at 4˚C. BCA Protein Assay kit was used to determine the protein concentrations. Gel electrophoresis was performed using 10% sodium dodecyl sulphate-polyacrylamide gel and transferred to 0.45 μm polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Chalfont, UK). The membranes were immersed in 5% non-fat milk blocking buffer for 1 h. The membranes were incubated overnight at 4˚C with the primary antibodies (1:1,000), followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000) at room temperature for 1 h and Immobilon Western Chemiluminescent HRP substrate (EMD Millipore). Gel-Pro Analyzer version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to extract quantitative information from the electrophoretic gels.

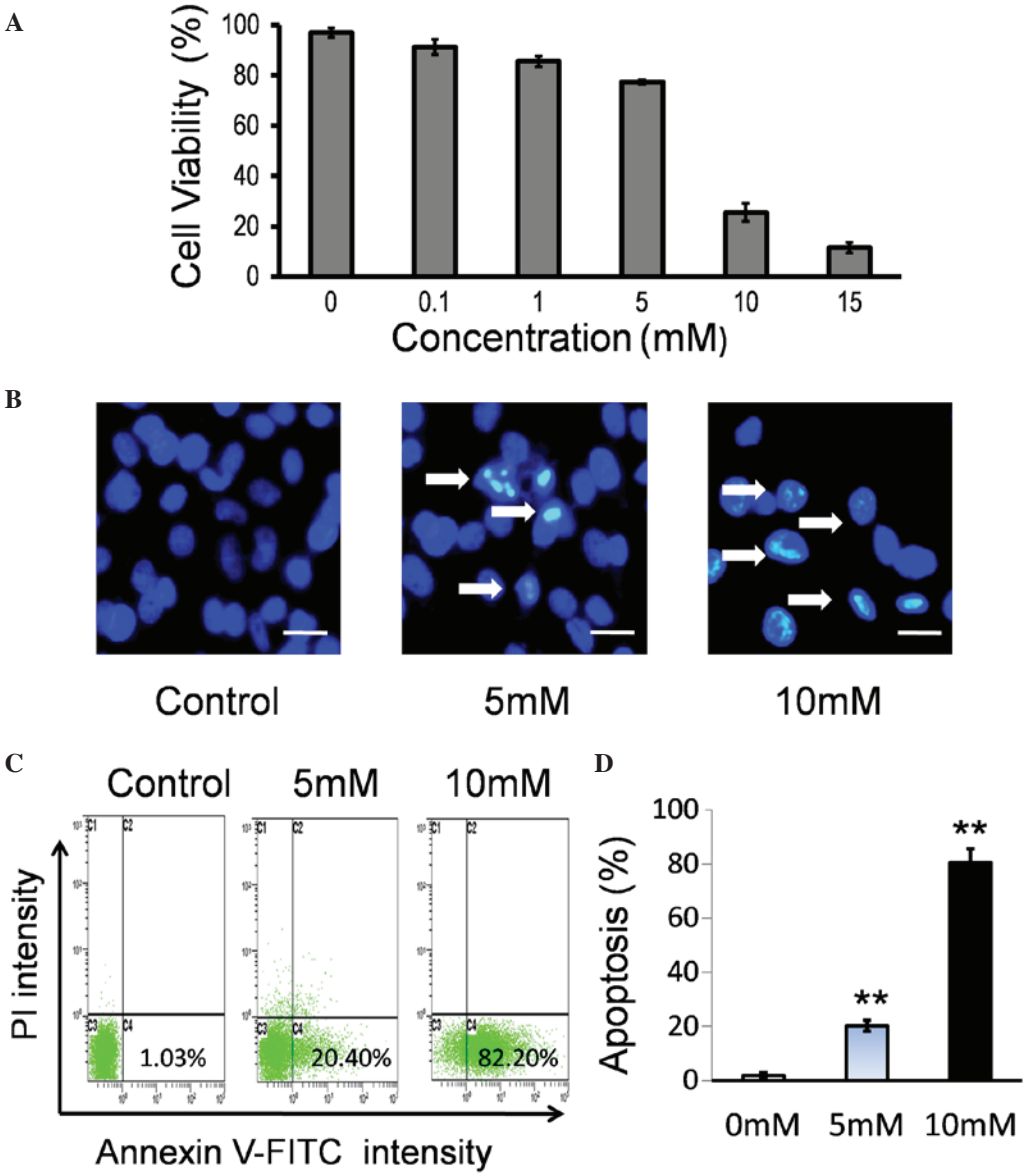

Figure 1. Hcy inhibited the growth and induced the apoptosis of HUVECs. (A) Hcy reduced the viability of HUVECs with increased concentration. (B) HUVECs exhibited apoptotic changes following exposure to Hcy, including condensed chromatin and fragmented nuclei, indicated by arrows. Scale bar, 50 μm. (C) Apoptosis was induced by Hcy in HUVECs as determined by flow cytometric analysis, with the quantification presented in (D). Data are expressed as the mean ± standard deviation of three independent experiments. **P<0.001 vs. the control. Hcy, homocysteine; HUVECs, human umbilical vein endothelial cells; PI, propidium iodide; FITC, fluorescein isothiocyanate.

Statistical analysis. Data are expressed as the mean ± standard deviation from at least three different experiments. Comparisons between groups were performed using one-way analysis of variance followed by Dunnett's test using using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Hcy reduces the survival of HUVECs and induces apoptosis. To examine the effect of Hcy on the viability of endothelial cells, HUVECs were treated with various concentrations (0.1-15 mM) of Hcy. No significant difference was identified between 0.1 mM Hcy and the control group (P>0.05; Fig. 1A). Hcy at 1, 5, 10 and 15 mM significantly inhibited cell viability in a dose-dependent manner compared with the control group (P<0.05; Fig. 1A).

The major features of apoptotic cell death are DNA fragmentation and loss of the asymmetry of the plasma membrane. To test the effect of Hcy on cell viability, morphological changes in the nuclei were observed using Hoechst 33342 staining and fluorescence microscopy. As presented in Fig. 1B, Hcy treatment induced nuclear morphological alterations in the HUVECs, including nuclear shrinkage and DNA fragmentation. Apoptosis induced by Hcy was additionally confirmed by Annexin V-FITC/PI staining. HUVECs were treated with Hcy for 24 h and the percentages of cells undergoing apoptosis/necrosis were determined by flow cytometry. It was identified that Hcy may induce cell apoptosis in a dose-dependent manner (Fig. 1C). A significant increase in early apoptosis of the Hcy 5 mM and 10 mM treatment groups

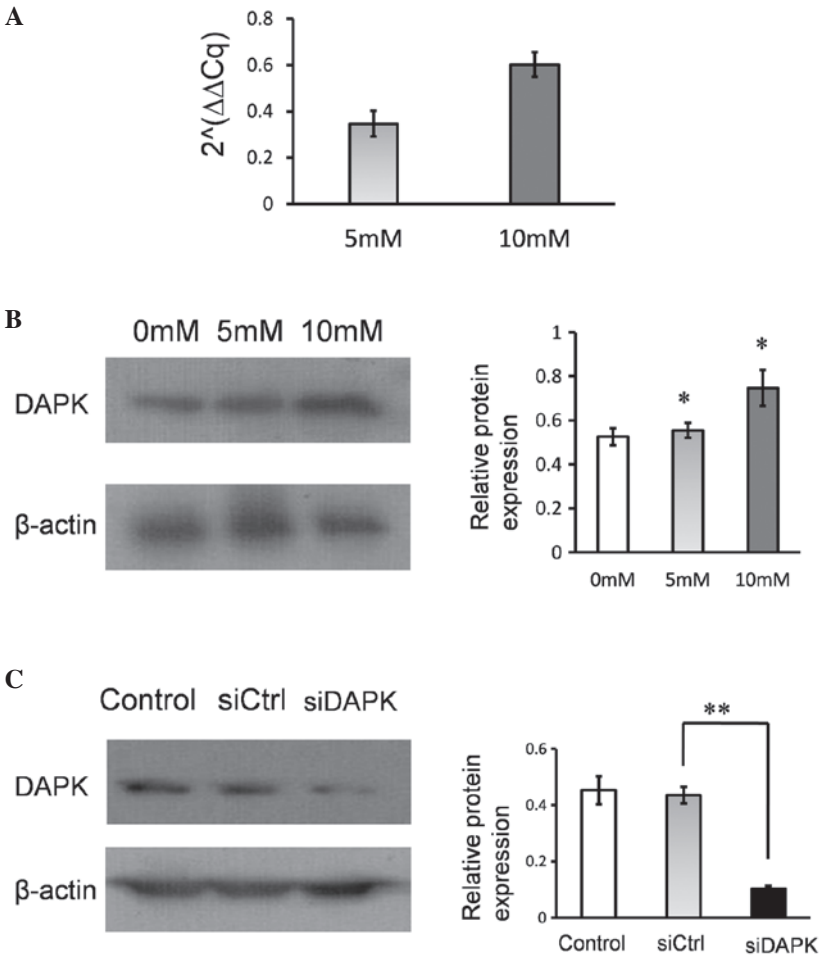

Figure 2. DAPK expression levels in HUVECs following exposure to Hcy and knockdown of DAPK. (A) DAPK mRNA expression levels in HUVECs following exposure to different concentrations of Hcy. (B) Western blot analysis of the DAPK protein expression levels in Hcy-treated HUVECs. *P<0.05 vs. ctrl. (C) Cells were transfected with siCtrl or DAPK-specific siRNA. DAPK knockdown efficiency was determined 48 h subsequent to transfection using western blotting. Data are expressed as the mean ± standard deviation of three independent experiments. **P<0.001 vs. siCtrl. DAPK, death-associated protein kinase; HUVECs, human umbilical vein endothelial cells; Hcy, homocysteine; ctrl, control; siRNA, small interfering RNA.

was observed compared with the control group (P<0.001; Fig. 1D).

Hcy increases DAPK expression in HUVECs. Following treatment with Hcy, the mRNA and protein expression levels of DAPK were determined using RT-qPCR and western blotting. It was identified that the mRNA and protein levels of DAPK were significantly upregulated in response to the 5 mM and 10 mM Hcy treatment groups, when compared with the control (P<0.05; Fig. 2A and B). Hcy treatment increased DAPK mRNA and protein expression levels in HUVECs.

DAPK knockdown reduced the Hcy-induced apoptosis. To determine the effect of DAPK expression on Hcy-induced apoptosis, DAPK expression was inhibited using siRNA. DAPK expression was significantly suppressed in HUVECs compared with the non-specific control siRNA group (P<0.001; Fig. 2C). Following treatment with Hcy, the apoptotic rate of DAPK siRNA-transfected HUVECs was significantly reduced from 88.59 to 51.40%, compared with the control group (transfected with non-specific siRNA) (P<0.05; Fig. 3A and B). These findings provide additional evidence that DAPK is important for the mediation of endothelial apoptosis induced by Hcy. Additionally, cell viability was significantly greater in the siDAPK group compared with the 10 mM Hcy group (P<0.001; Fig. 3C)

DAPK knockdown attenuates the effect of Hcy on $\Delta\psi m$. Hcy endothelial cells was associated with DAPK. In order to determine whether reduction of DAPK expression levels may alleviate Hcy-induced mitochondrial dysfunction, DAPK siRNA-transfected cells were treated with Hcy and the $\Delta\psi m$ was evaluated. Following treatment with Hcy, an increase of $\Delta\psi m$ was observed in the DAPK knockdown group from 22.98 to 53.12% (Fig. 3D). This indicated that DAPK was associated with the Hcy-induced mitochondrial dysfunction.

Knockdown of DAPK attenuates the effect of Hcy on the expression levels of apoptosis regulators. The data indicated that DAPK was involved in Hcy-induced apoptosis and with $\Delta\psi m$ disruption in HUVECs. The Bcl2 protein family, a large family of apoptosis regulating proteins, modulated the mitochondrial pathway investigated in the present study. In order to characterize the function of DAPK in Hcy-induced apoptosis, the impact of reduced DAPK expression levels via siRNA transfection on Bcl2 family proteins was investigated

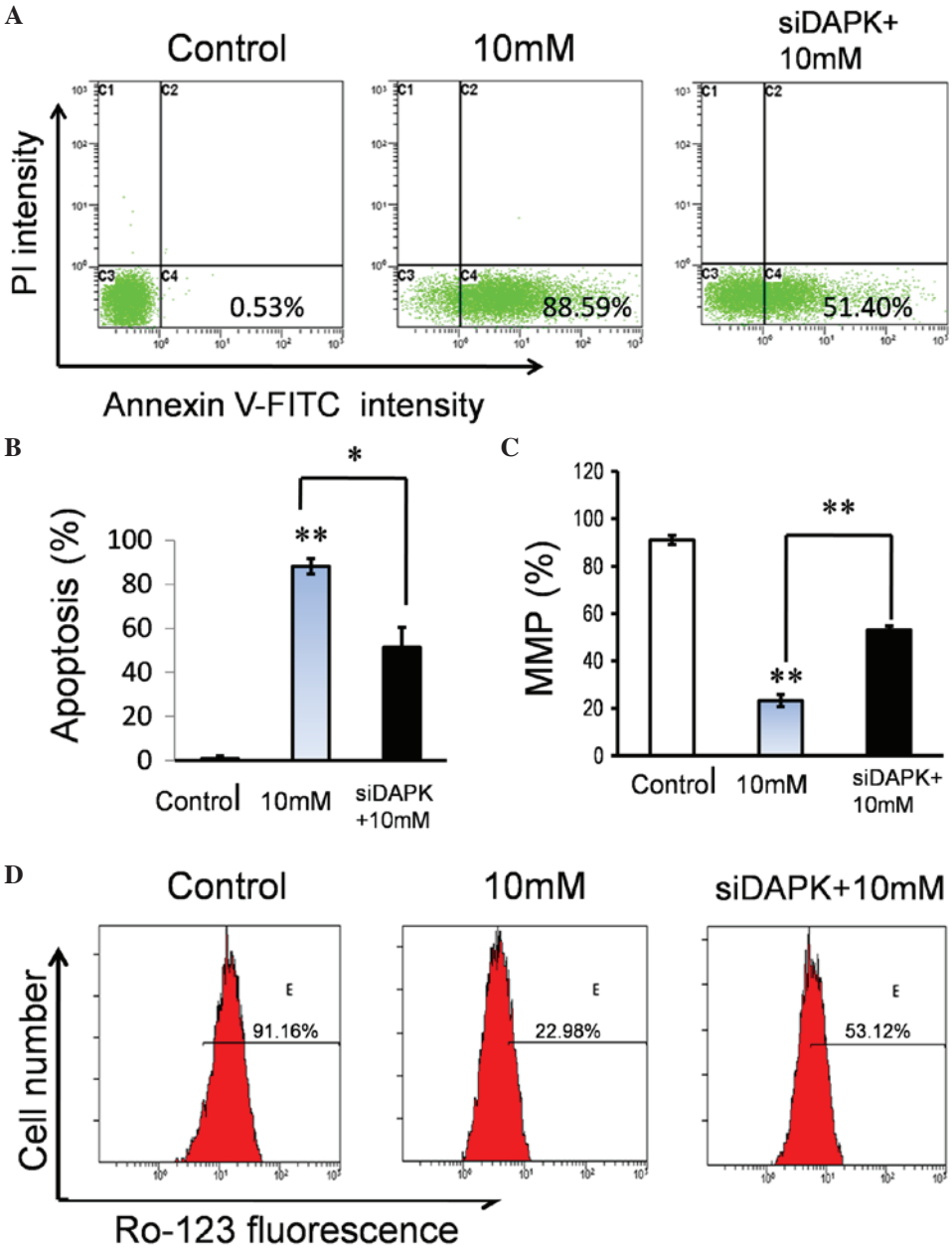

Figure 3. DAPK is required for a Hcy-induced apoptosis and Δψm change. (A) Transfection with siDAPK reduced the apoptosis induced by Hcy. Quantification of the (B) apoptotic rate and (C) Δψm following siDAPK transfection. Data are expressed as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.001 vs. control. (D) Flow cytometric analysis. The values indicate the percentages of rhodamine 123 fluorescence on HUVECs subsequent to treatment. Data shown are representative of three independent experiments. DAPK, death-associated protein kinase; Hcy, homocysteine; Δψm, mitochondrial membrane potential; siDAPK, small interfering DAPK; HUVECs, human umbilical vein endothelial cells; PI, propidium iodide; FITC, fluorescein isothiocyanate.

in HUVECs treated with Hcy using western blot analysis (Fig. 4). As presented in Fig. 4A and C, Hcy treatment significantly reduced the ratio of Bcl2 to Bax (P<0.05), whereas the knockdown of DAPK significantly reversed the effect of Hcy treatment on Bax and Bcl2 levels (P<0.05). In addition, caspase 3 and PARP activation was examined. Hcy treatment resulted in significantly increased caspase 3 cleavage and reduced PARP expression levels in HUVECs (P<0.01; Fig. 4C). Caspase 3 cleavage, characterized by the appearance of 17 and 19 kDa protein band and PARP cleavage characterized by a 89 kDa protein band reduced in cells transfected with siDAPK compared with the cells transfected with non-specific siRNA. Cleaved caspase 3 expression levels were significantly reduced in the siDAPK group compared with the 10 mM Hcy treatment group (P<0.05; Fig. 4C).

Discussion

The present study investigated the importance of DAPK for the possible mechanism that triggers Hcy-induced apoptosis in HUVECs. Initially, it was confirmed that Hcy reduced the viability of HUVECs and induced apoptosis. In addition, it was determined that Hcy upregulated DAPK expression in a dose-dependent manner. Additionally, it was revealed that inhibition of DAPK may attenuate apoptosis and dissipation of the Δψm. The underlying molecular mechanisms were also

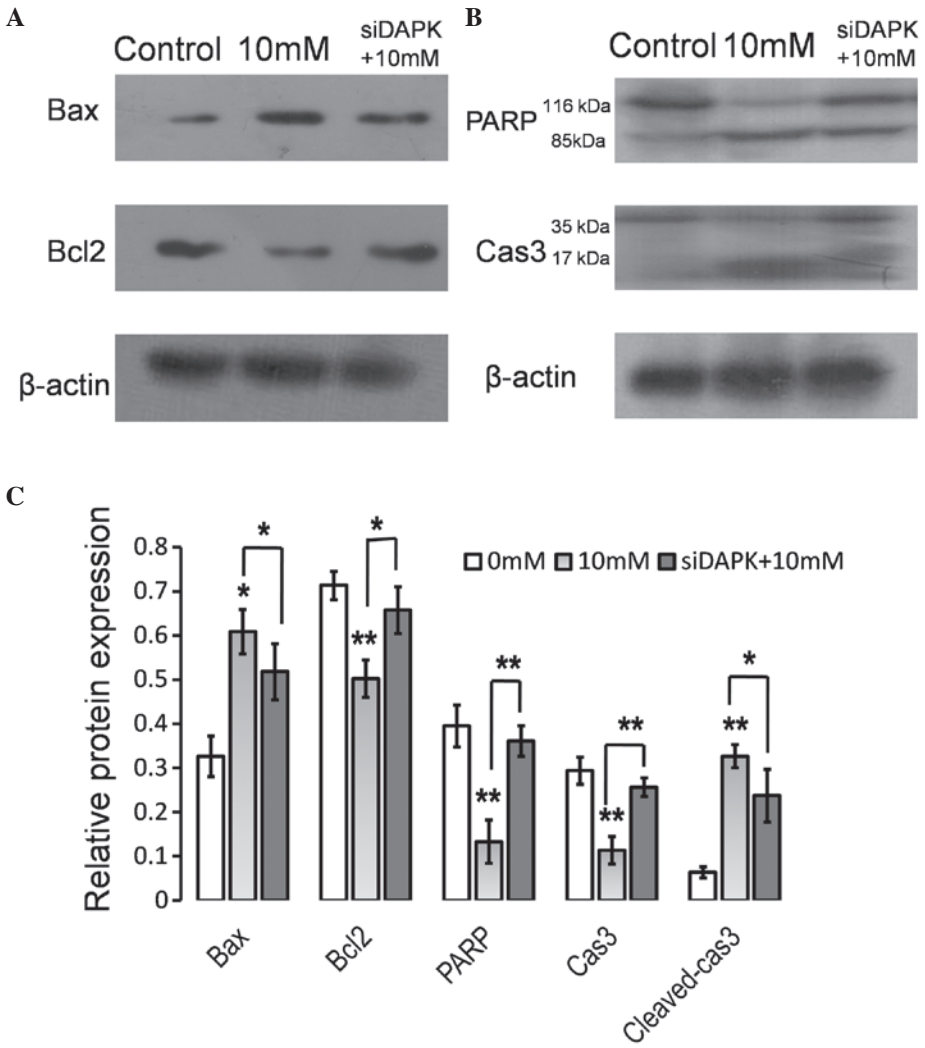

Figure 4. Suppression of DAPK attenuated the effect of Hcy on the expression levels of apoptosis regulators. HUVECs were transfected with DAPK-specific siRNA and 48 h subsequent to transfection, cells were treated with Hcy (10 mM) for 24 h. Whole-cell extracts were prepared and probed for (A) Bcl2, Bax, (B) PARP and Cas3 by western blot analysis. (C) Quantification of the western blotting. Data are expressed as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. control. DAPK, death-associated protein kinase; Hcy, homocysteine; HUVECS, human umbilical vein endothelial cells; siRNA, small interfering RNA; Bcl2, B cell leukemia/lymphoma 2; Bax, Bcl2-associated X protein; PARP, poly ADP-ribose polymerase; Cas3, caspase 3.

investigated and it was determined that DAPK participated in Hcy-induced apoptosis by reducing the ratio of Bcl2/Bax and activation of caspase 3 in HUVECs.

Increased levels of Hcy in blood plasma have been identified as an independent risk factor for the development of atherosclerosis (1-3). In atherosclerosis, increased apoptosis has been established to contribute to prolonged inflammatory response, plaque instability, rupture and thrombus formations (11). Previous studies have also reported apoptosis of endothelial cells associated with Hcy (24-27). The results of the present study are in agreement with this, as it was evident that Hcy reduced cell viability and induced apoptosis in a dose-dependent manner.

DAPK is an important protein kinase for the modulation of apoptotic pathways (28). A previous study identified that DAPK functioned as a positive mediator of apoptosis induced by various stimuli, including TNF-α, interferon-γ and p53 (29). It was previously reported that DAPK activity may be involved in TNF-α-induced apoptotic pathways in bovine aortic endothelial cells (20). To the best of our knowledge, the present study is the first to reported that Hcy treatment increased mRNA and protein DAPK expression levels. This suggested that DAPK may contribute to the apoptotic effect of Hcy in endothelial cells. When inhibition of DAPK was induced, a reduction in Hcy-induced apoptosis was observed in endothelial cells, indicating that DAPK is crucial for the mediation of Hcy-induced apoptosis.

A previous study determined that Δψm loss, an early occurring event, may be directly associated with apoptosis (30). As Δψm is reduced, mitochondrial permeability transition pores are opened and in turn release cytochrome *c* and other pro-apoptotic molecules from the intermembrane space into the cytosol. It has been previously demonstrated that Hcy activates the mitochondrial pathway leading to a reduction in Δψm and apoptosis in cardiac microvascular endothelial cells (26). The current study determined that Hcy treatment resulted in a reduction of Δψm in HUVECs, which is consistent with previous studies (26,31). It is of note, that the reduction of Δψm may be attenuated by the knockdown of DAPK, as it has been identified

to be important for Hcy-induced mitochondrial dysfunction. Therefore, this may be the possible mechanism behind endothelial apoptosis induced by Hcy.

Bcl2 family proteins are involved in the mitochondria-dependent apoptosis pathway, which includes anti-apoptotic proteins and pro-apoptotic proteins such as Bcl2 and Bax (32). The present study demonstrated that following Hcy treatment the protein expression levels of Bax were increased, whereas the expression levels of Bcl2 were reduced. It is of note that downregulation of DAPK expression reversed this response. Caspase activation is one of the processes that signify the onset of apoptosis, and caspase 3 has been considered to be a central component of the proteolytic cascade during apoptosis, as it may cleave various nuclear proteins, including PARP, which may lead to atypical apoptotic DNA fragmentation (33). The present study demonstrated that exposure to Hcy activated caspase 3, whereas the knockdown of DAPK reduced this activation. Therefore, it is possible that Hcy-induced apoptosis in HUVECs is associated with modulation of the Bcl2/Bax ratio and activation of caspase-3 by DAPK.

Acknowledgements

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