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Effect of Hyperhomocysteinemia on a Murine Model of Smoke-Induced Pulmonary Emphysema.

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Abstract

Hyperhomocysteinemia (HHcy) was reported to enhance endoplasmic reticulum (ER) stress and subsequent apoptosis in several cells. However, the precise mechanisms of smoking susceptibility associated with HHcy has not been fully elucidated. This study included seven- to nine-week-old C57BL6 male mice induced with HHcy and were exposed to cigarette smoke (CS). A549 cells (human alveolar epithelial cell line) were cultured with homocysteine and were exposed to cigarette smoke extract (CSE) to observe cell viability and expression of proteins related to the ER stress. After 6 months of CS exposure, pulmonary emphysema was more severely induced in the group under the condition of HHcy compared to that in the control group. The apoptotic A549 cells increased as Hcy concentration increased and that was enhanced by CSE. Protein expression levels of ER stress markers were significantly increased after simultaneous stimulation. Notably, vitamin B12 and folate supplementation improved ER stress after simultaneous stimulation of A549 cells. HHcy exacerbated smoking-induced pulmonary emphysema and ER stress-induced alveolar cell apoptosis. ER stress in alveolar cells was reversed by vitamin B12 and folate supplementation, suggesting that HHcy could be the new therapeutic target to improve smoking susceptibility.

Introduction

According to the Global Initiative for Chronic Obstructive Lung Disease 2020 Report, chronic obstructive pulmonary disease (COPD) is defined as a common, preventable, and treatable disease characterized by persistent respiratory symptoms and airflow limitation due to airway and/or alveolar abnormalities (1). The World Health Organization reported that COPD is the third leading cause of death worldwide and is likely to increase in the coming years due to higher smoking prevalence and aging populations in many countries (2). The main risk factor for COPD is cigarette smoking (3). Only a fraction of smokers was often acknowledged to develop airflow obstruction (4, 5), and they are called susceptible smokers. Although genetic predisposition was reported to be associated with smoking susceptibility (6), the risk factors predisposing to smoking susceptibility are not yet fully elucidated.

Histopathologic features of COPD include emphysema (7), which results from alveolar cell apoptosis (8). Emphysematous lung destruction reduces maximum expiratory flow by decreasing the elastic recoil force available to drive air out of the lungs (9). An inverse correlation between emphysema lesions and forced expiratory volume in one second (FEV_1) was reported (10).

Moreover, higher homocysteine (Hcy) level in the blood was reported to be associated with a rapid decline in FEV_1 among male smokers in a general Japanese population (11). Hcy is a sulfur-containing amino acid formed during methionine metabolism, an essential amino acid (12). Recently, Hcy was reported to be involved in lung diseases (13). A meta-analysis demonstrated that serum Hcy could be a useful predictor for COPD development (14). High-concentration Hcy stimulation induces endoplasmic reticulum (ER) stress in cells such as the human umbilical vein endothelial cell and neural cell (15, 16), leading to apoptosis (17).

The ER plays several roles in the biosynthesis, folding, assembly, and modification of numerous soluble proteins and membrane proteins (18). Physiological states that increase the demand for protein folding, or stimuli that disrupt the reactions by which proteins fold, resulted in an imbalance between the protein folding load and the ER capacity, causing misfolded or unfolded proteins to accumulate in the ER lumen, so-called ER stress (19). The ER responds to the burden of unfolded proteins by activating intracellular signal transduction, or unfolded protein response (20), to restore cellular homeostasis or induce apoptosis if ER stress remains unmitigated (21).

Cell injury secondary to chronic ER stress was reported to contribute to the pathophysiology of a wide range of human diseases including diabetes mellitus, stroke, heart disease, and cancer. Recently, ER stress is focused in the field of lung disease, particularly COPD (22, 23). Since CS contains a complex mixture of over 7,000 potentially harmful components (24), cigarette smoking was suggested to induce ER stress (25, 26). These findings suggested the involvement of Hcy in smoking susceptibility and in COPD pathogenesis.

According to these findings, this study was conducted to examine whether hyperhomocysteinemia (HHcy) exacerbate lung emphysematous changes by cigarette smoke (CS) exposure via ER stress and subsequent alveolar cell apoptosis.

Result

Plasma Hcy concentrations in mice.

Plasma Hcy level was significantly elevated 5 days after 1% methionine (Met) water feeding (Fig. 1A). After 5 days of administration of water or 1% of methionine solutions, plasma Hcy levels in mice treated with Met were significantly higher than those in mice treated with water (Met; 22.1 ± 0.7 μmol/L, water; 3.8 ± 0.1 μmol/L). To clarify the impact of cigarette smoke on the plasma Hcy level, plasma levels of Hcy in mice exposed to CS/room air for 6 months in the condition with/without 1% Met water feeding were measured. As shown in Fig. 1B, the significant increases in plasma Hcy level were observed in Met+/CS- group (19.1 (8.3–35.1) μmol/L) and Met+/CS + group (11.5 (8.9–24.1) μmol/L) compared to that in the control groups (Met-/CS- (4.4 (4.0–4.8) μmol/L) and Met-/CS+ (4.7 (4.3–5.2) μmol/L)). Conversely, no significant difference was found in plasma Hcy level between Met-/CS- group and Met-/CS + group, indicating that 6 months of cigarette smoke exposure alone did not elevate plasma Hcy level.

Cigarette Smoke Exposure With Hhcy Enhanced Lung Emphysematous Changes

To examine the impact of HHcy in the development of emphysematous change in the lungs (Fig. 2A, B), the difference of mean linear intercept (MLI) among four groups (Fig. 2C) was assessed. After 6 months of CS exposure, emphysematous changes were observed in the CS with control water group, but deteriorated in the CS with 1% Met water group. The MLI was significantly increased in the Met-/CS + group (47.98 (46.69 – 50.29) μm) compared with that in the Met-/CS- group (43.40 (40.94 – 44.11) μm), and further increase was observed in the Met+/CS + group (53.37 (51.36 – 57.38) μm, respectively. P =

0.0171, median (interquartile range)) (Fig. 2C). Conversely, MLIs in the Met+/CS- group (43.77 (41.95 – 44.88) μm) were not increased compared to those in the control (Met-/CS-) group. Therefore, HHcy alone did not induce pulmonary emphysema, but it enhanced emphysematous changes induced by CS exposure, suggesting the synergic effect of HHcy and CS exposure in the development of emphysematous changes.

Combination of Hcy and cigarette smoke extract augmented A549 cell apoptosis.

To clarify the synergic effects of Hcy and cigarette smoke exposure on alveolar cell apoptosis in vitro, apoptosis in the cultured alveolar cells were assessed using flow cytometry after the treatment with Hcy, CSE, or both (Fig. 3A). Sole addition of CSE into the cultured media did not induce the increase of apoptosis in this experiment. However, the percentage of apoptotic cells gradually increased as Hcy concentration increased (Fig. 3B), and significant elevation was observed in 10 mM of Hcy. Notably, simultaneous stimulation with Hcy more than 5 mM and 20% CSE significantly increased the percentage of apoptotic cells. These results showed that combination of Hcy and CSE enhanced an alveolar cell apoptosis.

Synergic induction of endoplasmic reticulum stress caused by Hcy and cigarette smoke extract.

The impact of simultaneous stimulation of Hcy and CSE on the protein levels of ER stress-related molecules was examined (Fig. 4A). The protein expression levels of 78-kDa glucose-regulated protein (GRP78) (Fig. 4B), phosphorylation of inositol-requiring enzyme 1 α (IRE1α) (Fig. 4C), and protein expression levels of CCAAT/enhancer binding protein homologous protein (CHOP) (Fig. 4F) were significantly elevated in response to Hcy stimulation. On the other hand, no significant differences were found in the protein expression levels of GRP78 (Fig. 4B), the phosphorylation of IRE1α (Fig. 4C) and eukaryotic translation initiation factor 2α (eIF2α) (Fig. 4D), protein expression levels of activating transcription factor 6 (ATF6) (Fig. 4E), and CHOP (Fig. 4F) in response to CSE stimulation. The combined stimulation of Hcy and CSE significantly elevated the expression levels of GRP78 (Fig. 4B), phosphorylation of IRE1α (Fig. 4C), and CHOP (Fig. 4F) in A549 cells, compared to those with sole stimulation. Moreover, the phosphorylation of eIF2α (Fig. 4D) and the protein level of ATF6 (Fig. 4E) did not change with sole stimulation of Hcy or CSE but were enhanced with combined stimulation compared to those in the control group. These results indicated that CSE under the high Hcy condition enhanced ER stress in A549 cells.

Vitamin B12 and folate cotreatment attenuated endoplasmic reticulum stress induced by cigarette smoking exposure and Hcy.

The effect of vitamin B12 and folate supplementation on the protein and phosphorylation levels of ER stress-related molecules was investigated (Fig. 5A). The protein expression levels of Hcy, GRP78, ATF6, and CHOP and the phosphorylation of IRE1α and eIF2α were decreased by pretreatment of vitamin B12 and folate in a dose-dependent manner (Figs. 5B, C, D, E, F, G). These findings demonstrated that vitamin B12 and folate restore ER stress caused by Hcy/CSE stimulation.

Cotreatment with vitamin B12 and folate reduced the proportion of apoptotic A549 cells induced by cigarette smoking exposure and Hcy.

Whether vitamin B12 and folate supplementation attenuated the apoptosis of A549 cells induced by Hcy/CSE was evaluated. Flow cytometry showed the increased percentage of apoptotic cells induced by CSE/Hcy improved by the pretreatment of vitamin B12 and folate in a dose-dependent manner (Fig. 6A, B).

Discussion

The new and important findings from this study included the following: [1] Although HHcy mice did not develop pulmonary emphysema without CS exposure, HHcy mice exposed to CS for 6 months developed more severe emphysema compared to non-HHcy mice exposed to CS. [2] The proportion of alveolar cell apoptosis was significantly increased in cells with simultaneous Hcy and CSE stimulation than in those with sole stimulation. [3] The ER stress assessed using GRP78, phosphorylation of IRE1α, phosphorylation of eIF2α, ATF6, and CHOP deteriorated by simultaneous stimulation. [4] Lastly, ER stress and subsequent alveolar cell apoptosis caused by simultaneous stimulation were restored by vitamin B12 and folate supplementation. These results indicate that HHcy exacerbates lung emphysematous changes by CS exposure via ER stress and subsequent alveolar cell death.

Several clinical studies have reported a positive correlation between cigarette smoking and blood Hcy levels (27, 28), but these studies did not consider factors that increase plasma Hcy levels such as blood vitamin B level and eating habits. On the other hand, an experimental report examined the thiol status in the blood of different mouse strains exposed to CS and demonstrated no increase in blood Hcy levels after cigarette smoke exposure in all tested mice including C57BL/6J (29). In accordance with this report, this study showed that plasma Hcy concentrations in mice were not increased by CS exposure (Fig. 1B). Therefore, no causal relationship between CS exposure and plasma Hcy levels in mice was found.

In this study, a proportion of apoptotic cells and expression of CHOP, a transcription factor related to apoptosis (30, 31), were increased in A549 cells with high Hcy state, but lung emphysematous changes were not formed by HHcy alone in mice. Plasma Hcy levels in mice induced by giving 1% L-methionine water corresponded to almost moderate HHcy in human, and these levels of Hcy alone was not assumed to lead to emphysema formation. Conversely, HHcy in mice enhanced the pulmonary emphysema after 6 months of CS exposure (Fig. 2). Considering these findings, HHcy may play an important role in smoking susceptibility for inducing emphysematous lesion in the lung.

In mammalian cells, three ER transmembrane transducers have been elucidated as unfolded protein response sensors: PERK, IRE1α and ATF6 (20, 32). PERK pathway activates the apoptosis cascade by upregulating CHOP transcription. IRE1α also activates an apoptotic kinase, c-jun N-terminal kinase, through the signal cascade of the tumor necrosis factor receptor-associated factor 2-apoptosis signal-regulating kinase 1 pathway (33). This study showed that phosphorylation of IRE1α and eIF2 and protein levels of CHOP were significantly increased in response to simultaneous Hcy and CSE stimulation,

suggesting the involvement of PERK and IRE1α pathway in alveolar cell apoptosis (Figs. 5 and 6). Recently, Hcy-induced ER stress and apoptosis were rescued by blocking the PERK pathway, suggesting the importance of PERK pathway under the HHcy (34). In contrast, in this study, CSE alone did not induce statistically significant apoptosis or CHOP expression in A549 cells. Although the concentration of CSE was considered to be enough to induce apoptosis (35), this CSE concentration (20%) was not sufficient to cause apoptosis or ER stress alone in this study. Under the hyperhomocysteinemic condition, the effects of CS exposure on the induction of ER stress may be greatly enhanced through activating ER transmembrane transducers, finally leading to pulmonary emphysema. Hcy may enhance smoking susceptibility via ER stress-induced alveolar cell apoptosis.

A recent review reports that the most frequent causes of HHcy in adult life are the genetic enzyme deficits involved in Hcy metabolism, mainly 5,10-methylenetetrahydrofolate reductase, methionine synthase, and cystathionine-β-synthase, and the nutritional deficiencies of folate, vitamin B6, and B12 (36). Smokers tend to consume fewer fruits and vegetables as a source of these vitamins and have a lower consumption of food items rich in antioxidants (37, 38), suggesting that smokers may have HHcy due to poor intake of vitamin B and folate-rich foods. In this study, ER stress and subsequent alveolar cell apoptosis were restored by vitamin B12 and folate supplementation (Fig. 6). Interestingly, a report showed that vitamin intake inhibits blood Hcy elevation even if the cause was genetic (39). Our epidemiological study showed that respiratory function of male smokers with higher Hcy declined more rapidly (11). Additionally, a meta-analysis showed that higher Hcy was thought to be a predictive marker for COPD development (14). These results indicate that CS exposure in the HHcy state more strongly enhanced ER stress-induced apoptosis than CS exposure only in alveolar epithelial cells and exacerbated emphysematous change in the lungs. Future studies are needed to reveal that vitamin intake can improve smoking susceptibility.

This study has several limitations. First, although the importance of ER stress in the alveolar cell apoptosis in vitro was shown in this study, an in vivo study was not assessed. Because the adverse effects of CS were assumed to affect many systems in vivo, this study focused on alveolar cells to clarify the mechanism. Second, oxidative stress and inflammation were not investigated, which are inducible factor for ER stress in this study. Finally, the regulation of thiol metabolism including Hcy was suggested to differ between mouse and human. As regards basal status, mice have lower blood Hcy levels than humans (40). And in the response of oral methionine loading, glutathione increases in mice but decreases in humans (41). Although the mechanisms underlying such differences between species remain unknown, since the antioxidant glutathione is low in humans, HHcy may be more harmful in humans than in mice.

In conclusion, this study demonstrated that HHcy increased alveolar epithelial cell death caused by CS exposure through ER stress-induced apoptosis and enhanced lung emphysematous changes for the first time. This study indicates that HHcy is a risk factor influencing smoking susceptibility. HHcy could be the new target for the prevention and treatment of COPD.

Materials And Methods

Animal welfare

All experimental procedures were performed according to the animal welfare regulations of Yamagata University Faculty of Medicine.
The study protocol was approved by the Animal Subjects Committee of Yamagata University Faculty of Medicine (Approval number 29050, Date of approval March 9th, 2017, Approved by Hiroshi Iizuka (Chairman of Animal Research Committee Director (Research) Yamagata University)).
The investigation conformed to the Guide for the Care and Use of Laboratory animals published by the U.S. National Institutes of Health (NIH Publication, 8th Edition, 2011).

Materials and reagents

L-methionine, D,L-Hcy, vitamin B12, and folate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit monoclonal anti-phospho- eIF2α and rabbit polyclonal anti-β-Tubulin were purchased from Cell Signaling Technologies (Danvers, MA, USA). Rabbit polyclonal anti-phospho- IRE1α and mouse monoclonal anti-ATF6 were purchased from Novus Biologicals (Littleton, CO, USA). Rabbit polyclonal anti-t-IRE1α, rabbit polyclonal anti-GRP78, mouse monoclonal anti-CHOP, and rabbit polyclonal anti-Hcy were purchased from Abcam (Cambridge, MA, USA). Annexin V-FITC Kit was purchased from MBL (Nagoya, Japan).

Animal maintenance and diet-induced HHcy mice model

Seven- to nine-week-old male C57BL/6 mice were housed in standard cages (2–5 per cage) in a temperature-controlled room with a 12/12-h light/dark cycle and were fed a normal diet (Oriental Yeast Co., Ltd, Tokyo, Japan). A report showed that methionine supplementation at concentrations of 1% in water induced HHcy in male C57BL/6 mice (42). The mice were administered to normal water or L-methionine (Met) at 1% concentrations in drinking water and were exposed to the air or CS of five nonfiltered cigarettes (Peace; Japan Tobacco Inc., Japan) for 30 min, twice per day, 5 days per week, using a whole-body smoking exposure apparatus (INH03-CIGR01A; MIPS, Osaka, Japan). For sample size determination, we determine a reasonable number of animals that can be statistically tested, do not involve excessive animal sacrifice, and refer to similar experimental reports in the previous study (43). All mice that had completed drug administration or smoking exposure were included in the analysis.

Measurement of plasma Hcy

Blood was collected from the inferior vena cava to the tubes containing K2 EDTA. Plasma was separated by centrifugation at 4°C, 1300 × g for 15 min. Plasma Hcy level was measured using high-performance liquid chromatography (44), with the cooperation of the Japan Institute for the Control of Aging, NIKKEN SEIL Co., Ltd.

Histological analysis

To examine the development of pulmonary emphysema, the lungs were fixed with intratracheal instillation with 4% buffered formalin at a constant pressure of 25 cmH2O, and paraffin-embedded lung blocks were prepared. Three-μm-thick lung sections were stained with hematoxylin and eosin. The mean linear intercept (MLI), as a measure of the interalveolar septal wall distance (45), was measured using light microscopy at 400× magnification. The MLI was obtained by dividing the length of a line drawn across the lung section by the total number of intercepts encountered in 50 lines per mouse lung, as described previously (43).

Cell culture

Human lung alveolar epithelial A549 cells were purchased from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Japan, and were cultured in DMEM supplemented with 10% fetal calf serum, L-glutamine, and an antibiotic-antimycotic solution (100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.025 μg/ml of amphotericin B; Gibco) in a humidified incubator at 37°C with 5% CO2. To examine the cytotoxic effects of Hcy and cigarette smoke extract (CSE), A549 cells were stimulated using varying concentrations of Hcy (0—10 mM) and/or 20% of CSE.

Preparation of cigarette smoke extract

CSE was produced as described previously (35, 46). Briefly, smoke from one stick of cigarette was bubbled through 25 ml of Hanks' Balanced Salt Solution. The resultant product was defined as 100% CSE.

Vitamin treatment

In the previous study, vitamin B12 and folate supplementations have been reported to reduce ER stress caused by Hcy (16). Vitamin B12 were dissolved in water. Folate was dissolved in 0.1 M NaOH. Compounds with the same vitamin B12 and folate concentration ranging 5—50 μM were diluted in a cultured medium to examine whether these compounds can restore ER stress in a dose-dependent manner.

Flow cytometry

A549 cell apoptosis was investigated following the addition of fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide according to the manufacturer's protocol. Apoptosis was regarded as the cell percentage in the Q3 quadrant. The results were analyzed using flow cytometry (EC800 Flow Cytometry Analyzer; Sony Biotechnology Inc. Tokyo, Japan).

Western blotting analysis

A549 cells were lysed in ice-cold radioimmunoprecipitation assay lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 0.1% NP-40, 1 mM DTT, 0.1% SDS, 100 mM PMSF, 100 mM NEM, 100

mM iodoacetamide, and 1% phosphatase inhibitor. The protein concentration of each sample was determined using the BCA protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of protein were electrophoresed on 8–10% sodium dodecyl sulfate-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes (GE Healthcare UK Ltd, Little Chalfont, UK). Membranes were blocked with 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.1% Tween (TBS-T), and 5% milk or 5% BSA at room temperature for 1 h. Then the membranes were probed with primary antibodies diluted in TBS-T. After incubation with horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T containing 5% milk or 5% BSA, immunoreactive bands were detected using an ECL kit (Amersham Biosciences, Piscataway, NJ, USA) (47). The expression levels of GRP78, IRE1α, eIF2α, ATF6 and CHOP were examined. GRP78 (also known as BiP and HSPA5) is sequestered by binding to unfolded or misfolded polypeptide chains and/or unassembled multisubunit proteins, thereby leading to the release and, consequently, the activation of the ER-stress sensors such as IRE1α and PKR-like ER kinase (PERK) (48). Each sensor introduces its own unfolded protein response signaling pathways, activated PERK phosphorylate eIF2α, causing apoptosis cascade by upregulating CHOP transcription in chronic ER stress (49). The expression of CHOP, a pro-apoptotic protein, is currently used as a marker of chronic ER stress and subsequent apoptosis (50).

Statistical analysis

Data are expressed as the mean ± standard error or median and interquartile range. Differences between groups were assessed using analysis of variance, which was followed by the Tukey–Kramer test or Steel–Dwass test. Significance was inferred for P values < 0.05. Statistical analyses were performed using JMP version 12.2 software (SAS Institute, Cary, NC, USA).

Declarations

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Autor contributions

Hiroshi Nakano: Data curation, Original draft preparation. Sumito Inoue: Conceptualization, Study design. Akira Igarashi, Yoshikane Tokairin, Keiko Yamauchi, Tomomi Kimura, and Michiko Nishiwaki performed experiment of cultured cell lines. Takako Nemoto, Masamichi Sato, and Hiroyoshi Machida, Hiroaki Murano performed animal experiments. Kento Sato, Sujeong Yang, Yoichiro Otaki contributed to analysis and interpretation of data. Masafumi Watanabe contributed to analysis and interpretation of data and assisted in the preparation of the manuscript. Yoko Shibata got the fund for the study, designed the study and wrote the final draft of the manuscript.

All other authors have contributed to data collection and interpretation, and critically reviewed the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Declaration of Competing Interest

The authors declare no competing interests.

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Figures

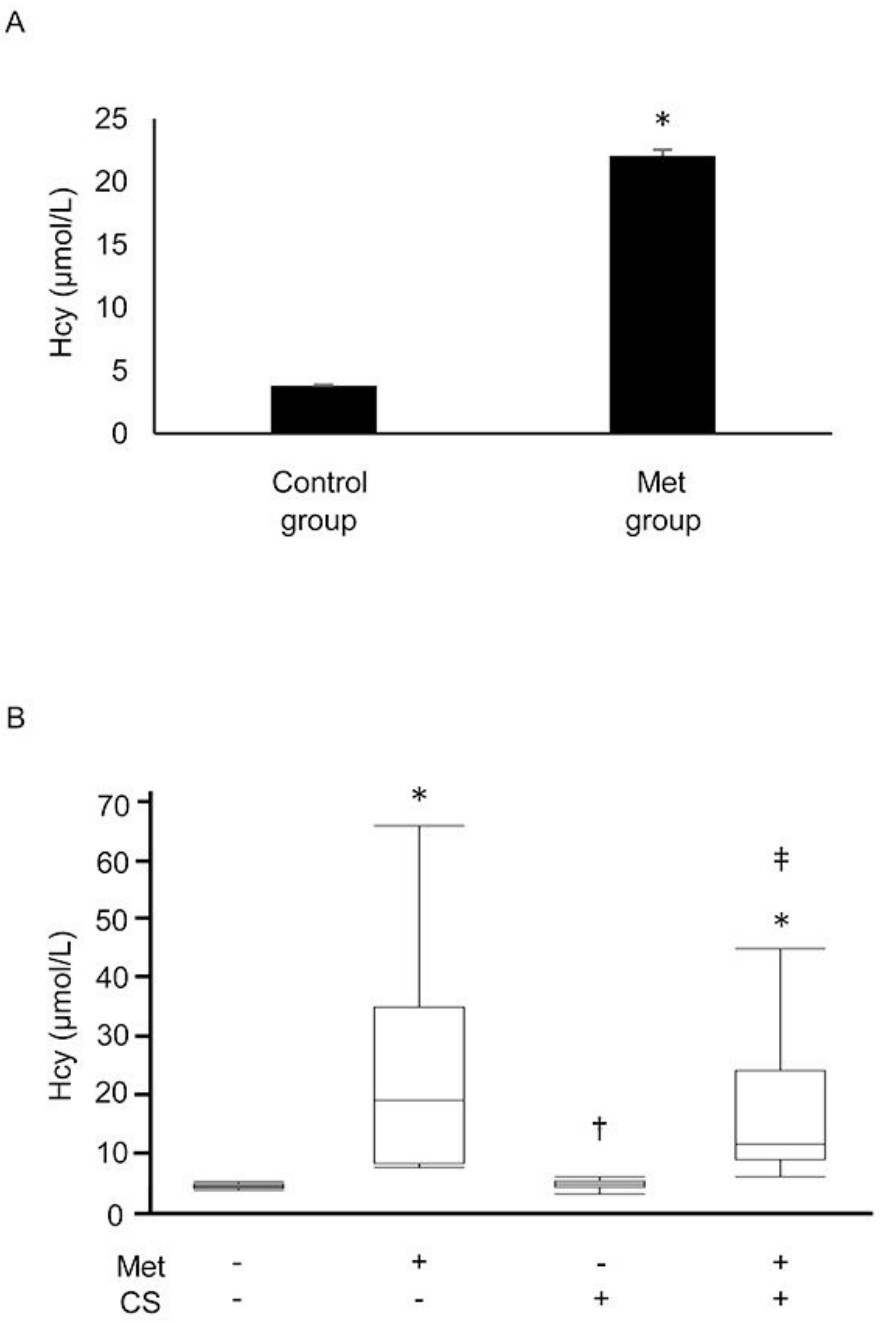

Figure 1

1A. Plasma homocysteine (Hcy) level in mice After 5 days of administration of water or 1% of methionine solutions, plasma Hcy levels in mice treated with methionine were significantly higher than those in mice treated with water. n = 3 in each group. Data are presented as the mean ± standard error (SE). Differences between groups were examined using t-test. *p < 0.0001 vs control group. Met = L-Methionine at 1% concentrations in water. 1B. Difference in plasma homocysteine (Hcy) level between water and 1%

methionine water-fed mice after 6 months of cigarette smoke exposure Met- group, normal water administration; Met+ group, 1% methionine water administration; CS- group, air exposure; and CS+ group, cigarette smoke exposure. Plasma Hcy concentrations were compared after 6 months of treatment. Significant increases in plasma Hcy level were observed in Met+/CS- group and Met+/CS+ group compared to Met-/CS- group. In contrast, no significant difference was found in plasma Hcy level between Met-/CS- group and Met-/CS+ group. n = 8 in each group. Data are presented as the median and interquartile range. Differences between groups were examined using Steel–Dwass test. *P < 0.05 vs Met-/CS-, †P < 0.05 vs Met+/CS-, ‡ P < 0.05 vs Met-/CS+.

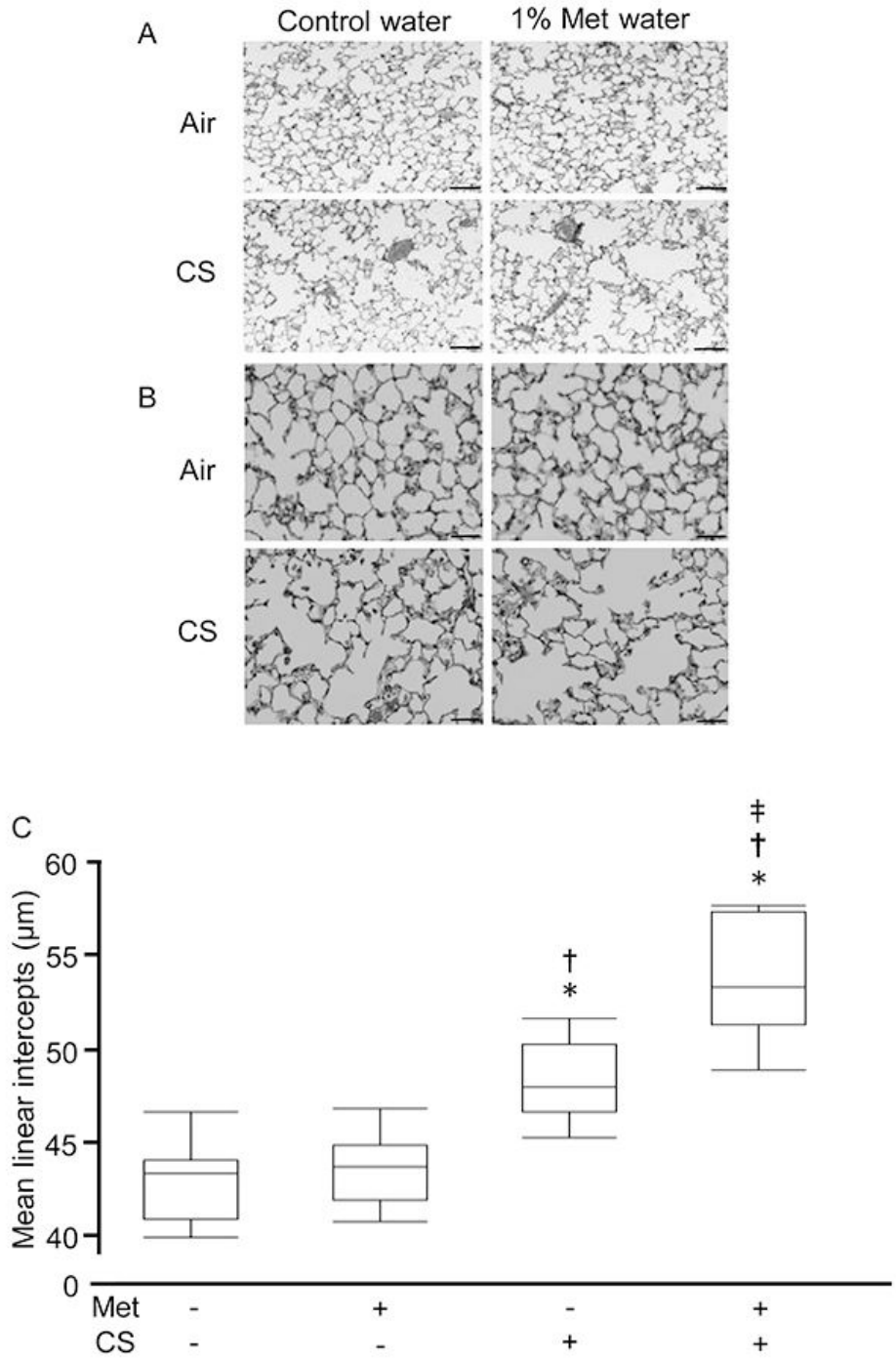

Figure 2

Pathological examination of lung emphysematous changes in mice by hematoxylin and eosin (H&E) staining (A) At 200× magnification. Scale bar means 100 μm. (B) At 400× magnification. Scale bar means 40 μm. Lung sections stained with H&E were shown. Dense alveolar structure is observed in the lung of mice given with normal water and exposed to air. Histological images of mice given with 1% methionine water and exposed to air is not different from histological images of the lung in mice given

with normal water and exposed to air. Emphysematous changes and inflammatory cells are observed in the lung of mice given with normal water and exposed to cigarette smoke (CS). In mice given with 1% methionine water and exposed to CS, enhanced emphysematous changes compared with histological images of the lung in mice given with normal water are observed. Also, inflammatory cells and alveolar-wall destruction are observed. 2C. Difference in mean linear intercept (MLI) in the lung section between water and 1% methionine water-fed mice 6 months after cigarette smoke exposure Met- group, normal water administration; Met+ group, 1% methionine water administration; CS- group, air exposure; and CS+ group, cigarette smoke exposure. The MLI was significantly increased in Met-/CS- group, and further increase was observed in Met+/CS+ group (47.98 (46.69–50.29) μm, 53.37 (51.36–57.38) μm, respectively. P = 0.0171, median (interquartile range)). Significant increase in MLI was not observed in Met+/CS- group compared to Met-/CS- group. n = 8 in each group. Data are presented as the median and interquartile range. Differences between groups were examined using Steel–Dwass test. *P < 0.05 vs Met-/CS-, †P < 0.05 vs Met+/CS-, ‡P < 0.05 vs Met-/CS+. CS: cigarette smoke Met: methionine

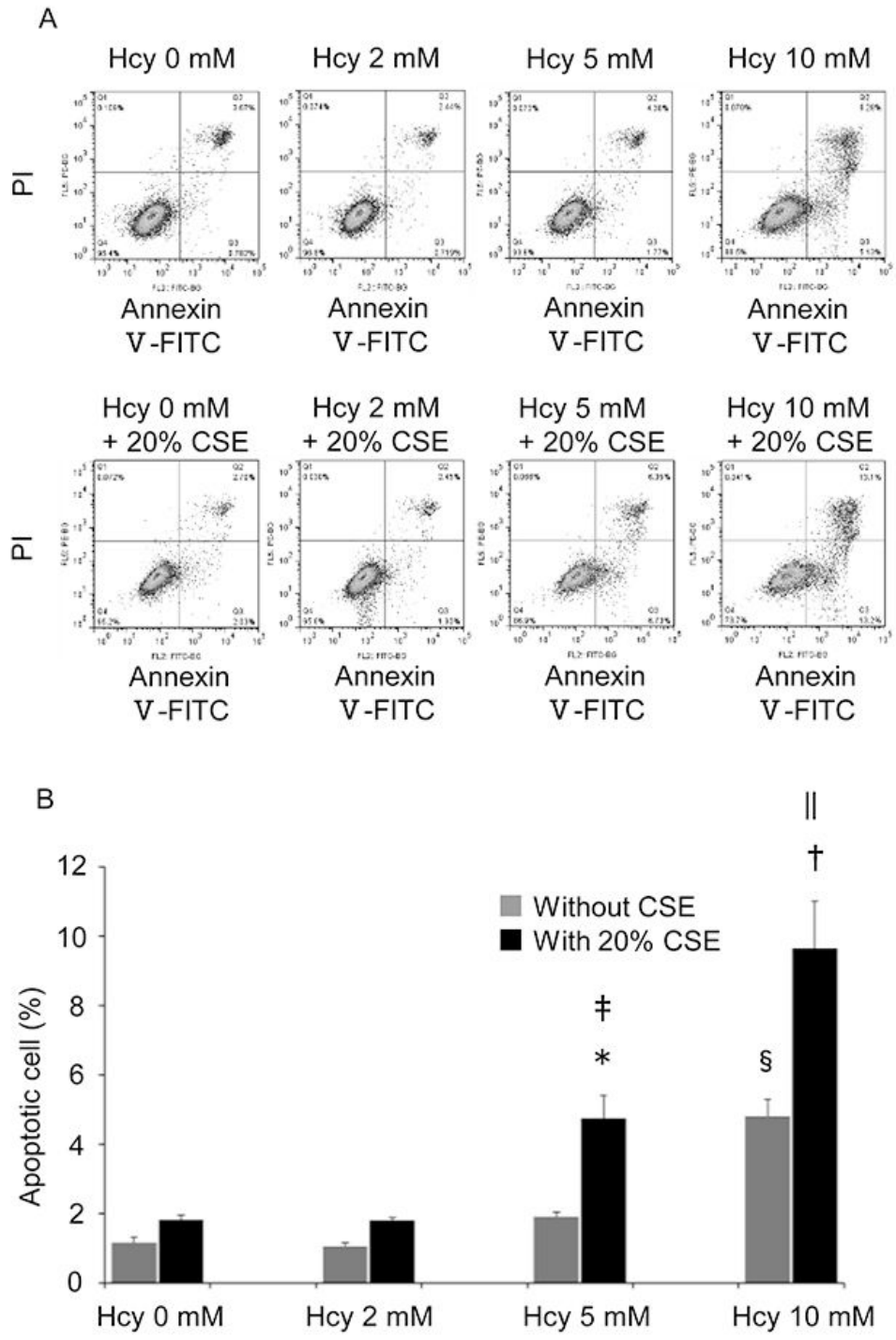

Figure 3

Effects of homocysteine (Hcy) and cigarette smoke extract (CSE) on the induction of A549 cells apoptosis To investigate the synergic effect of Hcy and cigarette smoke exposure on alveolar cell apoptosis in vitro, A549 cells were treated with 2 mM, 5 mM, or 10 mM Hcy and 20% CSE for 24 h, followed by apoptosis assays using flow cytometry. (A) The apoptotic rates of A549 cells were evaluated using Annexin V/propidium iodide double staining. Apoptosis was regarded as the cell percentage in the

Q3 quadrant. (B) Quantified data of flow cytometry. Quantified data of flow cytometry showed no significant difference in the percentage of apoptotic cells after only CSE administration. On the other hand, the percentage of apoptotic cells was gradually increased as Hcy concentration increased, and significant elevation was observed in 10 mM of Hcy. Simultaneous stimulation with Hcy more than 5 mM and 20% CSE dramatically increased the percentage of apoptotic cells. Data were expressed as mean ± standard error (SE). n = 4 in each group. Differences between groups were examined using Tukey–Kramer test. *P < 0.05 vs Hcy 5 mM without 20% CSE, †P < 0.05 vs Hcy 10 mM without 20% CSE, ‡P < 0.05 vs Hcy 2 mM with 20% CSE, §P < 0.05 vs Hcy 5 mM without CSE, ||P < 0.05 vs Hcy 5 mM with 20% CSE. CSE: cigarette smoke extract Hcy: homocysteine PI: propidium iodide

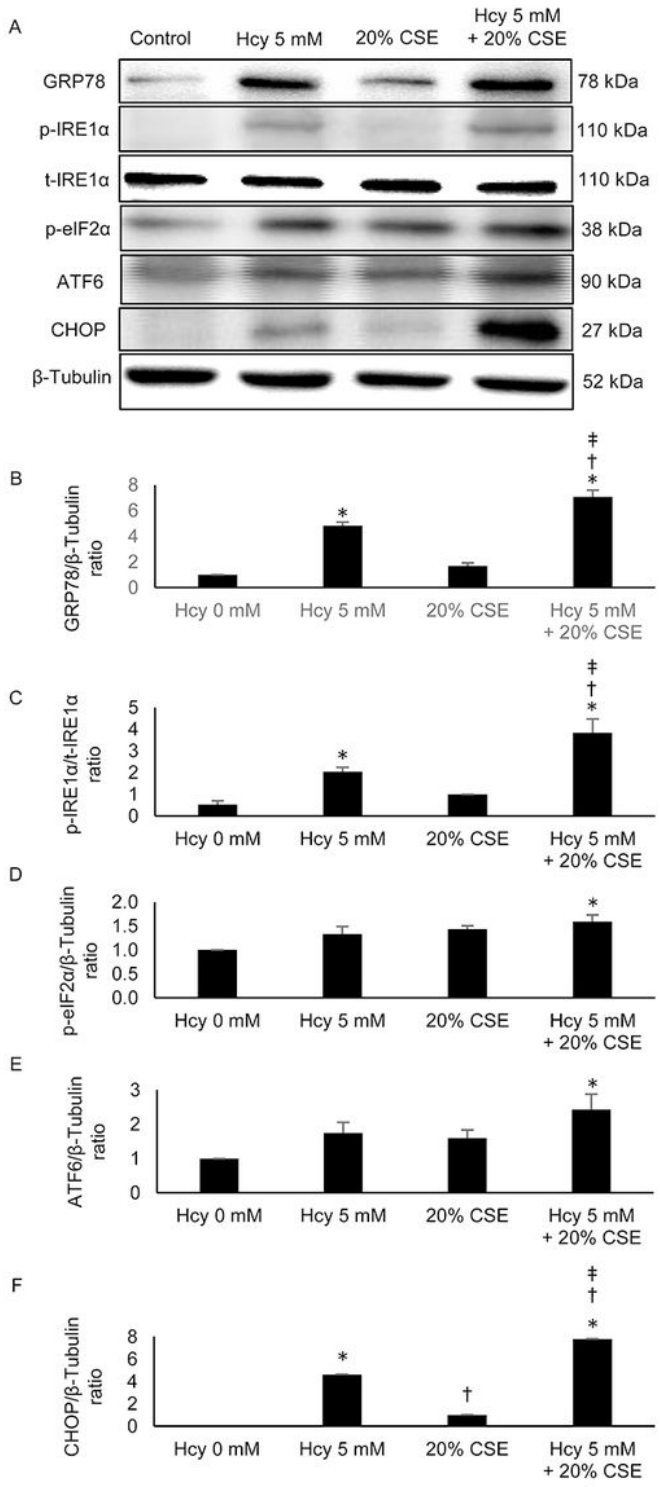

Figure 4

Synergic induction of endoplasmic reticulum (ER) stress by homocysteine (Hcy) and cigarette smoke extract (CSE) in A549 cells To examine the impact of simultaneous stimulation of Hcy and CSE on the ER stress, A549 cells were treated with 20% CSE and 5 mM Hcy, and then the protein levels of GRP78, phosphorylation of IRE1α and eIF2α, protein levels of ATF6 and¬ CHOP were examined using western blotting. (A) Graphic depictions of western blotting (B, C, D, E, and F) Quantitative analysis for protein

expression of GRP78, phosphorylation of IRE1α, phosphorylation of eIF2α, protein expression of ATF6 and CHOP by western blotting The protein expression levels of GRP78 and CHOP were significantly elevated in response to Hcy stimulation. Also, phosphorylation of IRE1α was enhanced after Hcy stimulation. In contrast, no significant differences were found in the phosphorylation of IRE1α and the protein expression levels of GRP78, phosphorylation of eIF2α, and protein expression of ATF6 and CHOP in response to CSE stimulation. The expression levels of GRP78, phosphorylation of IRE1α, and CHOP in A549 cells stimulated by Hcy and CSE simultaneously were significantly increased compared to those in A549 cells with sole stimulation. Also, the phosphorylation of eIF2α and protein expression of ATF6 were significantly increased after simultaneous stimulation compared to that in the control group. n = 5 in each group. Data were expressed as mean ± standard error (SE). Differences between groups were examined using Tukey–Kramer test. *P < 0.05 vs Hcy 0 mM, †P < 0.05 vs Hcy 5 mM, ‡P < 0.05 vs 20% CSE. ATF6: activating transcription factor 6 CSE: cigarette smoke extract CHOP: CCAAT/enhancer binding protein homologous protein Hcy: homocysteine p-IRE1α: phosphorylation of inositol-requiring enzyme 1 α p-eIF2α: eukaryotic translation initiation factor 2α

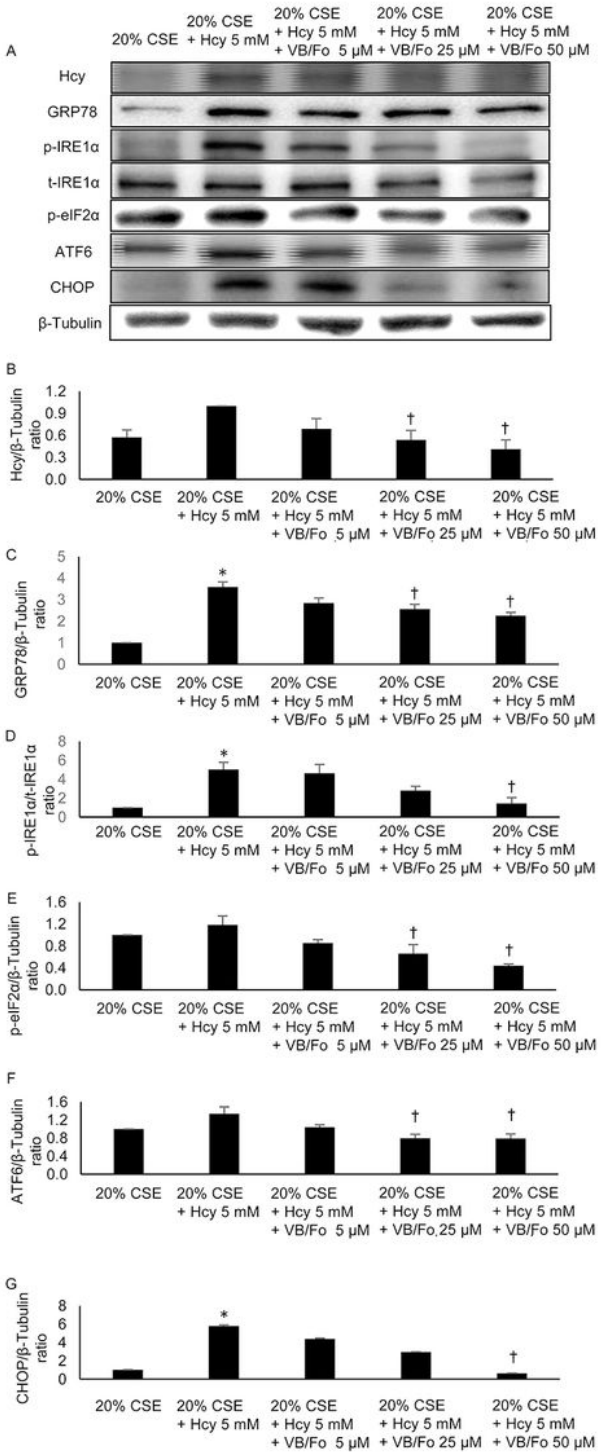

Figure 5

Vitamin B12 (VB) and folate (Fo) improved endoplasmic reticulum (ER) stress induced by homocysteine (Hcy) and cigarette smoke extract (CSE) in A549 cells To investigate the effect of vitamin B12 and folate supplementation on the protein levels of ER stress-related molecules, A549 cells were pretreated with the same concentration of vitamin B12 and folate adjusted to 5–50 μM for 12 h, followed by stimulation with 5 mM Hcy plus 20% CSE. (A) Graphic depictions of western blotting (B, C, D, E, F, and G) Quantitative

analysis for protein expression of Hcy, GRP78, phosphorylation of IRE1α, phosphorylation of eIF2α, ATF6, and CHOP by western blotting The protein expression levels of GRP78 and CHOP were decreased by administration of vitamin B12 and folate in a dose-dependent manner. n = 4–6 in each group. Data were expressed as mean ± standard error (SE). Differences between groups were examined using Tukey–Kramer test. *P < 0.05 vs 20% CSE. †P < 0.05 vs Hcy 5 mM with 20% CSE. ATF6: activating transcription factor 6 CSE: cigarette smoke extract CHOP: CCAAT/enhancer binding protein homologous protein Fo: folate Hcy: homocysteine p-IRE1α: phosphorylation of inositol-requiring enzyme 1 p-eIF2α: eukaryotic translation initiation factor 2α VB: vitamin B12

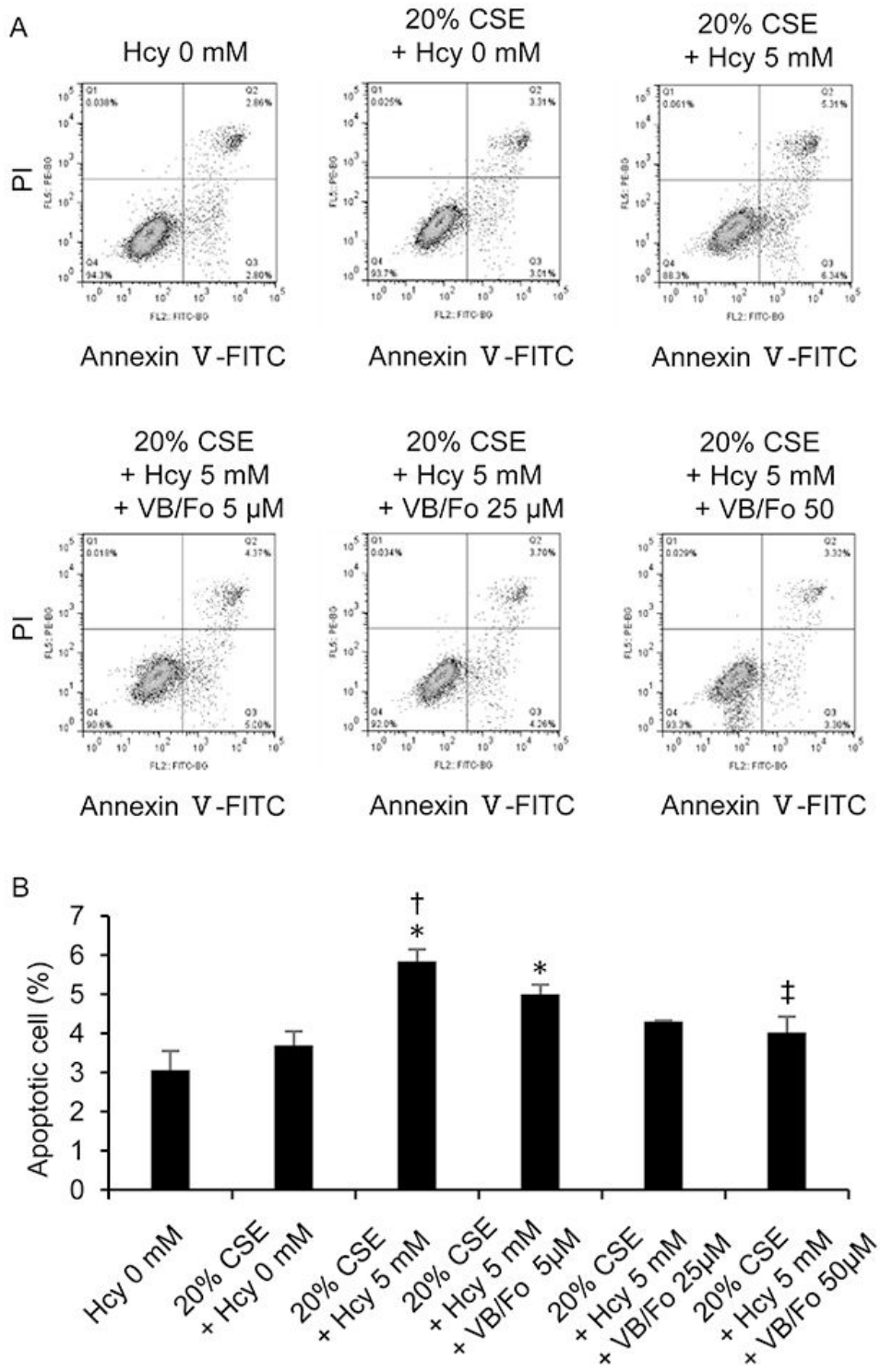

Figure 6

Vitamin B12 (VB) and folate (Fo) reduced the proportion of apoptotic A549 cells induced by homocysteine (Hcy) and cigarette smoke extract (CSE) To investigate the effect of vitamin B12 and folate supplementation on the apoptosis induced by Hcy/CSE, A549 cells were pretreated with the same concentration of vitamin B12 and folate adjusted to 5–50 μM for 12 h, followed by stimulation with 5 mM Hcy plus 20% CSE. (A) The proportion of apoptotic A549 cells were evaluated using Annexin

V/propidium iodide double staining. Apoptosis was regarded as the cell percentage in the Q3 quadrant. (B) Quantified data of flow cytometry Quantified data of flow cytometry show that simultaneous stimulation with Hcy 5 mM and 20% CSE increased the proportion of apoptotic cells. However, the proportion of apoptotic cells was decreased as the concentration of Vitamin B12 and folate increased. Data were expressed as mean ± standard error (SE). n = 3 in each group. Differences between groups were examined using Tukey–Kramer test. *P < 0.05 vs Hcy 0 mM, †P < 0.05 vs Hcy 0 mM with 20% CSE, ‡P < 0.05 vs Hcy 5 mM with 20% CSE. CSE: cigarette smoke extract Fo: folate Hcy: homocysteine PI: propidium iodide VB: vitamin B12