

# ABCG1 deficiency promotes endothelial apoptosis by endoplasmic reticulum stress-dependent pathway

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**Abstract** The present study was focused on whether ABCG1 deficiency was involved in endothelial apoptosis and its possible mechanism. Human umbilical artery endothelial cells were transfected with ABCG1 siRNA and/or ABCG1 expression plasmid. We observed that silencing of endothelial ABCG1 reduced cholesterol efflux to HDL and increased intracellular lipid content. Moreover, reduction of ABCG1 promoted endothelial apoptosis and expression of endoplasmic reticulum (ER) stress-related molecules GRP78 and CHOP. In contrast, transfection of ABCG1 overexpression plasmid reversed endothelial apoptosis and intracellular lipid accumulation as well as decreased expression of GRP78 and CHOP in ABCG1-deficient endothelial cells. Furthermore, endothelial apoptosis and ER stress-related molecules were induced by repletion of endothelial cells with cholesterol-loaded cyclodextrin, otherwise endothelial apoptotic response and expression of GRP78 and CHOP were suppressed by depletion of cellular cholesterol in ABCG1-deficient endothelial cells. The present results suggest that reduction of ABCG1 induces endothelial apoptosis, which seems associated with intracellular free cholesterol accumulation and subsequent ER stress.

**Keywords** ATP-binding cassette transporter G1 · Endothelial apoptosis · Endoplasmic reticulum stress · Cholesterol efflux

## Introduction

Atherosclerotic lesions are determined by the balance of a series of pro- and antiatherogenic events. In recent years, apoptosis has been implicated as one of the key factors influencing the cellularity, stability, and thrombogenicity of atherosclerotic lesions [1–5]. It has been reported that apoptosis of endothelial cells (ECs) and smooth muscle cells favors plaque instability [2–4], and macrophage apoptosis seems to be a double-edged sword. It means that macrophage apoptosis is beneficial in early atherosclerotic lesions, whereas loss of macrophage becomes detrimental in advanced lesions [1, 2, 5].

In the control of cell death and apoptosis, alteration in the function of the endoplasmic reticulum (ER), also known as ER stress, has been considered to play a crucial role [6]. It is well known that ER stress response is an adaptive mechanism by which cells react to perturbations in ER homeostasis through the upregulation of ER resident chaperons such as 78-kDa glucose-regulated protein (GRP78). However, when ER function is severely impaired, the organelle spreads apoptotic signals, such as activation of C/EBP homologous protein (CHOP) [6].

ATP-binding cassette transporter G1 (ABCG1) has been shown to promote cholesterol efflux to high-density lipoprotein (HDL) particles in reverse cholesterol transport [7, 8]. Deficiency of ABCG1 in macrophages and smooth muscle cells lead to reduced cholesterol efflux and increased intracellular cholesterol accumulation [7–10]. It has been reported that intracellular cholesterol accumulation is a potent inducer of apoptosis in macrophages [11]. In addition, intracellular free cholesterol (FC) trafficking to the ER triggers the unfolded protein response (UPR), an ER stress response pathway [12, 13]. ABCG1 has also been proved to highly express in EC [14–16], and to aid in

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cholesterol homeostasis to prevent endothelial activation in vessel walls [15, 16]. Based on the fact that lipid content can induce ER stress in macrophages, we were focused on whether ABCG1 deficiency was involved in endothelial cell apoptosis, and on whether it was associated with regulation of ABCG1 to cholesterol efflux.

## Methods

### Cell culture

Human umbilical artery endothelial cells (HUAEC; ScienCell) were cultured in Endothelial Cell Medium (No.1001; ScienCell) in a CO<sub>2</sub>/O<sub>2</sub> incubator at 37 °C. Cells were subcultured every 72 h. HUAEC were transfected with ABCG1 siRNA or scramble siRNA and ABCG1 expression plasmid or empty vector. Then, 24 h after transfection, cells were observed. All experiments were repeated at least three times.

### Western blot

Whole cell lysates were generated with RIPA buffer. Equal amounts of protein extracts (50 µg) were separated by 10 % SDS-PAGE gel and then transferred onto nitrocellulose membranes using a Bio-Rad transfer blotting system. The membranes were incubated with antibodies against ABCG1, GRP78, and CHOP (all Proteintech, USA). Proteins were visualized using an enhanced chemiluminescence detection system (ECL; Cell Signaling Technology). Anti-β-actin (Santa Cruz, USA) was used to control for equal protein loading.

### Real-time RT-PCR

Total RNA and first-strand cDNA was produced using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Real-time quantitative PCR analysis was used to measure the relative levels of ABCG1 mRNA expression. The nucleotide sequences of the primers were as follows: ABCG1, forward primer 5'-GGTGATGCCGAGGTGAAC-3' and reverse primer 5'-CAATGTGCGAGGTGATGC-3'; β-actin, forward primer 5'-ATCGTGCGTGACA TTAAGGAGAAG-3' and reverse primer 5'-AGGAAGG AAGGCTGGAAGAGTG-3'. Levels of ABCG1 mRNA were normalized to beta-actin mRNA levels.

### Detection of apoptosis by flow cytometry analysis

Annexin V-FITC/PI double-staining assay was used to quantify apoptosis, according to the manufacturer's protocol (Calbiochem). Following treatment, cells were

centrifuged, washed with PBS, resuspended in 195 µl binding buffer, and incubated with 5 µl Annexin V-FITC and 10 µl propidium iodide (PI) solution for 10 min at room temperature in the dark. The samples were analyzed on a FAC scan flow cytometer using CELL-Quest software (BD, CA). Double staining of cells with Annexin V-FITC and PI permits the identification of different cell populations based on their staining patterns as follows: live cells (FITC -PI-), early apoptotic (FITC +PI-), late apoptotic (FITC +PI+), and necrotic cells (FITC -PI+).

### Intracellular cholesterol content

Intracellular lipids were extracted with isopropyl alcohol (including 5α-cholestane as an internal standard) at room temperature overnight and analyzed for cholesterol content by gas-liquid chromatography as described previously [15, 17].

### Lipid efflux assay

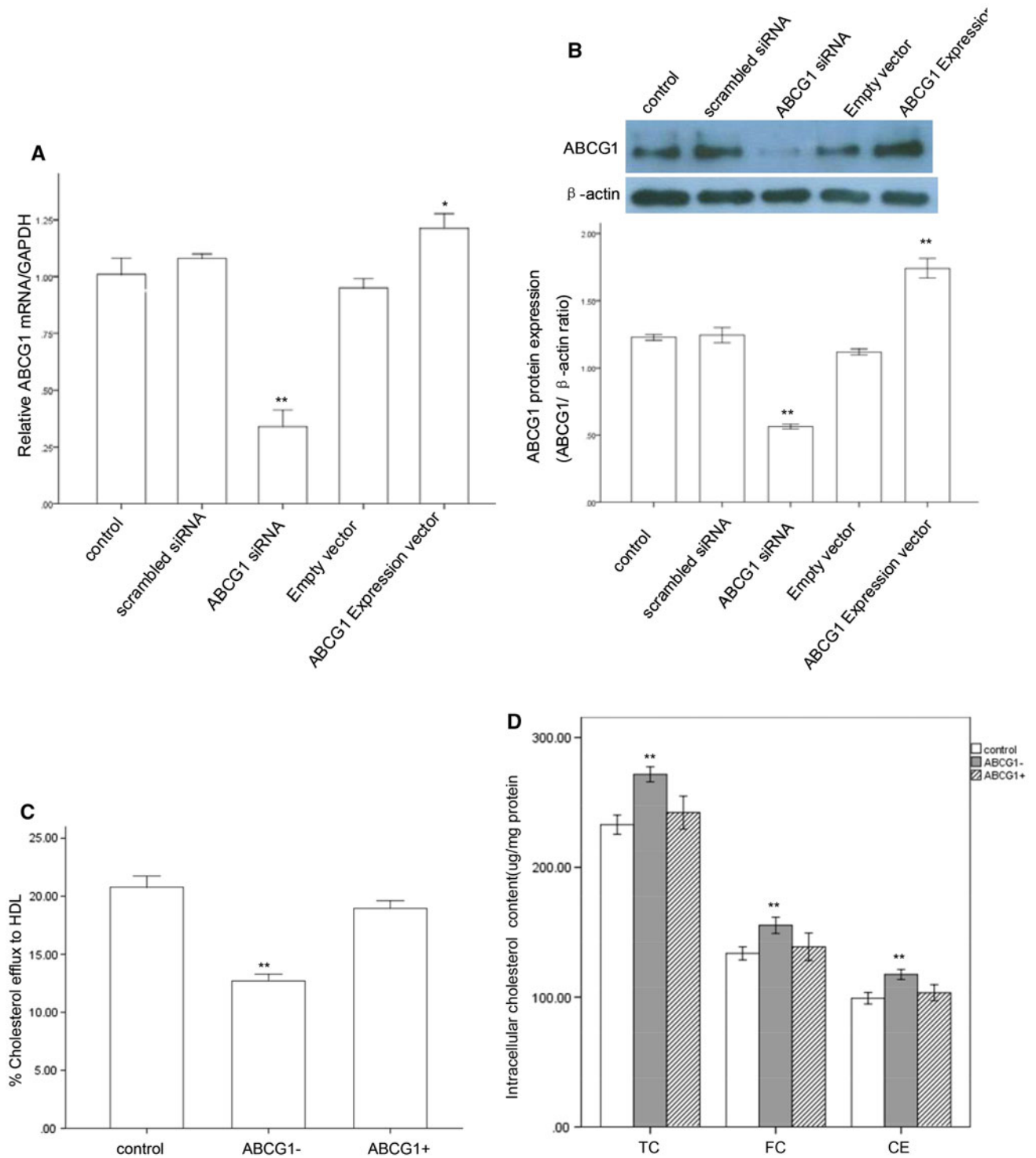
HUAEC were plated in 12-well plates and radiolabeled for 48 h in serum-free medium containing 1 µCi/mL [<sup>3</sup>H] cholesterol (Sigma-Aldrich). After 48 h, cell layers were rinsed. Cholesterol efflux was conducted for 12 h at 37 °C in media containing 0.2 % BSA and 50 µg/mL of human HDL. At the end of this incubation, the supernatant was collected and centrifuged at 13,000 rpm for 10 min to remove debris. Cells were lysed with 0.5 mL of 0.1 N NaOH. The radioactivity in both the supernatant and cellular lipid was measured by scintillation counting. The data were normalized by total [<sup>3</sup>H]-cholesterol radioactivity in the supernatant and cell pellet.

### Cholesterol repletion and depletion of endothelial cells

Cholesterol repletion and depletion of EC with methyl-β-cyclodextrin (MβCD, sigma) were performed as described previously [18]. Briefly, EC were incubated with or without prewarmed 10 mM MβCD at 37 °C for 30 min to deplete cholesterol. EC were then washed with PBS and incubated in the absence or presence of cholesterol (80 µg/mL) and 1.5 mM MβCD at 37 °C for 1 h to reload EC with cholesterol.

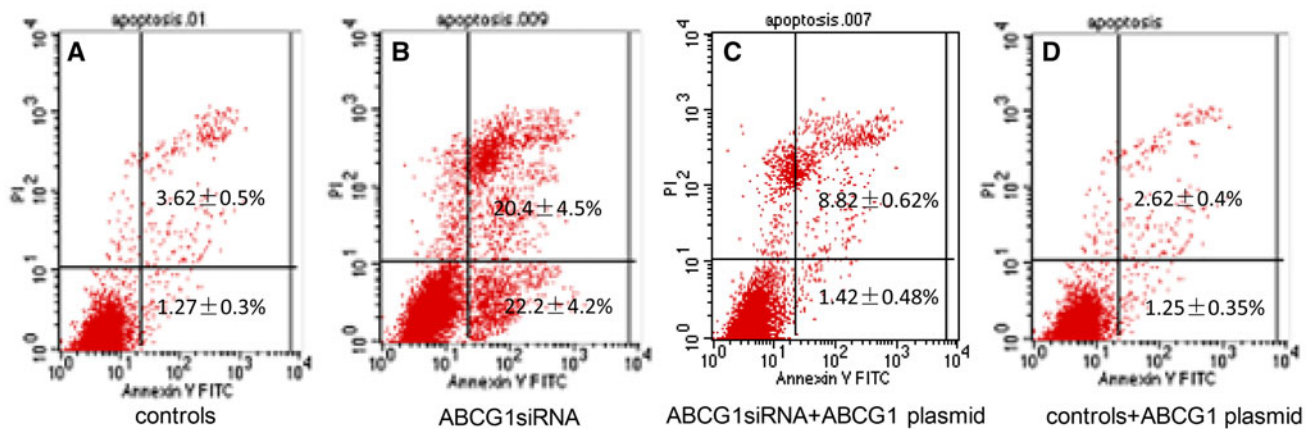
### RNA interference

The following ABCG1 siRNA sequences were designed: forward, 5'-GAGUCUUUCUUCGGGAACATT-3' and reverse, 5'-UGUCCCCGAAGAAAGACUCTT-3' (Shanghai GenePharma Co., Ltd). SiRNA for ABCG1 or random siRNA were transfected into HUAEC using TurboFect siRNA Transfection Reagent (Thermo Scientific, USA) for 24 h.



**Fig. 1** Endothelial ABCG1 expression was correlated with cholesterol efflux and intracellular lipid content. **a, b** HUAEC were transfected with scrambled siRNA, ABCG1 siRNA and either pcDNA (empty vector) or a pReceiver-ABCG1 expression vector. ABCG1 mRNA (**a**) and protein expression (**b**) were measured by real time PCR and western blotting. **c** Cholesterol efflux to HDL was decreased

in ABCG1-deficient EC (ABCG1<sup>-</sup>), and reversed in ABCG1 overexpression plasmid transfection (ABCG1<sup>+</sup>). **d** Intracellular cholesterol content was increased in ABCG1-deficient EC while decreased in ABCG1 overexpression plasmid transfection. *TC* total cholesterol, *FC* free cholesterol, *CE* cholesterol ester. Data represent mean ± SD (*n* = 3). \**P* < 0.05, \*\**P* < 0.001 vs. EC control

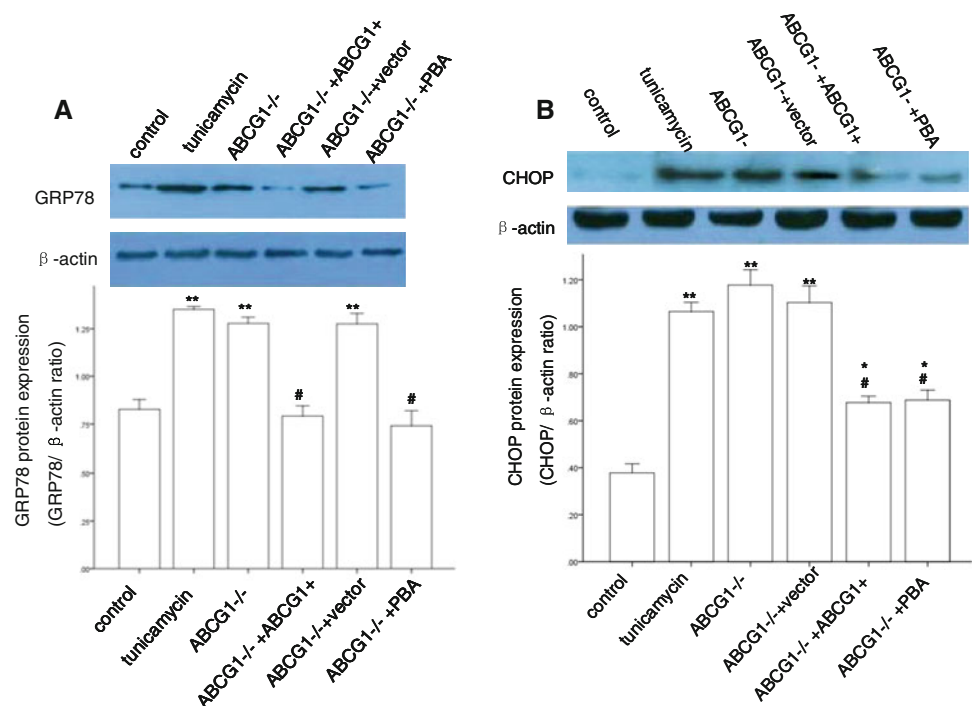


**Fig. 2** Downregulation of ABCG1 promoted endothelial apoptosis. Annexin V-FITC/PI double-staining assay was used to quantify apoptosis in HUAEC by means of flow cytometry. Endothelial apoptosis includes early apoptotic (FITC +PI<sup>-</sup>) and late apoptotic

(FITC +PI<sup>+</sup>). **a** Apoptosis in endothelial control, **b** increased apoptosis in ABCG1-deficient EC, **c** apoptosis in ABCG1-deficient EC transfected with ABCG1 plasmid, **d** apoptosis in endothelial control transfected with ABCG1 plasmid

**Fig. 3** The relationship between ABCG1 expression and ER stress-related molecules GRP78 and CHOP.

Downregulation of ABCG1 by siRNA increased the protein expression of GRP78 (**a**) and CHOP (**b**), which is similar to the effect of tunicamycin, a representative ER stress inducer. Increased expression of GRP78 and CHOP were reversed by restoration of ABCG1 expression using ABCG1 overexpression plasmid. Expression of GRP78 and CHOP were decreased in ABCG1-deficient EC treated with PBA, an ER stress inhibitor. Data are mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.001$  vs. EC control; # $P < 0.001$  vs. ABCG1-deficient EC. Representative images are shown for three independent experiments



Then, siRNA-targeted cells were subjected to various treatments.

#### Plasmid

The OmicsLink<sup>TM</sup> ORF Expression plasmid of ABCG1 (pReceiver-ABCG1 Expression vector, NO: EX-Z0509-M61) and pEZ-M61 with eGFP as control vector (NO: EX-EGFP-M61) were obtained from GeneCopoeia<sup>TM</sup>. Plasmid DNA was transfected into HUAEC with the use of a cationic polymer-based transfection reagents TurboFect<sup>TM</sup> in vitro Transfection Reagent (Thermo Scientific)

according to the manufacturer's instructions. At 48 h after transfection, cell lysates were harvested for subsequent analyses. The infected EC were subjected to various treatments.

#### Statistical analysis

The results were reported as mean  $\pm$  standard deviation (SD) of at least three measurements. One-way analysis of variance (ANOVA) was used to compare the means, and the least significant difference (LSD) test showed the statistical significance of differences. Differences were

considered significant at  $P \leq 0.05$ . All statistical analyses were performed with SPSS 19.0.

## Results

Silencing of endothelial ABCG1 resulted in reduced cholesterol efflux, increased intracellular lipid content

To determine whether silencing of ABCG1 in EC resulted in increased lipid content, we transfected EC with scramble siRNA or ABCG1 siRNA. ABCG1 mRNA and protein expression were respectively decreased by 67 and 54 % in cells transfected with ABCG1 siRNA relative to the endothelial controls (Fig. 1a, b). Silencing of ABCG1 expression in EC resulted in a 40 % reduction of HDL-mediated cholesterol efflux (Fig. 1c), a statistically significant 14.5 % increase in FC content, and a 23 % increase in cholesterol ester (CE) content (Fig. 1d). In contrast, transfection of ABCG1 overexpression plasmid to ABCG1-deficient EC reversed the case, as shown in Fig. 1a–d.

Downregulation of endothelial ABCG1 promoted endothelial apoptosis

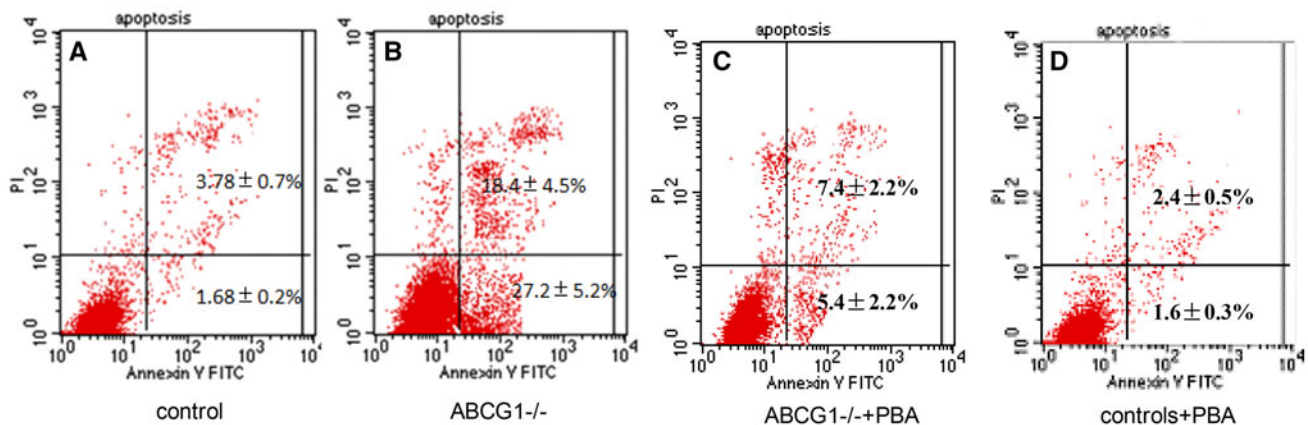
Compared with controls, endothelial apoptosis was significantly induced in EC transfection with ABCG1 siRNA (from  $4.89 \pm 0.8$  to  $42.6 \pm 8.7$  %,  $P < 0.05$ ). However, restoration of ABCG1 expression in ABCG1-deficient EC by transfection ABCG1 overexpression plasmid resulted in significant reduction in endothelial apoptosis. Transfection ABCG1 overexpression plasmid to control cells caused a small reduction in endothelial apoptosis (Fig. 2).

Downregulation of endothelial ABCG1 increased the expression of GRP78 and CHOP

To determine what mechanism involved in endothelial apoptosis in ABCG1-deficient EC, we explored the expression of GRP78 and CHOP, two important markers of ER stress. As seen in Fig. 3, the results showed that GRP78 was present in untreated control cells and increased in response to treatment with tunicamycin, a representative ER stress inducer. Similarly, silencing of endothelial ABCG1 by ABCG1 siRNA increased the level of GRP78 protein expression by 45 % compared with controls. Additionally, we examined the expression of another ER stress-related protein, CHOP, which has been shown to induce apoptosis [6]. We found that CHOP was barely present in untreated cells, but was induced by tunicamycin and ABCG1 siRNA in EC. Conversely, ABCG1 overexpression plasmid transfection significantly suppressed the expression of GRP78 and CHOP, suggesting that the loss of ABCG1 was involved in ER stress (Fig. 3).

Blocking ER stress reduced endothelial apoptosis in ABCG1-deficient EC

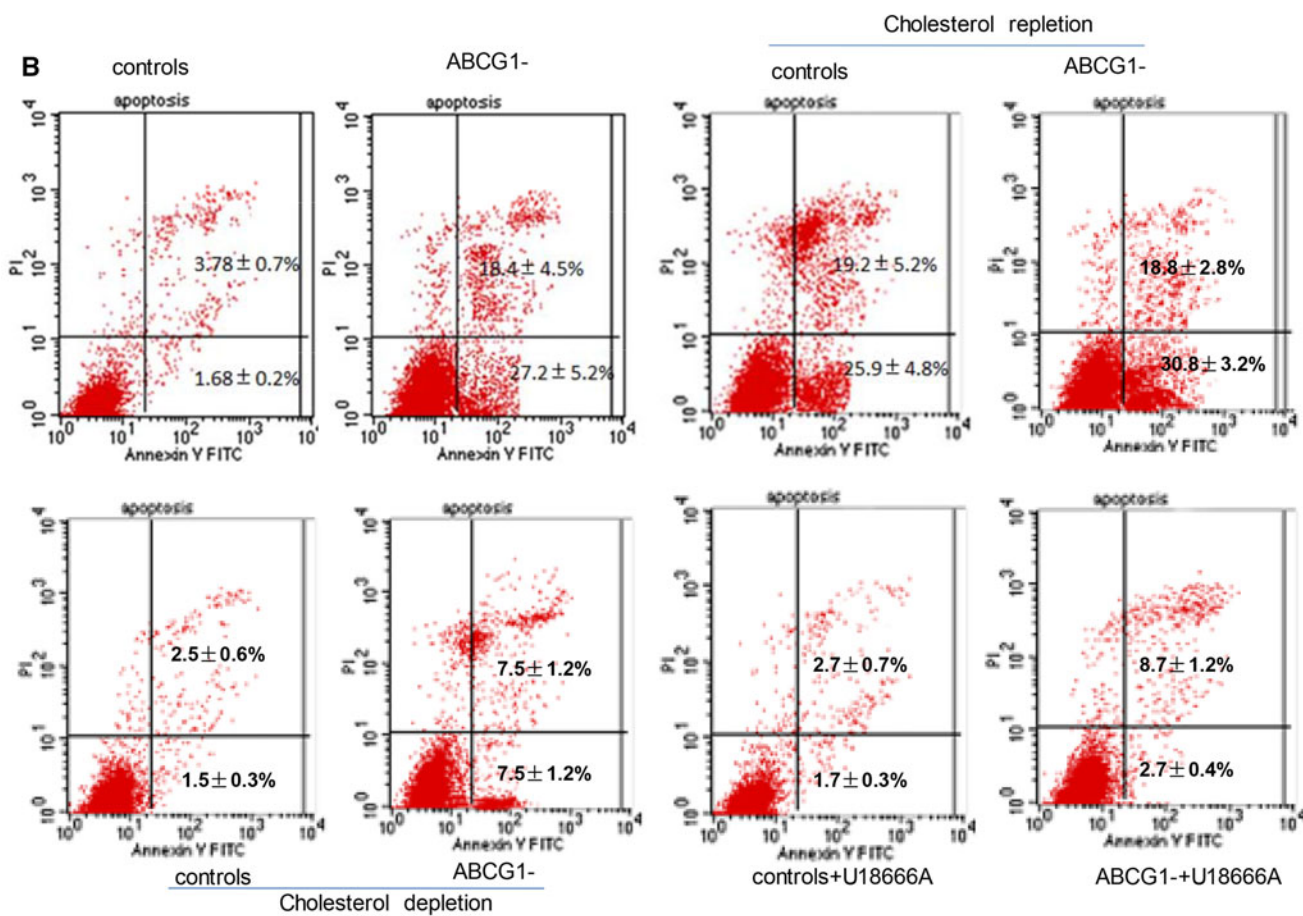
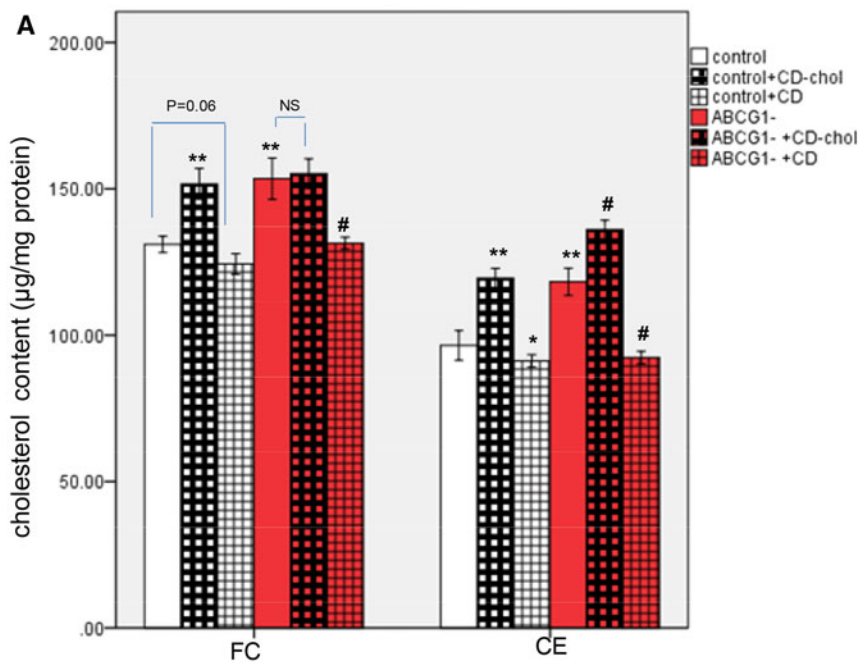
The chemical chaperone, 4-phenyl butyric acid (PBA), has been proven to alleviate ER stress [19–21]. In the present study, we showed that addition of PBA to ABCG1-deficient EC significantly reduced the expression of GRP78 and CHOP, nearly to the levels of controls (Fig. 3). We also observed that endothelial apoptosis was reduced (from  $45.6 \pm 9.7$  to  $12.8 \pm 4.4$  %,  $P < 0.05$ ) in ABCG1-deficient EC cultured with PBA (Fig. 4). It was suggested that apoptosis in ABCG1-deficient EC was associated with activation of ER stress.



**Fig. 4** Blocking ER stress reduced endothelial apoptosis in ABCG1-deficient EC. Compared with control cells (a), endothelial apoptosis was significantly induced in ABCG1-deficient EC (b). c Endothelial apoptosis was partly reversed in ABCG1-deficient EC treated with

PBA. d Endothelial apoptosis was moderately but not statistically decreased in control cells treated with PBA. Endothelial apoptosis includes early apoptotic (FITC +PI–) and late apoptotic (FITC +PI+) as described in “Methods”





**Fig. 5** Cholesterol repletion and depletion were correlated with endothelial apoptosis and activation of ER stress. **a** Cyclodextrin (CD)-cholesterol loading of control cells increased FC and CE content. CD-cholesterol loading of ABCG1-deficient EC statistically increased CE content but not FC content. Cholesterol depletion with CD lowered FC and CE content in both endothelial control and ABCG1-deficient EC. **b** Cholesterol repletion induced endothelial apoptosis in endothelial controls. Cholesterol depletion significantly reversed endothelial apoptosis in ABCG1-deficient EC. Apoptosis was suppressed in ABCG1-deficient EC treated with U18666A, a cholesterol transport to ER inhibitor. **c, d** Cholesterol repletion increased the expression of GRP78 and CHOP, and cholesterol depletion reduced the expression of GRP78 and CHOP in ABCG1-deficient EC and control cells. **e, f** Addition of U18666A to ABCG1-deficient EC suppressed expression of GRP78 and CHOP. Data are mean  $\pm$  SD ( $n = 3$ ). \* $P = 0.05$ , \*\* $P < 0.001$  vs. EC controls, # $P < 0.001$  vs. ABCG1-deficient EC. Representative images are shown for three independent experiments

### Cholesterol repletion and depletion correlated with ER stress and apoptosis of EC

Intracellular FC accumulation is a potent inducer of ER stress and subsequent apoptosis in macrophages [6, 11–13]. To focus on modulation of lipid content via ABCG1 as a possible cause of endothelial apoptosis, we loaded control EC with soluble cholesterol using cholesterol-loaded cyclodextrin (CD). As shown in Fig. 5a, CD-cholesterol loading led to a significantly higher amount of FC and CE, reaching equivalent levels for ABCG1-deficient EC. Cholesterol repletion also resulted in increased apoptotic response (Fig. 5b). However, CD-cholesterol loading of ABCG1-deficient EC resulted in a statistically increase in CE content but not FC content, and endothelial apoptosis was not further induced (Fig. 5b). In contrast, cholesterol depletion with free CD either reduced intracellular cholesterol content or reversed the percentage of endothelial apoptosis in ABCG1-deficient EC (Fig. 5a, b). This confirmed previous reports that accumulation of FC not CE is an inducer of apoptosis [11–13]. Moreover, ER stress-related molecules of GRP78 and CHOP were both enhanced in control EC loaded with CD-cholesterol while it decreased in ABCG1-deficient EC treated with CD (Fig. 5c, d). We then pretreated EC for 24 h with U18666A, a cholesterol transport to ER inhibitor, and Fig. 5 shows that protein expression of GRP78 and CHOP were decreased and endothelial apoptosis was reversed in ABCG1-deficient EC. The results suggest that accumulation and transport of FC to ER is correlated with ER stress, which is involved in endothelial apoptosis.

### Discussion

Endothelial cells form the inner lining of all blood vessels and function to maintain vascular tone and anticoagulant

properties of vessels. Endothelial cell injury is a key event in the pathogenesis of atherosclerosis [2, 3, 22]. In the present study, we found that downregulation of ABCG1 induced endothelial apoptosis in vitro, which seems to be associated with intracellular cholesterol accumulation regulated by ABCG1 deficiency and subsequent ER stress.

ABCG1, an ATP-binding cassette transporter, has a major role in inducing cellular cholesterol efflux to HDL particles. Deficiency of ABCG1 in macrophages and smooth muscle cells lead to reduced cholesterol efflux and increased CE accumulation in macrophages and smooth muscle cells, promoting foam cell formation [7–10]. ABCG1 is also highly expressed in EC, in which it is likely to aid in cholesterol homeostasis to prevent endothelial activation in the vessel wall [14–16]. In the present study, we showed that lack ABCG1 expression accumulated lipid and induced apoptosis in EC. Moreover, restoration of ABCG1 expression in ABCG1-deficient EC was able to rescue both the lipidosis and apoptosis. Similarly, there is evidence of increased numbers of apoptotic macrophages in the lung, heart, and lesions of ABCG1 $^{-/-}$  and ABCA1 $^{-/-}$  ABCG1 $^{-/-}$  mice [23–26]. Overexpression of ABCG1 in cultured macrophages attenuates cell death by stimulating the efflux of cholesterol or oxysterol to exogenous HDL [27, 28]. So, it is suggested that ABCG1-deficient EC are more prone to apoptosis, likely due to the toxic effect of lipid accumulation. However, László Seres et al. [29] recently demonstrated that functional ABCG1 expression induced cell apoptosis in macrophages. This apparent contradiction was explained by the different subcellular localization of ABCG1. When ABCG1 transporter is located in the plasma membrane, it can inhibit apoptosis by expelling cholesterol from the cell. ABCG1 can stimulate apoptosis when it locates in the intracellular membrane compartments by transport cholesterol to ER, which in turn, led to apoptosis [29]. Therefore, it is suggested that apoptosis induced by ABCG1 deficiency in the current study is likely related to regulation for cholesterol efflux of ABCG1.

Endoplasmic reticulum is now recognized as an important organelle in deciding cell life and death [6]. GRP78 is a central regulator of ER homeostasis, and CHOP is a part of the ER stress, and its prolonged expression is a potent inducer of apoptosis [6]. There is now increasing in vitro and in vivo evidence that prolonged ER stress is an important cause of macrophage apoptosis [6, 11–13]. In the present study, we showed that ABCG1-deficient EC promoted upregulation of GRP78 and CHOP, whereas upregulation of ABCG1 reversed the increased expression of both GRP78 and CHOP. This indicates that loss of ABCG1 is a potent inducer of ER stress in EC. Previous reports have shown that intracellular accumulation of excess FC induces significant macrophage death via

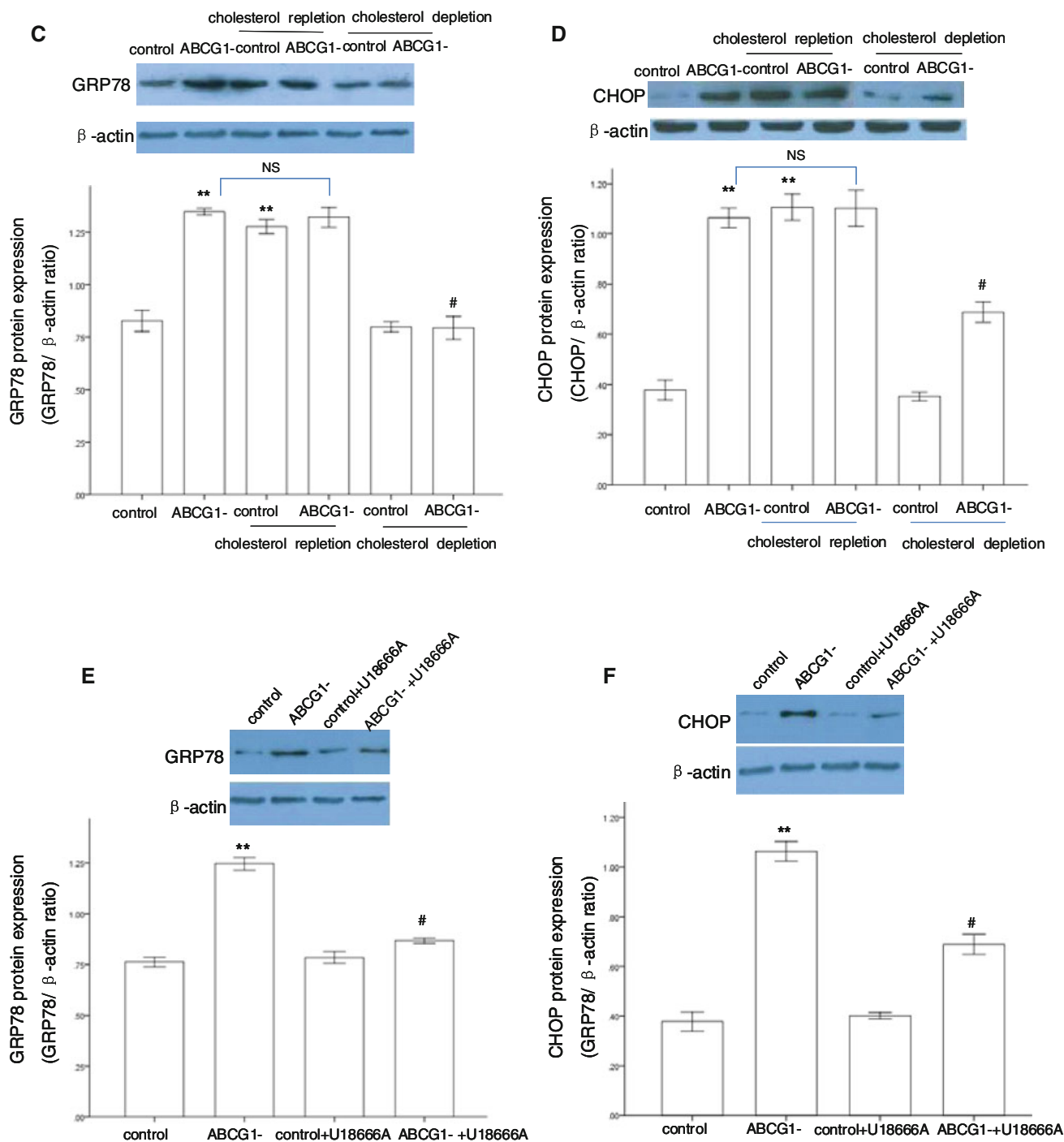


Fig. 5 continued

ER-mediated cell apoptosis [6, 11–13, 27, 28]. The current data has extended previous reports and shown that ABCG1 deficiency promoted intracellular FC accumulation and apoptosis of EC. In addition, to further confirm the role of lipid content in activation of ER stress, we loaded EC with cholesterol-loaded CD. This method is commonly used to load cells with cholesterol, and the cholesterol is rapidly

distributed into cellular pools [30]. We noticed a marked increase in cholesterol content (both of FC and CE) accompanied by increased ER stress-related factors and endothelial apoptosis in CD-cholesterol-loaded EC. However, no statistically significant higher levels of FC content were observed in ABCG1-deficient EC loaded with CD-cholesterol, although CE content was shown to be



enhanced. Similarly, neither ER stress-related molecules nor endothelial apoptosis was significantly induced. Therefore, we speculated that it was increased FC content but not CE content which trafficked to ER membrane and led to ER stress. This speculation agreed with what was previously published by Maxfield and colleagues [13], who showed that, when FC accumulated in the macrophages, the ratio of FC to phospholipids was disturbed in the ER membrane. The “stiffening” of the ER membrane bilayer leads to ER protein dysfunction and ER stress, resulting in apoptosis of the cell [13]. Furthermore, U18666A, which selectively blocked cholesterol trafficking to the ER [12, 31], was shown to reverse the increased protein expression of GRP78 and CHOP and endothelial apoptosis in ABCG1-deficient EC, further supporting the fact that activation of ER stress depends on trafficking of FC to the ER membrane.

To summarize, our results have extended previous reports in which it has been shown that ABCG1-deficient macrophages were more apoptotic. We have now demonstrated that the endothelial apoptotic effect was at least partly due to the accumulation of FC and subsequent activation of ER stress by a loss of ABCG1 transporter. Combined with previous studies, this has shown that ABCG1 knock-out contributed to atherosclerosis progression [15, 25, 32], and it is suggested that the apoptotic effect induced by ABCG1 downregulation may be one of the key factors in promoting atherosclerosis progression in the ABCG1-deficient state.

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**Conflict of interest** There are no financial or other relationships with industry that could lead to a conflict of interest.

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