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Original Article

Endoplasmic reticulum oxidoreductin 1α mediates hepatic endoplasmic reticulum stress in homocysteine-induced atherosclerosis

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Endoplasmic reticulum (ER) stress is emerging as an important modulator of different pathological process and as a mechanism contributing to homocysteine (Hcy)-induced hepar injury. However, the molecular event that Hcy-induced ER stress in the hepar under the atherosclerosis background is currently unknown. Endoplasmic reticulum oxidoreductin 1α (ERO1α) plays a crucial role in maintaining ER stress function. In this study, we determined the expression of ERO1α in the hepar in hyperhomocysteinemia and the effect of ERO1α in hepacytes ER stress in the presence of Hcy. HHcy model was established by feeding the methionine diet in apolipoprotein-E-deficient (ApoE-/-) mice, and the hepatocytes were incubated with folate and different concentrations of Hcy. Our results showed that Hcy triggered ER stress characterized by an increased contents of glucose-regulated protein 78 (GRP78), protein kinase RNA-like ER kinase (PERK), activating transcription factor (ATF) 6 and X-box binding protein-1 (XBP-1). The ERO1α expressions in HHcy mice and Hcy-treated hepatocytes were decreased compared with those in ApoE-/- group and control hepacytes ($P < 0.05$), respectively. Knocking-down the expression of ERO1α with small-interfering RNA significantly augmented Hcy-induced ER stress. Meanwhile, the expressions of ER stress-related factor including GRP78, PERK, ATF6 and XBP-1, were significantly decreased when the ERO1α gene was over-expressed in hepacytes. Our results suggested that ERO1α may be involved in Hcy-induced hepar ER stress, and the inhibition of ERO1α expression can accelerate this process.

Keywords endoplasmic reticulum stress; endoplasmic reticulum oxidoreductin 1α; homocysteine; atherosclerosis

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Introduction

In eukaryotic cells, the endoplasmic reticulum (ER) is the site of protein transportation, folding, and assembly, and it involves lipid metabolism and steroid synthesis. Hypoxia, oxidative stress and hyperhomocysteinemia (HHcy) may induce physiological dysfunction of ER and trigger ER stress [1,2]. ER stress is a kind of cellular reaction for improving survival ability in harmful conditions by stimulating unfolded protein response (UPR) signal pathway. Adapted ER stress makes for protein renovating and boosts the ability to tolerate stress and stimulation, but severe and persistent ER stress has been regarded as a novel cell apoptosis signal besides death receptor activation and mitochondria injury [3]. When cells engage the ER stress, gene expression is modulated to promote either cell survival or apoptosis, depending on the nature, extent, and duration of the stress [4]. As a typical molecular pathophysiological process, ER stress is activated in many cardiovascular, endocrine, and hepatic diseases [5]. Hepatic cells contain abundant ER and are easy to trigger ER stress in the presence of various kinds of pathophysiological factor, and as a result, hepar lipid metabolism balance is broken and hepatic function is decreased.

HHcy has long been considered as an important independent risk factor of atherosclerosis (AS), which is companied by other organ injury during its development [6,7]. Increased homocysteine (Hcy) levels has been observed in experimental animal models of ethanol and CCl_4-induced hepatotoxicity [8], and elevation of Hcy has also been found in patients with cirrhosis and chronic alcohol consumption [9]. Excessive superoxide anion that is generated by Hcy causes hepacyte oxidative stress and hepar injury [10]. Therefore, HHcy is involved in a wide range of impaired liver function. Hcy contains a free sulfhydryl group, which

can be oxidized with other thiols to form mixed disulfides. In this process, the sulfhydryl group is oxidized and reactive oxygen species (ROS) are generated. Consequently, the proteins are misfolded and ER stress occur [11]. When the misfolded proteins are formed, these proteins get through the ER membrane and trigger ER stress, consequently, hepar is impaired [11]. Hepar is the central organ in the synthesis and metabolism of lipoproteins and Hcy [12]. Hepar injury would affect the pathogenesis of AS [13]. To our knowledge, Hcy is involved in both AS and hepar ER stress. It is likely that Hcy-induced AS is closely related to hepar ER stress. Thus, alleviating hepar ER stress resulted from Hcy may be a better way to attenuate the development and progression of AS caused by HHcy. But up to now, the mechanism responsible for Hcy-induced liver ER stress, especially in AS background, is poorly understood.

ERO1α that locates on human chromosome 14 and encodes a flavoprotein oxidase is identified as the primary oxidative source in the ER [14]. It promotes the formation of the disulfide bond in the unfolded protein and serves as a redox-sensor to maintain the proper oxidative environment of the ER [15]. Mammals have two genes which encode proteins homologous to ERO1α, known as ERO1α and ERO1β [16]. Wright and colleagues [17] have found that ERO1α limits mutant proinsulin-induced ER stress. Modest inhibition of ERO1α promotes signaling in the UPR and precondition cells against severe ER stress [18]. Therefore, ERO1α is endowed with an important role for maintaining ER function and synthetic capacity. Because Hcy induces ER stress and UPR through disrupting disulfide bond formation and causing mis-folded proteins traversing the ER, we presume that ERO1α is involved in Hcy-induced hepar ER stress during AS process.

Our previous study has shown that feeding apolipoprotein-E-deficient (ApoE-/-) mice with a high methionine diet may induce HHcy, hypertriglyceridemia and AS [6]. In this study, we further observed the hepar ER stress during this process and tested the hypothesis that Hcy-induced hepar ER stress was mediated by down-regulation of ERO1α.

Materials and Methods

Animal model

Male ApoE-/- mice (Animal Center of Peking University, Beijing, China) aged 6 weeks were divided into three groups ($n = 10$ each): (1) hyperlipidaemia (HLA) group: mice were fed with regular mouse diet (0% cholesterol, 5.23% fat, 0.37% methionine, 2.39 mg/g choline, 3.19 mg/kg folate, 54.6 μg/kg B12, 14.5 mg/kg B6); (2) HHcy group: mice were fed with regular mouse diet with 1.7% methionine supplement; (3) HHcy + folate + vitamin B12 group (HHcy + FA + VB) group: mice were fed with regular mouse diet plus 1.7% methionine, folate, and vitamin B12. Wild-type C57BL/6J mice fed with regular mouse diet were used as control group. The animals were housed in a standard controlled animal care facility (six mice/cage) and were maintained in a temperature controlled room (22–25°C, 45% humidity) on a 12 : 12-h dark-light cycle for 15 weeks. The experimental procedures used on the mice were approved by the Ningxia Medical University Animal Care and Use Committee and was conducted according to the Ningxia Medical University Animal Care Committee guidelines.

Electron microscopy

Conventional electron microscopy (EM) was performed as described [19]. In brief, hepar tissues were cut into ~1 mm pieces, and placed immediately in 2.5% glutaraldehyde/1.5% paraformaldehyde solution at 4°C for 2 h. The pieces were post-fixed in 1% osmium tetroxide/1.5% potassium ferrocyanide solution. Then samples were dehydrated in ethanolacetone solution, and embedded in Epon 618. Afterwards, the epoxy blocks were sliced at 70–80 nm thickness and stained with uranyl acetate and lead citrate. All sections were observed with a Hitachi Hu-12A transmission electron microscope equipped with a Megaview III digital camera and Soft Imaging System (Olympus Corporation, Tokyo, Japan).

Cell culture and homocysteine treatment

Mouse hepatocytes HL-7702 (Institute of Biochemistry and Cell Biology, Shanghai, China) were prepared and characterized as described previously [20]. In brief, hepatocytes were cultured in serum-free William's medium E (Gibco, Gaithersburg, USA), including 20 mU/ml insulin (Sigma, St Louis, USA), 50 ng/ml epidermal growth factor and fetal calf serum (FBS, Gibco). The medium was changed every two days. The aliquot cell suspensions were seeded into 24-well plates. After incubated with different concentrations (0, 50, 100, 200, 500 μM) of Hcy (Sigma) and 30 μM folate for 18 h, cells were collected for further use.

Reverse transcription quantitative real-time polymerase chain reaction analysis

Total RNA from hepar tissues and hepatocytes were isolated using Trizol Reagent (Gibco) according to the manufacturer's instructions. First strand cDNAs were synthesized using SuperScript II reverse transcriptase (Promega, Southampton, UK). A reverse transcription quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) using SYBR® Premix-Ex Tag™ (Takara, Dalian, China). *β-actin* was used as the internal control. A Ct value was obtained for each amplification curve and a ΔCt value was calculated by subtracting the Ct value for the corresponding internal control cDNA from the Ct value for each sample and transcript. Fold changes compared with the internal control were determined by calculating

the $2^{-\Delta\Delta Ct}$, and expression results are given as the expression ratio relative to *β-actin*.

Western blot analysis

Hepar tissues and cells were lysed on ice for 30 min with lysis buffer (containing 0.15 M NaCl, 30 mM Tris, 1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, 1 mM EDTA, 10 μg/ml leupetin, 2 μg/ml pepstatin, 2 μg/ml aprotinin and 2 mM Na_3VO_4). Lysates containing equal amounts of protein (20−35 μg, measured using BCA protein assay kit; Pierce, Rockford, USA) was mixed with 6 × sample buffer and then loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and blotted onto a PVDF membrane. After blocking with TBST plus 5% (w/v) dry non-fat milk, membranes were probed with primary antibodies (1 : 2000, anti-ERO1α, anti-GRP78, anti-PERK, anti-ATF6 and anti-XBP-1; Sigma) overnight at 4°C. Subsequently, membranes were washed with TBST and probed with horseradish peroxidase conjugated anti-goat IgG secondary antibody (1 : 5000, Amersham Biosciences, Piscataway, USA) for 1 h at room temperature. After three times wash, the membranes were developed using an enhanced chemiluminescence detection kit (ECL Plus, Biyuntian, Shanghai, China). Band intensities were analyzed using a BioRad Gel Doc Imaging System with Quantity One software (BioRad, Hercules, USA) and normalized to the housekeeping band intensity (β-actin) on the same blot.

Plasmid construction and transfection of *ERO1α*

The cDNA of *ERO1α* gene was amplified using the primers 5′- CCGCTCGAGATGGGCCGCGGCTGGGGATTCTTG T-3′ (forward) and 5′-AATTCTGCAGATGAATATTCTG TAACAAGTTCCTGAAGTTTTC-3′ (reverse) and cloned into the vector pEGFP-N1 using the restriction sites *Xho*I and *Pst*I (underlined in the primer sequences). The amplified fragment was inserted in the *Xho*I/*Pst*I sites of pEGFP-N1 vector to create the recombinant plasmid, rp-ERO1α. When hypacytes were in a subconfluent state, the recombinant plasmid was transient transfected into cells using the polycationic lipid Roti-Fect (Roth, Lactan, Graz, Austria) or Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were collected to determine the transfection efficiency by qRT-PCR and western blot analysis.

siRNA silencing of *ERO1α*

Hepacytes were seeded at 70% confluence for siRNA transfection. siRNA targeting *ERO1α* was composed of three different sequences (ON-TARGET plus set of 4 duplexes, Dharmacon, Beijing, China) that match different parts of *ERO1α* mRNAs. The purpose of using three different siRNA sequences for a target gene was to cut down target effects and keep high silencing potency. The final concentration of used siRNAs was 100 nM, and siRNA transfection was performed in accordance with Lipofectamine 2000 introductions. Nucleotide sequences of siRNA are listed below: *ERO1α*-689(siRNA1): (sense) 5′-GACCAAGCAUG AUGAUUCUTT-3′, (anti-sense) 5′-AGAAUCAUCAUGC UUGGUCTT-3′; *ERO1α*-436 (siRNA2): (sense) 5′-GUGAC UACUUUAGGUAUUATT-3′, (anti-sense) 5′-UAAUACC UAAAGUAGUCACTTA-3′; *ERO1α*-958 (siRNA3): (sense) 5′-GAGCAUUCUACAGACUUAUTT-3′, (anti-sense) 5′-A UAAGUCUGUAGAAUGCUCTT-3′. Silencer negative control *FAM* siRNA (sense 5′-UUCUCCGAACGUGUC ACGUTT-3′, anti-sense: 5′-ACGUGACACGUUCGGAGA ATT-3′) was used as control. After overnight transfection, medium was substitute with complete William's medium E medium containing 10% FBS & 1% penicillin/strepeomycin. The cells were cultured for 48 h until further use. Knockdown efficiency was examined by qRT-PCR or western blot analysis.

Statistical analysis

The data were shown as mean ± standard error of the mean (SEM). For the comparisons among the experimental groups, when data presented a normal distribution and variance homogeneity, one-way analysis of variance and additional analysis of variance test were used. Otherwise, the corresponding nonparametric test was used. Statistical analysis was performed using Microsoft Excel spreadsheets and SPSS 13.0 for Windows. $P < 0.05$ was considered statistically significant.

Results

HHcy induced hepar ER structure change

To evaluate whether HHcy led to serious ER stress in hepar, we used the scanning EM to observe the cell morphology. The results showed that in the hepar ER of HLA group, there were slight pathological lesions characterized by hyperplasia and irregular arrangement of rough ER, reducing the number of smooth ER (**Fig. 1A,B**). In the HHcy group, we found an obvious swell and degranulation of rough ER, expansion of ER lumen, formation of vacuole and ecclasis of ribosome from rough ER (**Fig. 1C**). In the HHcy + FA + VB group (**Fig. 1D**), although rough ER hypertrophy was observed, these pathological damages were lighter than those of HHcy group, while the control group displayed a consistent and compact structure, with the ribosome on the ER. These results indicated that HHcy may aggravate the pathological damage of hepar ER in ApoE-/- mice.

HHcy promoted hepar ER stress

Because glucose-regulated protein 78 (GRP78) is one of the ER chaperones and is an important molecular marker of ER

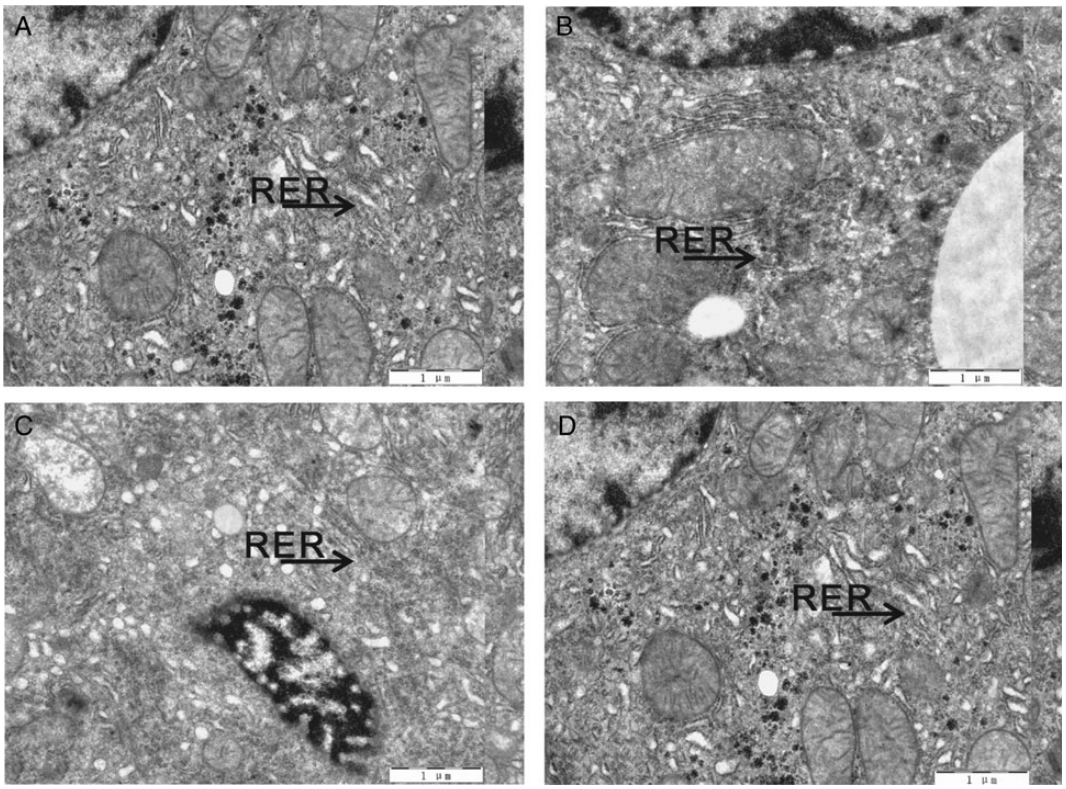

Figure 1. The morphological changes of endoplasmic reticulum in mice of each group Representative electron microscopy shows normal ultrastructure in control (A), HLA (B), HHcy (C), or HLA + FA + VB group (D) ($n = 10$/group). Arrows show hyperplasia of rough endoplasmic reticulum (RER) in hepatocyte image (×3000).

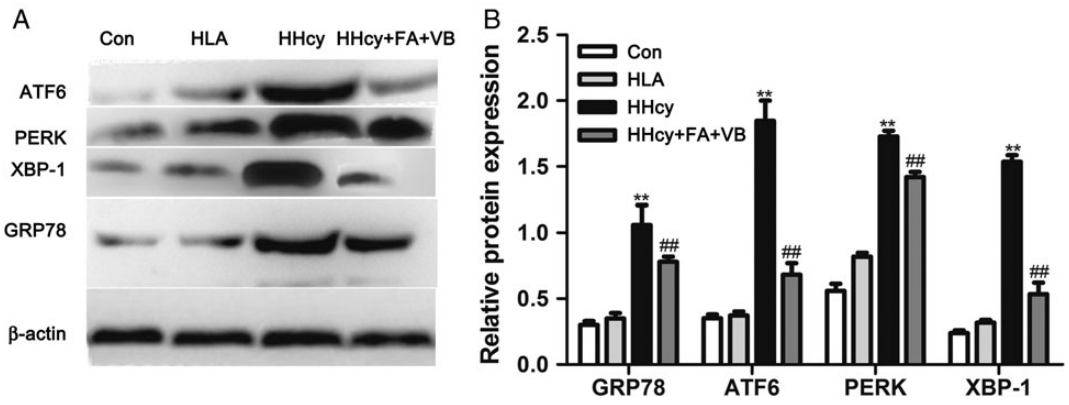

Figure 2. Expressions of GRP78, ATF6, PERK, XBP1 in the hepar of mice 15 weeks later, mice were killed and the hepar tissues were collected and lysated for determining the expression of GRP78, ATF6, PERK, and XBP1 by western blot analysis. Data were expressed as mean ± SEM ($n = 10$). $^{**}P < 0.01$ vs. Con and HLA; $^{\#\#}P < 0.01$ vs. HHcy.

stress, we further detect the expression of GRP78 in hepar by western blot analysis. Our results showed that the expression of GRP78 was increased to 2-fold times in the HHcy group when compared with the control and HLA groups, respectively. The expression of GRP78 in HHcy + FA + VB group was lower compared with that in HHcy group. There was no significant difference between the control and HLA groups. Morphological changes and a significant increase of GRP78 expression indicated aggravating ER stress in hepar from HHcy-induced AS (**Fig. 2**).

Protein kinase RNA-like ER kinase (PERK), activating transcription factor (ATF) 6 and X-box binding protein-1 (XBP-1) are three downstream targets of GRP78. In order to further confirm HHcy-induced ER stress, we then detected the expressions of these three ER stress protein in hepar. We found that ATF6 level in HHcy group was significantly elevated when compared with that in the control and HLA group. ATF6 level in HHcy + FA + VB group was lower than that in HHcy group ($P < 0.01$, **Fig. 2**). Consistent with the result from ATF6, levels of XBP-1 and PERK in HHcy

group were higher than that in HLA group, respectively. In HHcy + FA + VB group, XBP-1 and PERK levels were lowered as compared with the HHcy group ($P < 0.01$). However, there were no significant differences of PERK, XBP-1, and ATF6 between the control and HLA group ($P > 0.05$, **Fig. 2**). These results indicated that these three downstream signals were involved in hepar ER stress in HHcy-induced AS.

ERO1α expressions were up-regulated in hepar

ERO1α is a key gene in UPR and formation of disulfide bonds and is closely related to ER stress. We then observed the expression of ERO1α in hepar in HHcy-induced AS. **Figure 3** showed that the relative mRNA and protein expression of ERO1α in HLA group were decreased by 23.38% and 16.08% when compared with the control group ($P < 0.01$, **Fig. 3A,B**). In HHcy group, the mRNA and protein expression of ERO1α was decreased by 73.35% and 33.03% as compared with the HLA group. In HHcy + FA + VB group, ERO1α mRNA and protein expression was 96.42% and 29.03% more than that in the HHcy group ($P < 0.01$, **Fig. 3A,B**). Our findings suggested that ERO1α expression in hepar was decreased at both transcriptional and translational level in HHcy-induced AS.

Hcy induced down-regulation of ERO1α expression in hepacytes

We also investigated the mRNA and protein expression levels of ERO1α in hepacytes in the presence of Hcy. Consistent with the results from the mice, ERO1α expressions in hepacytes were substantially down-regulated after incubation with Hcy at various doses (50, 100, 200 and 500 μM) in a dose-dependent manner (**Fig. 4A,B**). After folate and vitamin B12 treatment, *ERO1α* mRNA

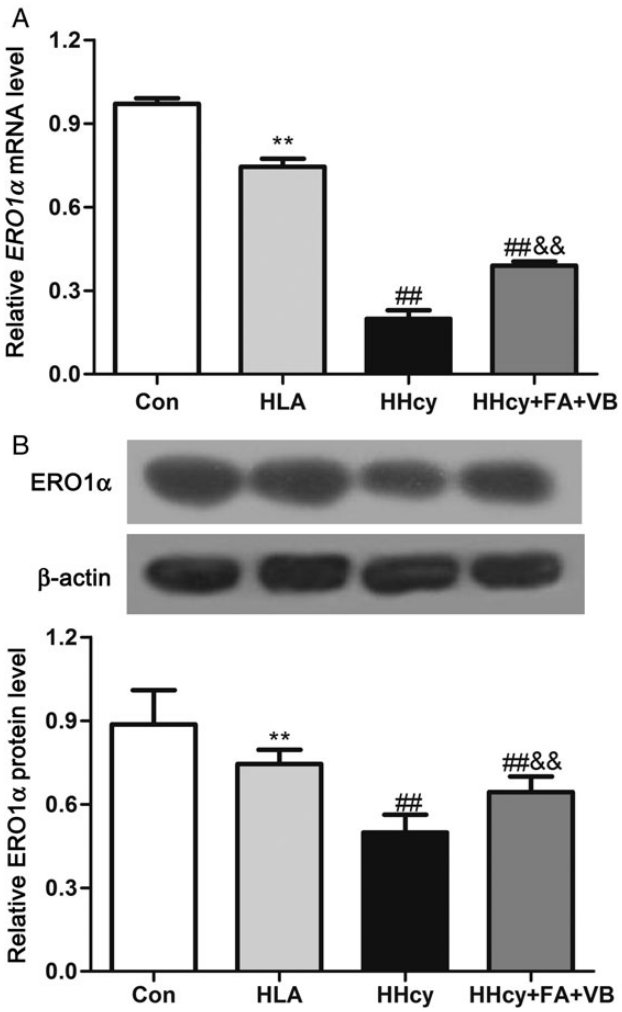

Figure 3. ERO1α mRNA and protein expressions in the hepar of mice Total RNA and protein in the hepar tissues were extracted. *ERO1α* mRNA (A) and protein (B) expression were detected by qRT-PCR and western blot analysis. Data were expressed as mean ± SEM ($n = 10$). $^{**}P < 0.01$ vs. Con; $^{\#\#}P < 0.01$ vs. HLA; $^{\&\&}P < 0.01$ vs. HHcy.

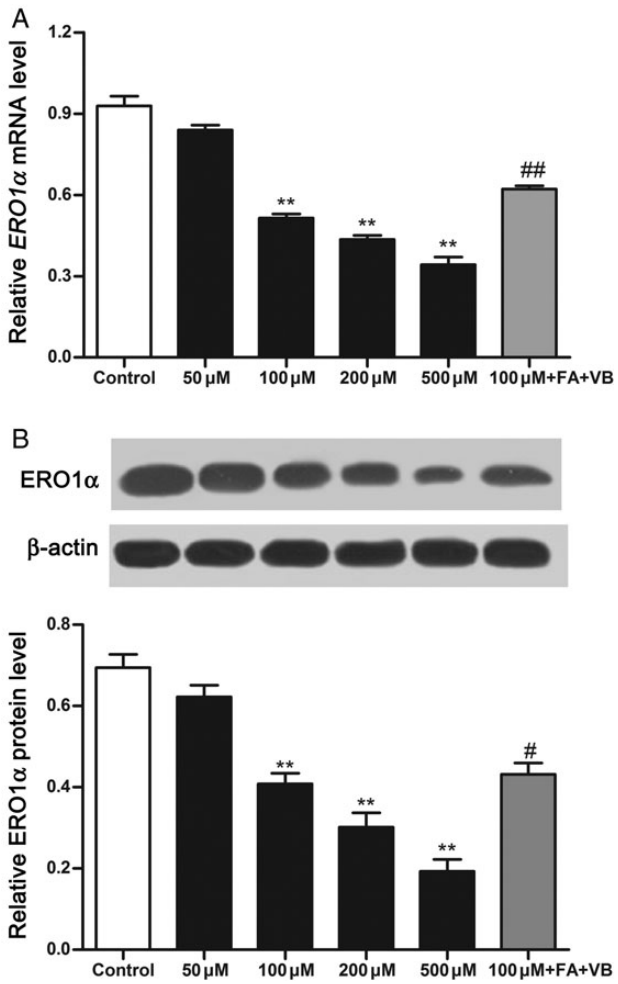

Figure 4. Effects of Hcy on ERO1α expression in hepacytes After cells were treated with Hcy for 24 h, *ERO1α* mRNA (A) and protein expression (B) in hepacytes were detected by qRT-PCR and western blot analysis. Data were expressed as mean ± SEM. $^{**}P < 0.01$ vs. Con; $^{\#}P < 0.05$ vs. 100 μM Hcy; $^{\#\#}P < 0.01$ vs. 100 μM Hcy.

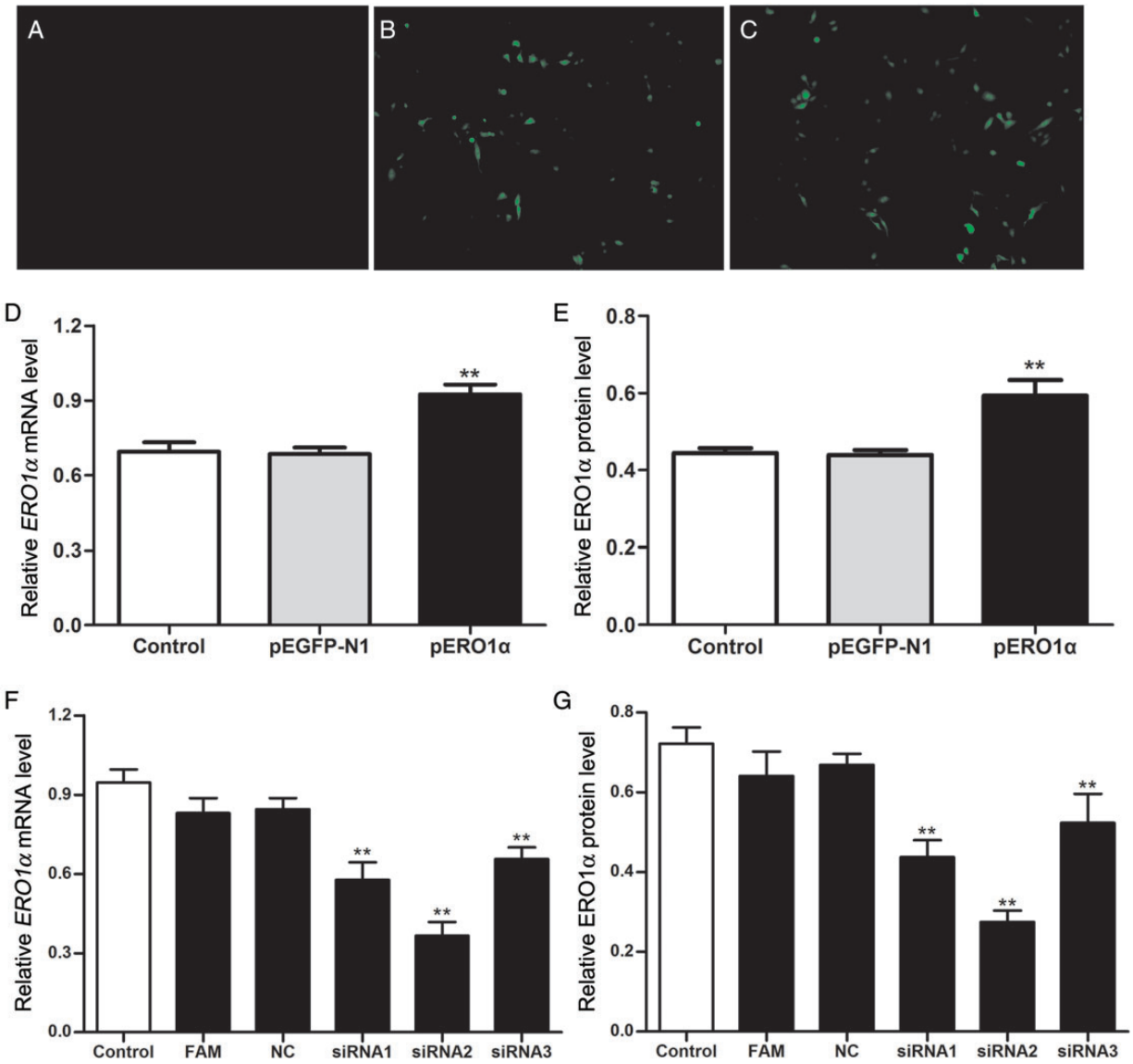

Figure 5. ERO1α expression in hepacytes When cells were in logarithmic growth phase, pEGFP-N1 plasmid which carried *ERO1α* cDNA was transfected into hepacytes, and the expression of fluorescence intensity (A, B, C), mRNA (D) and protein (E) were determined by fluorescence microscopy, qRT-PCR, and western blot analysis. Meanwhile, siRNA fragments were also transfected into hepacytes to detect the *ERO1α* mRNA (F), protein (G) expression. Data were expressed as mean ± SEM. $^{**}P < 0.01$ vs. control.

expressions were 20.73% more than that of 100 μM Hcy. The effect of Hcy on the protein levels of ERO1α in hepacytes was similar to its effect on mRNA expression. Protein level of ERO1α in 100, 200, and 500 μM Hcy groups were decreased by 41.19%, 56.51%, and 72.22% when compared with that in the control group ($P < 0.01$), respectively. Folate and vitamin B12 also affected ERO1α protein expression in the presence of Hcy. We found that the relative expression of ERO1α in 100 μM + FA + VB group were increased by 5.81% compared with the 100 μM Hcy group ($P < 0.05$, **Fig. 4A,B**). These results indicated that Hcy affected the expression of ERO1α at both transcriptional and translational levels in hepacytes.

Effect of ERO1α on Hcy-induced ER stress

In order to determine the exact function of ERO1α in hepacytes, we over-expressed *ERO1α* gene in hepacytes. *ERO1α* cDNA was introduced into the HL-7702 cells using pEGFP-N1 plasmid that carried an enhanced green fluorescence protein (EGFP). Then we observed an obvious expression of green fluorescent in the tranfected cells, but no fluorescence was found in the control (Fig. 5**A–C**). We found that mRNA expressions of ERO1α were significantly increased by 33.22% and 34.88% in recombinant plasmid group compared with the control and blank vector groups ($P < 0.01$, **Fig. 5D**). In blank vector group, there was no significant difference of ERO1α expression either at

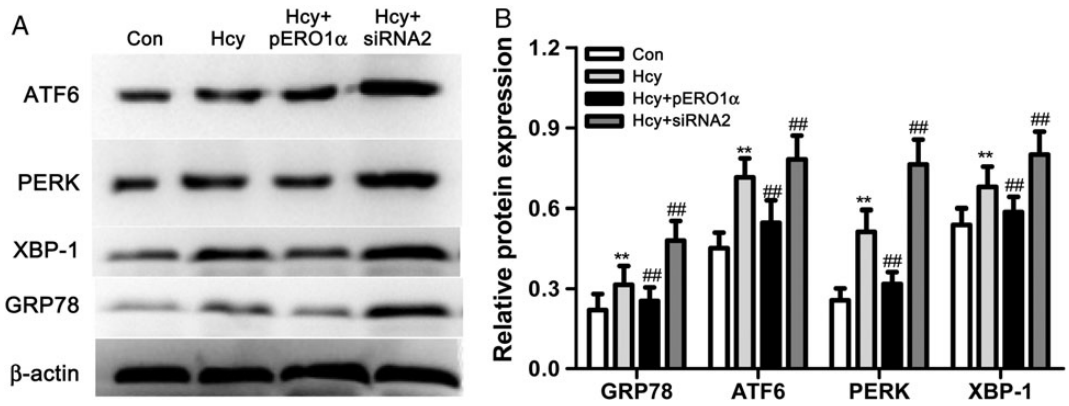

Figure 6. Involvement of ERO1α in Hcy-induced ERS After cells were transfected with p-ERO1α and si-ERO1α, Hcy was added, protein levels of ERS related factor including GRP78, ATF6, PERK, and XBP-1 were assayed by western blot anaysis. Data were expressed as mean ± SEM. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. Hcy.

transcriptional or at translational level compared with the control. Consistent with the result from mRNA, the protein expression of ERO1α in recombinant plasmid group was also increased by 33.55% and 35.13% compared with the control and blank vector groups (**Fig. 5E**). In addition, three siRNAs were transfected into hepacytes. As expected, *ERO1α* expression was blocked after siRNAs incubation. mRNA expression in hepacytes were decreased by ~38.94% ($P < 0.01$), 61.30% and 30.59% for siRNA1, siRNA2, and siRNA3, respectively (**Fig. 5F**). Similarly, its protein expression also decreased by 39.38%, 61.88%, and 27.47% for siRNA1, siRNA2, and siRNA3, respectively ($P < 0.01$, **Fig. 5G**). Because the interfering effect of siRNA2 on ERO1α expression was the highest among these three siRNAs, we chosen siRNA2 for further study.

We then observed the effects of ERO1α in hepacytes ER stress in the presence of Hcy. Consistent with results from *in vivo* experiment, after Hcy incubation, GRP8, ATF6, PERK, and XBP-1 were increased by 1.43, 1.58, 2.00, and 1.26-folds compared with the control group. Over-expression of ERO1α in hepacytes in the presence of Hcy resulted in 19.36%, 23.53%, 38.14%, and 13.55% decrease of GRP78, ATF6, PERK, and XBP-1, respectively, when compared with Hcy incubation alone, meanwhile, knockdown of *ERO1α* by siRNA2 resulted in 1.52, 1.09, 1.49, and 1.18-folds increase in the levels of GRP78, ATF6, PERK, and XBP-1, as compared with the Hcy group ($P < 0.01$, **Fig. 6**). These results indicated that ERO1α might restrain ER stress and it might be a key factor in Hcy-induced hepacytes ER stress.

Discussion

In this study, we demonstrated the involvement of ERO1α in Hcy-induced ER stress both *in vivo* and *in vitro*. In ApoE-/- mice, HHcy induced hepar ER stress which was characterized by the activation of GRP78, ATF6, XBP-1, and PERK, and ER stress could be attenuated by folate and vitamin B12. After exposure to Hcy, ER stress-related factors were also activated, while folate and vitamin B12 contributed to relieve this process in hepacytes. Over-expression or knockdown of *ERO1α* in hepacytes also induced changes of ER stress-related factors contents. These results proved for the first time that Hcy-induced ER stress in hepacytes is mediated, at least in part, by ERO1α.

Hepar is a major metabolic organ of Hcy. Evidence has shown that chronic ethanol administration alters methionine in the liver, resulting in Hcy release into the plasma. Our previous study also proved that serum Hcy levels were significantly increased in ApoE-/- mice after feeding with high methionine diet [6]. An excess of Hcy may promoted URP and induce accumulation of misfolded proteins under ER stress [21]. Wei *et al.* [22] also reported that methionine overload induced HHcy and caused a marked ER stress. Transcriptional activation of ER chaperone protein is the marker of ER stress, and GRP78 is the typical one that binds to ATF6, PERK, and IRE1 in normal condition [23]. When the cells are under stress, these transmembrane transducers will be separated from GRP78 and activate downstream signal pathway to initiate UPR [24]. Activation of PERK causes phosphorylation of the eukaryotic translation initiation factor 2a and then inhibits protein synthesis. Activated IRE1 catalyzes the removal of a small intron from the *XBP-1* mRNA, which triggers the induction of ER chaperones and other genes involved in ER-associated protein degradation [23]. ATF6 and XBP-1 may combine to the ER stress response element and the UPR element, leading to GRP78 expression [25]. Our results showed that the expressions of GRP78, ATF6, XBP-1, and PERK in hepar were increased in HHcy group. By using the EM, we also found a severe swell and degranulation of rough ER, expansion of ER lumen. Moreover, in our *in vitro* experiment, we also found the increased expressions of ER stress-related proteins including GRP78, ATF6, XBP-1, and PERK in the presence of Hcy. So we concluded that Hcy might induce hepar ER stress in AS background and it might be a strong activator of ER stress.

ERO1α plays an essential role in maintaining the redox potential in the ER, and the regulation of ERO1α activity is the central to keep redox homeostasis and proper ER folding activity [26]. Dysfunction of ERO1α leads to a rapid decline in oxidative protein folding, strong activation of the UPR, and potential source of ER-derived oxidative stress [27]. In the mammalian ER, ERO1α oxidized PDI, which, in turn, introduces disulfides into ER-related proteins to trigger URP [15]. Specific and limited PDI oxidation by ERO1α is essential to avoid ER hyperoxidation [28]. A study has shown the increased transient protein expression levels in Chinese hamster ovary cells engineered to express ERO1α [29]. Lowered levels of ERO1α can protect prokaryocyte and cultured eukaryotic cells against severe ER stress [18]**.** Here, we showed that ERO1α levels were increased in HHcy mice hepar, which was consistent with that ERO1α expression in mice were up-regulated during a much later stage of tunicamycin-induced stress response [28]. Meanwhile, we also observed a similar result of ERO1α expression in hepacytes after Hcy stimulation. When the cells were treated with vitamin B12 and folic acid, a recovery of ERO1α expression was observed. These data indicated that Hcy promoted the expression of ERO1α both *in vivo* and *in vitro*.

Over-expression of ERO1α has been shown to induce a significant increase in ROS, whereas lowering of ERO1α level by RNAi enhanced resistance to the lethal effects of high levels of ER stress [30]. Moreover, the combined elevation of ERO1α with an increased ER client protein load can have lethal consequences [28]. In order to explicitly analyze the exact effect of ERO1α in Hcy-induced ER stress, we further over-expressed *ERO1α* gene or knockdown this gene in hepacytes to detect the ER stress. Our results revealed that, in the presence of Hcy, ER stress reaction was strengthened when transcriptional activation of ERO1α was boosted in hepacytes. In contrast, contents of ER stress-related protein were decreased in the ERO1α knockdown cells. Vitamin B12 and folic acid contributed to alleviate Hcy-induced ER stress in ERO1α transfected hepacytes. As one of the traditional metabolic pathways of Hcy is transulfenyl, and ERO1α has the ability to form disulfide bond, we proposed that Hcy may transfer sulfhydryl group to other substance such as ERO1α to cause ER stress, which might be a novel mechanism for Hcy to induce AS, which needs to be further explored.

In conclusion, these data demonstrated a definite role for ERO1α in promoting ER stress in the presence of Hcy, and suggested that ERO1α may mediate the effects of metabolic perturbations in Hcy-induced AS. This finding would provide a novel strategies in clinical treatment of AS. However, the underlying mechanism of ERO1α in Hcy-induced hepar ER stress is still not clear, which will be the focus of our further study.

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