

Homocysteine injures vascular endothelial cells by inhibiting mitochondrial activity

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Abstract. The aim of the present study was to investigate the role of homocysteine (Hcy) in the pathogenesis of pulmonary embolism (PE) and the associated molecular mechanisms in human umbilical vein endothelial cells (HUVECs). Hcy contents were detected with high-performance liquid chromatography. Apoptosis was detected by flow cytometry using Annexin-V staining. Cytochrome *c* oxidase (COX) activity was assessed with an enzyme activity assay, and the expression levels of COX 17 were determined by western blot analysis. Intracellular reactive oxygen species levels were measured using a microplate reader with a fluorescence probe. The results demonstrated that, compared with the control group, the serum Hcy levels were significantly elevated in the PE group, suggesting that Hcy may be an indicator for PE. Following treatment with Hcy, the apoptosis rate was markedly elevated in HUVECs. Moreover, Hcy decreased COX activity and downregulated the expression of COX 17 in HUVECs. Furthermore, Hcy increased the ROS levels in these endothelial cells. However, all the above-mentioned physiopathological changes induced by Hcy in HUVECs could be restored by folic acid. In conclusion, the results of the present study demonstrated that Hcy inhibited COX activity, downregulated COX 17 expression, increased intracellular ROS levels and enhanced apoptosis in endothelial cells.

Introduction

Pulmonary embolism (PE) is a frequent clinical syndrome with abnormal pulmonary circulation, which is predominantly caused by the blockage of pulmonary and/or bronchial

arteries by thrombus shedding (1). Due to the sudden disease onset, PE is difficult to treat and usually results in the patient succumbing to the disease (2,3). Homocysteine (Hcy) is an intermediate product of methionine metabolism, and is an independent risk factor for cardiovascular diseases (4,5). Previous studies reported that serum Hcy levels are markedly elevated in patients with PE, which is implicated in the disease pathogenesis (6-8).

Vessel wall injury is one of the important factors for PE, as the pathological changes in endothelial cells may result in the formation of thrombosis (9,10). It has been demonstrated that high concentrations of Hcy induce apoptosis in endothelial cells (11,12), in which mitochondria are involved. Cytochrome *c* oxidase (COX) is a key enzyme in mitochondrial function (13-15). Enhanced COX activity may increase intracellular reactive oxygen species (ROS) levels, resulting in cellular apoptosis (16-21). Based on these reports, it would be of interest to define the association between Hcy, COX, and ROS in apoptosis, specifically in the context of PE pathogenesis.

In the present study, the effects of Hcy on endothelial cells and the associated molecular mechanisms were investigated.

Materials and methods

Patients. A total of 10 patients with PE, 4 males and 6 females, aged 50-67 years (with an average age of 60.4 years), were included in this study. These PE cases were diagnosed based on the clinical manifestations and the results from radiological and laboratory tests, in line with the standard literature (22). Furthermore, a further 30 healthy subjects, 15 males and 15 females, aged 30-88 years (with an average age of 56.5 years), were used as the control group. All subjects were recruited from the Laiwu City People's Hospital (Laiwu, China). Prior written and informed consent was obtained from each patient and the study was approved by the ethics review board of the Laiwu City People's Hospital.

Measurement of Hcy concentration. Blood samples were obtained from the PE patients and control subjects, and the concentration of Hcy was measured. Firstly, the column was washed with 95% methanol for 30 min, followed by 5%

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methanol for 30 min. The column was then equilibrated with the mobile phase, 0.05 M KH_2PO_4 (pH 2.1):acetonitrile (93:7). For fluorescence detection, the excitation wavelength was set as 385 nm, and the emission wavelength was set as 515 nm. A 10 μl sample was injected, with a detection duration of 10 min. The concentration was calculated using the peak area as the response value, and the results were normalized with the total protein concentration in cardiomyocytes.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were obtained from Beijing Yuhengfeng Biotech (Beijing, China). The cells were cultured in Endothelial Cell Medium (Beijing Yuhengfeng Biotech) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in an incubator containing 5% CO_2 . The control group was free from intervention; the folic acid group was treated with 100 $\mu\text{mol/l}$ folic acid; the Hcy group was treated with 1 mM Hcy; and the Hcy + folic acid group was treated with 1 mM Hcy and 100 $\mu\text{mol/l}$ folic acid.

High-performance liquid chromatography (HPLC). Hcy concentration was determined with HPLC. For serum sample preparation, anticoagulant blood (10 ml) was centrifuged at 200 x g at 4°C for 15 min, and the supernatant was used for measurement. For cell sample preparation, the cells were collected and washed with phosphate-buffered saline (PBS). Following the addition of 100 μl 1% sodium dodecyl sulfate (SDS), the cells were lysed with an ultrasonic cell disruptor, and centrifuged at 12,000 x g at 4°C for 10 min, and the supernatant was used for measurement. Prior to the measurements, protein concentrations were determined using a BCA kit (cat. no. BCA1; Sigma-Aldrich, St. Louis, MO, USA). Standard substance concentration series were set as 3.125, 6.25, 12.5, 25, 50, and 100 μM . For HPLC detection, 10 μl tris (2-carboxyethyl) phosphine (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was added into 90 μl protein sample, followed by vortex for 1 min and incubation at 4°C for 30 min. Subsequently, 100 μl methanol was added, and the sample was vortexed for 1 min and centrifuged at 20,000 x g for 10 min. A total of 150 μl supernatant was collected into an EP tube, and 20 μl 1.55 M NaOH, 250 μl borate buffer (containing 0.125 M H_3BO_3 and 4 mM ethylenediaminetetraacetic acid, pH 9.5), and 10 μl SBD-F (10 mg/ml in borate buffer; Sigma-Aldrich) were added to the sample prior to vortex for 1 min and incubation at 60°C for 60 min. Following centrifugation at 10,000 rpm for 10 min, 200 μl supernatant was collected and subjected to HPLC detection.

Flow cytometry. Cellular apoptosis was detected by flow cytometry and Annexin-V staining. HUVECs were first incubated with Hcy at 0, 0.01, 0.1, and 1 mM, respectively, for 24 h. The cells (3×10^6 cells) were then trypsinized, and collected through centrifugation at 200 x g at 4°C for 5 min. Following washing with PBS, the cells were incubated with 100 μl Annexin-V-FLUOS reagent (Roche Molecular Systems, Inc., Branchburg, NJ, USA) supplemented with 2% propidium iodide (PI) at room temperature for 10-15 min, and the fluorescence was detected by flow cytometry (FACScan; BS Biosciences, Franklin Lakes, NJ, USA). The apoptosis rate

was calculated as the percentage of Annexin-V and PI double positive cells out of the total cells. Experiments were repeated in triplicate.

Mitochondrial isolation. Cardiomyocytes (2.5×10^6 cells; purchased from Dashou Biotechnology Company, Chengdu, China), obtained from heart tissues in rats within 3 days of birth, were cultured in T-25 flasks at 37°C for 5 days containing Hepes-buffered Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS. The cells were then digested with trypsin and collected into EP tubes. A total of 1 ml mitochondrial separation medium buffer (containing 0.21 M mannitol, 0.07 M sucrose, 10 mM Tris base and 1 mM EGTA; pH 7.4) was added to re-suspend the cells, and the cell suspension was homogenized 200 times in a Dounce homogenizer (Active Motif, San Diego, CA, USA) on ice. Cell homogenates were transferred into a new 2 ml EP tube, and centrifuged at 600 x g for 5 min at 4°C. The supernatant was collected into a 1.5 ml EP tube, and centrifuged at 1,000 x g for 5 min at 4°C. Subsequently, the supernatant was collected into another 1.5 ml EP tube, and subjected to centrifugation at 7,000 x g for 10 min at 4°C. This time, the supernatant was discarded, and 1 ml mitochondrial separation medium buffer was used to re-suspend the precipitate, followed by centrifugation at 7,000 x g for 10 min at 4°C. The supernatant was then discarded, and the precipitate was re-suspended with 1 ml MSTE. Following centrifugation at 10,000 x g for 10 min at 4°C, the mitochondria were obtained.

COX activity assay. A total of 40 μl 1% SDS was added into the mitochondria in the EP tube on ice. Following vortexing 6 times within 30 min, the mitochondria were centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was collected into a new EP tube and contained the mitochondrial proteins. The protein concentration was determined using the DAC protein assay reagent (Bio-Rad Laboratories, Inc.), and the standard curve was obtained with bovine serum albumin in 1% SDS. COX activity was determined with a COX activity assay kit (cat. no. GMS100014.3.1; Shanghai Genmed Gene Pharmaceutical Technology Co., Ltd., Shanghai, China), according to the manufacturer's protocol.

Intracellular ROS level measurement. The cells (2×10^6 cells) were trypsinized and collected by centrifugation at 300 x g at 4°C for 5 min. An intracellular ROS red fluorescence determination kit (cat. no. GMS10111.1; Shanghai Genmed Gene Pharmaceutical Technology Co., Ltd.) was used to stain the cells. Fluorescence was detected using a microplate reader (iMark; Bio-Rad Laboratories, Inc.), with an excitation wavelength of 540 nm and an emission wavelength of 590 nm. The intracellular ROS levels were expressed as relative fluorescence unit values.

Western blot analysis. Protein expression levels were assessed by western blot analysis. Total proteins were extracted with a lysis buffer (Sigma-Aldrich). The protein concentration was measured with a DAC protein assay reagent. A total of 30 μg protein sample was separated by 12% SDS-PAGE, and then transferred onto the polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with non-fat milk

at room temperature for 1 h. Subsequently, goat anti-rat COX 17 primary polyclonal antibody (1:1,000 dilution; cat. no. sc-27533; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was added to the membrane at incubated at 4°C overnight. The secondary horseradish peroxidase-labeled rabbit anti-goat immunoglobulin G (1:2,000 dilution; cat. no. A0208; Beyotime Institute of Biotechnology, Haimen, China) antibody was then added for incubation at room temperature for 1 h. Expression levels were quantified using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc.). GAPDH was used as the internal reference.

Statistical analysis. Data were expressed as means \pm standard deviation. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for the statistical analyses. A t-test was performed for the group comparison. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Serum Hcy levels were elevated in patients with PE. The serum Hcy levels were determined in patients with PE and healthy controls. The results obtained from the HPLC demonstrated that, compared with the control group, the serum Hcy levels were significantly elevated in the PE group ($P < 0.05$; Fig. 1). These results suggest that the serum Hcy levels may be an indicator for PE.

Hcy induces apoptosis in endothelial cells. The effects of Hcy on apoptosis in endothelial cells were subsequently investigated. HUVECs were first incubated with Hcy at indicated concentrations (0, 0.01, 0.1 and 1 mM) for 24 h, and then the apoptotic rates were detected by flow cytometry. Compared with the control group, treatment with 0.01 mM Hcy marginally increased the apoptosis rate in HUVECs ($P > 0.05$; Fig. 2). Furthermore, at concentrations of 0.1 and 1 mM, Hcy significantly increased the apoptosis rate in HUVECs, compared with the control group ($P < 0.05$; Fig. 2). These results suggest that Hcy may induce apoptosis in HUVECs, which may contribute to the pathogenesis of PE.

Hcy decreases COX activity and inhibits COX 17 expression. To investigate the effects of Hcy on COX activity levels in endothelial cells, HUVECs were first treated with Hcy (0, 0.01, 0.1, and 1 mM) for 24 h, and then COX activity was assessed using the COX activity assay kit. Compared with the control group, Hcy decreased COX activity levels in HUVECs (for 0.01 mM, $P > 0.05$; for 0.1 and 1 mM, $P < 0.05$; Fig. 3A). In addition, the expression levels of COX 17 in these cells were detected by western blot analysis. As shown in Fig. 3B, 0.01 mM Hcy marginally decreased the protein expression levels of COX 17, whereas 0.1 and 1 mM Hcy significantly decreased the expression levels of COX 17 ($P < 0.05$). These results suggest that Hcy could decrease COX activity and inhibit COX 17 expression in endothelial cells.

Hcy increases intracellular ROS levels in endothelial cells. The effects of Hcy on the intracellular ROS levels in endothelial cells were then investigated. HUVECs were incubated with 0, 0.01, 0.1, and 1 mM Hcy for 24 h, and the intracellular

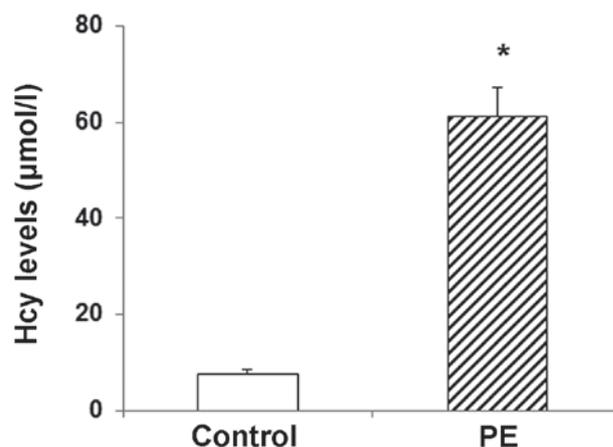


Figure 1. Serum Hcy levels are elevated in patients with PE. The serum Hcy levels were determined in the control and PE groups using high-performance liquid chromatography. * $P < 0.05$, vs. the control group. Hcy, homocysteine; PE, pulmonary embolism.

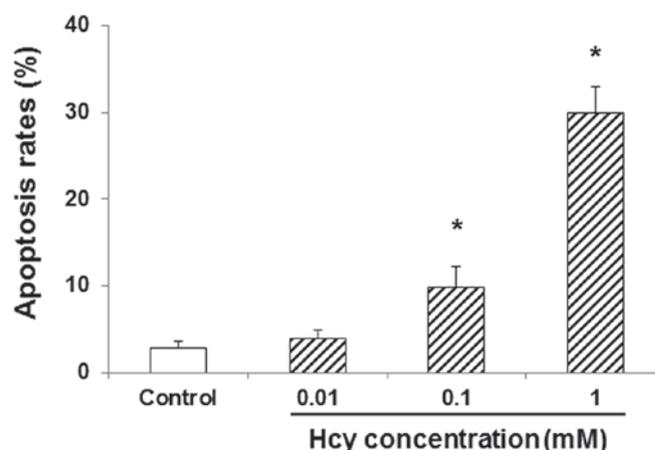


Figure 2. Hcy induces apoptosis in HUVECs. HUVECs were first incubated with Hcy at 0, 0.01, 0.1, and 1 mM for 24 h. The apoptotic levels were then assessed by flow cytometry with Annexin-V staining, and the apoptosis rates were calculated accordingly. * $P < 0.05$, vs. the control group. Hcy, homocysteine; HUVECs, human umbilical vein endothelial cells.

ROS levels were determined. Compared with the control group, 0.01 mM Hcy marginally increased the intracellular ROS levels in HUVECs ($P > 0.05$; Fig. 4). Furthermore, 0.1 and 1 mM Hcy significantly elevated the intracellular ROS levels in these cells ($P < 0.05$; Fig. 4). These results suggest that Hcy could increase the intracellular ROS levels in endothelial cells, which may be associated with its effects on apoptosis in these cells.

Folic acid alleviates the physiopathological changes induced by Hcy. The effects of folic acid on Hcy-induced pathological changes in endothelial cells were then investigated. HUVECs were treated with Hcy and/or folic acid for 24 h, and then the apoptosis levels, the COX activity and COX 17 expression levels, and the intracellular ROS levels were evaluated. Compared with the control group, folic acid treatment alone did not alter the apoptosis rate, COX activity and COX 17 expression, or the intracellular ROS levels in HUVECs ($P > 0.05$; Fig. 5). However, treatment with folic

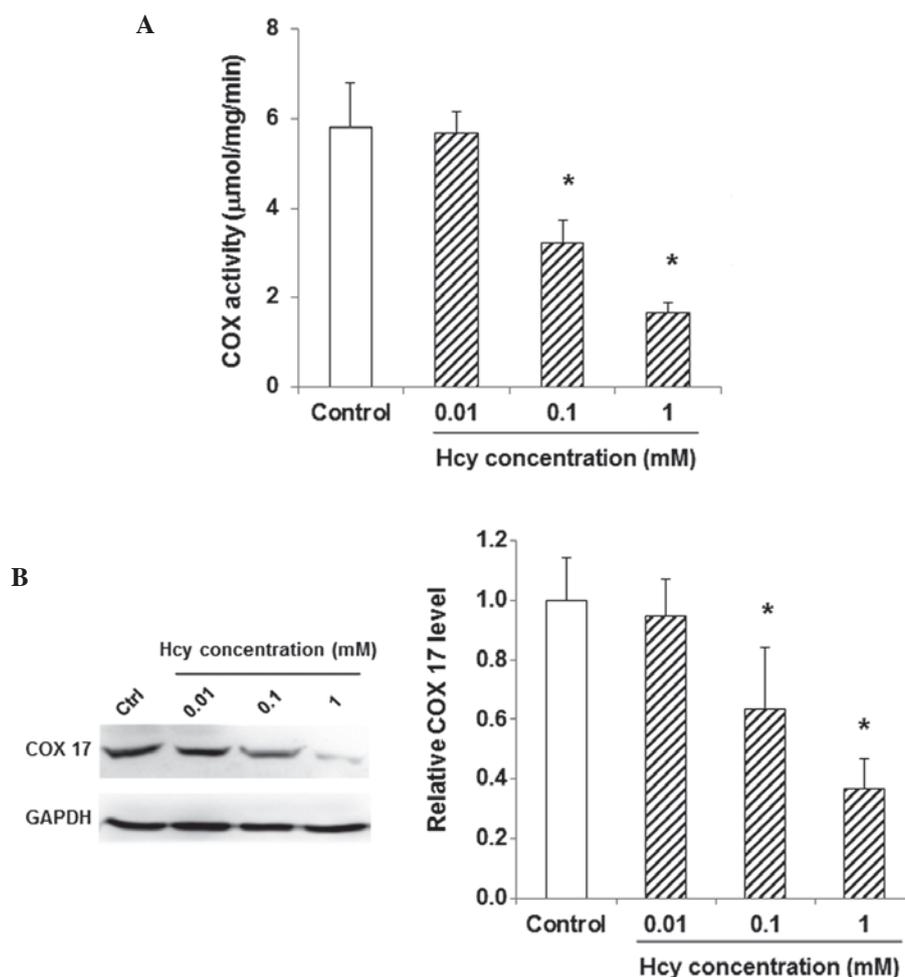


Figure 3. Hcy decreases COX activity and COX 17 expression levels in HUVECs. HUVECs were treated with Hcy at 0, 0.01, 0.1, and 1 mM for 24 h. (A) COX activity was assessed using a COX activity assay kit. (B) The protein expression levels of COX 17 were detected by western blot analysis. * $P < 0.05$, vs. the control group. COX, cytochrome *c* oxidase; Hcy, homocysteine; HUVECs, human umbilical vein endothelial cells.

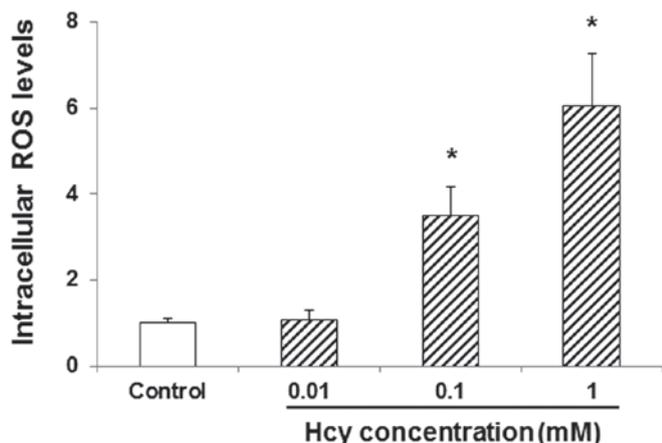


Figure 4. Hcy increases the intracellular ROS levels in HUVECs. HUVECs were incubated with 0, 0.01, 0.1 and 1 mM Hcy for 24 h. The intracellular ROS levels were then determined. * $P < 0.05$, vs. the control group. ROS, reactive oxygen species; Hcy, homocysteine; HUVECs, human umbilical vein endothelial cells.

acid inhibited the effects of Hcy in these cells. Compared with the Hcy group, folic acid treatment significantly

decreased the apoptosis rate, inhibited the Hcy-induced COX activity decline and COX 17 expression downregulation, and decreased intracellular ROS levels in HUVECs ($P < 0.05$; Fig. 5). These results suggest that folic acid could alleviate the physiopathological changes induced by Hcy in endothelial cells.

Discussion

PE is an acute pathological syndrome with a relatively high mortality (2,3). It has been reported that the serum Hcy levels are elevated in patients with PE, which is an independent risk factor for cardiovascular diseases, and is an important factor for the occurrence of PE (4-8). In the present study, the effects of Hcy on PE pathogenesis and the molecular mechanisms underlying these effects were investigated.

COX 17 is an important factor for COX activity, and is responsible for the transition of Cu^{2+} to the COX subunit (23,24). The results of the present study demonstrated that 1 mM Hcy significantly decreased COX activity, and downregulated the expression of COX 17 in HUVECs. Further studies are required in order to elucidate the underlying mechanisms. Decreased COX activity levels may lead to the elevation of

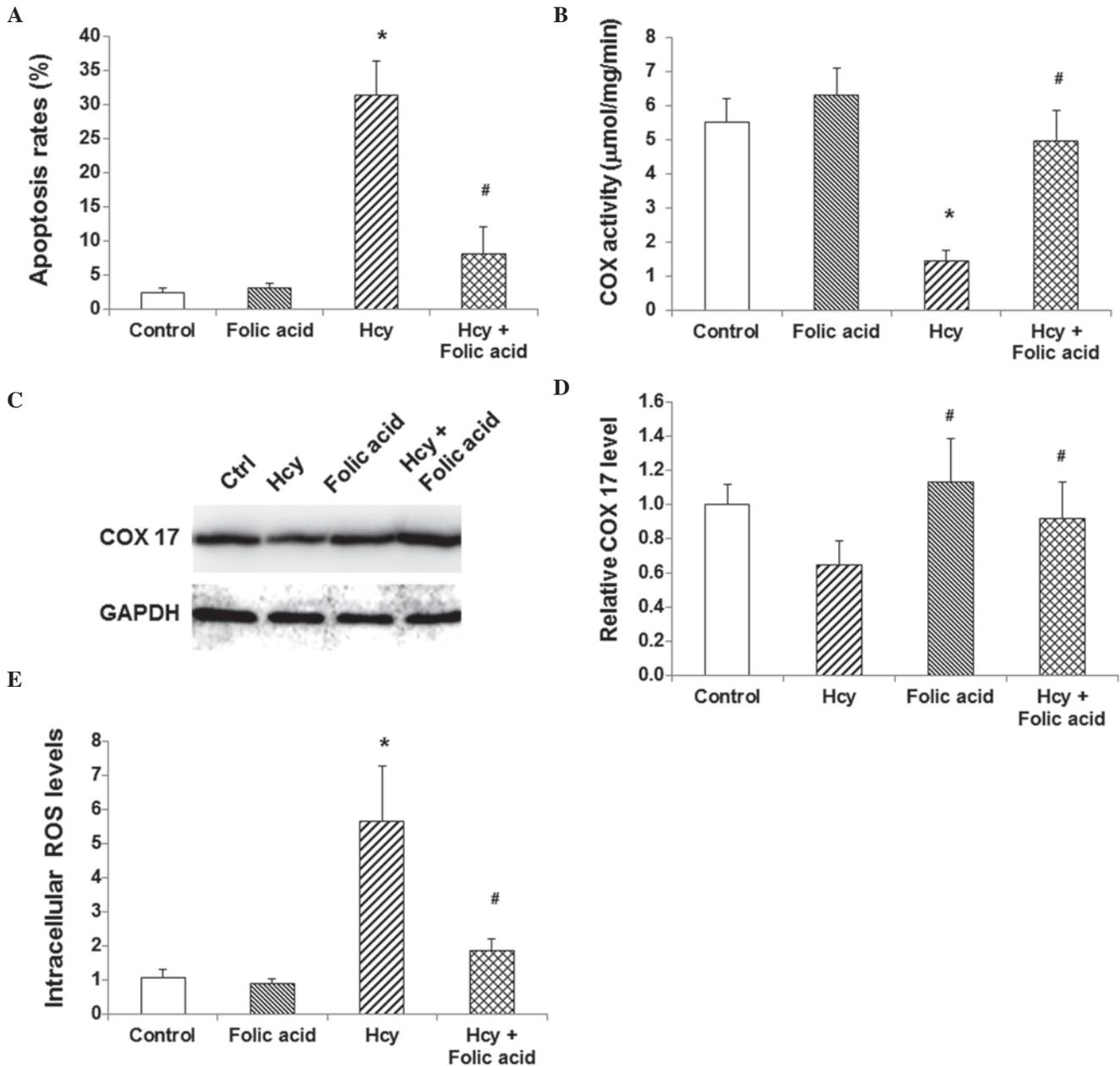


Figure 5. Folic acid alleviates the physiopathological changes induced by Hcy. HUVECs were treated with 1 mM Hcy and/or 100 µmol/l folic acid for 24 h, and then the (A) apoptosis rate, the (B) COX activity levels, the (C and D) COX 17 expression levels and the (E) intracellular ROS levels were determined in these cells. * $P < 0.05$, vs. the control group; # $P < 0.05$, vs. the Hcy group. ROS, reactive oxygen species; COX, cytochrome *c* oxidase; Hcy, homocysteine; HUVECs, human umbilical vein endothelial cells.

intracellular ROS levels, further inducing apoptosis (16,17). The results showed that 1 mM Hcy significantly increased the intracellular ROS levels and the apoptosis rate in endothelial cells.

Within cells, Hcy may be methylated into methionine when catalyzed with methionine synthase (MS), which involves folic acid (3,4). In the present study, folic acid was demonstrated to inhibit Hcy in endothelial cells. Moreover, treatment with folic acid treatment alleviated the physiopathological changes induced by Hcy in these cells.

In conclusion, the results demonstrated that Hcy inhibited COX activity and decreased the expression levels of COX 17, thereby increasing intracellular ROS levels, which eventually resulted in increased apoptosis rates in endothelial cells.

However, treatment with folic acid was able to alleviate the physiopathological changes induced by Hcy, restoring the COX activity and COX 17 expression levels, inhibiting intracellular ROS, and reducing the apoptosis rate in the endothelial cells. These findings suggest that the Hcy concentration in blood is important for the prevention of pulmonary embolism, which provides theoretical basis for the disease prevention in clinic. Further studies are required in order to explore the mechanisms underlying Hcy-induced COX activity decline.

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