Atorvastatin attenuates atherosclerotic plaque destabilization by inhibiting endoplasmic reticulum stress in hyperhomocysteinemic mice

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Abstract. Endoplasmic reticulum (ER) stress has been suggested to play a role in the progression of plaque vulnerability and the occurrence of acute complications of coronary atherosclerosis. Atorvastatin is known to exert pleiotropic effects on the cardiovascular system. The present study aimed to examine the stabilizing effects of atorvastatin on vulnerable plaques within hyperhomocysteinemic apolipoprotein E-deficient (ApoE⁻/⁻) mice, and to investigate the potential mechanisms underlying ER stress in ApoE⁻/⁻ mice and macrophages. In the present study, ApoE⁻/⁻ mice were administrated methionine or atorvastatin, and were sacrificed after 2 months. Necrotic core size, collagen content and inflammatory cytokine infiltration were subsequently measured in the aortic lesions, in order to investigate plaque stability. Treatment with atorvastatin decreased the number and size of necrotic cores, increased collagen content, and downregulated tumor necrosis factor (TNF)-α and matrix metalloproteinase (MMP)-9 mRNA expression, as compared with the methionine group. Immunohistochemical analysis indicated that atorvastatin administration prevented ER stress activation in aortic lesions of hyperhomocysteinemic mice. Furthermore, macrophages were challenged with homocysteine (Hcy) in the presence or absence of atorvastatin and thapsigargin (an ER stress inducer). Atorvastatin suppressed Hcy-induced ER stress, and downregulated TNF-α and MMP-9 mRNA expression in the macrophages. Conversely, thapsigargin attenuated the inhibitory effects of atorvastatin against Hcy-induced TNF-α and MMP-9 expression. These results indicated that hyperhomocysteinemia may promote atherosclerotic plaque development and instability. In addition, atorvastatin was able to improve atherosclerotic plaque stability in hyperhomocysteinemic mice by inhibiting ER stress.

Introduction

Atherosclerotic plaque destabilization and rupture are thought to account for the majority of acute coronary syndromes. Rupture-prone unstable plaques possess the following histological features: Lipid core formation, fibrous cap thinning, inflammatory cell infiltration of the fibrous cap and adventitia (1). Plaque destabilization may be induced by external factors, including increased blood pressure and shear stress, and/or by factors within the atherosclerotic plaque, including inflammation and intraplaque hemorrhage. Macrophages in atherosclerotic plaques have a marked impact on atherogenesis and plaque destabilization. During the development of atherosclerotic plaques, macrophages can initiate lesion progression, destabilization and rupture via the production and release of various cytokines and proteases, including tumor necrosis factor (TNF)-α and matrix metalloproteinases (MMPs) (2). Furthermore, exacerbated macrophage apoptosis may contribute to necrotic core expansion and fibrous cap thinning during advanced stages of the disease (3).

It has previously been suggested that endoplasmic reticulum (ER) stress may have a role in the progression of plaque vulnerability, and the occurrence of acute complications associated with coronary atherosclerosis (4). ER stress is chronically activated in atherosclerotic lesional cells, particularly in advanced lesions. Macrophages and endothelial cells. In vitro and in vivo studies have demonstrated that prolonged ER stress may result in macrophage-derived foam cell formation and proatherogenic cytokine expression in advanced atherosclerotic lesions (5,6). Atherosclerotic-relevant inducers of ER stress, including modified forms of low-density lipoprotein (LDL) and hyperhomocysteinemia (HHcy), have an important role in inflammation and cell apoptosis in atherosclerotic lesions (7,8). HHcy-induced ER stress has been reported to initiate and accelerate atherosclerosis via the
upregulation of genes associated with lipid biosynthesis and uptake, inflammation, collagen synthesis and apoptosis (8). Due to its indispensable role in the progression of atherosclerosis, ER stress is therefore considered an important molecular target for the treatment of HHcy.

Atorvastatin, which is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, exerts antioxidant and anti-inflammatory functions independent of its lipid-lowering abilities. Atorvastatin has previously been shown to attenuate macrophage foam cell formation by regulating scavenger receptor expression, oxidized-LDL uptake and cholesterol efflux (9,10). In addition, atorvastatin is able to alter the cytokine balance in the microenvironment of atherosclerotic plaques (11). In our previous studies, it was demonstrated that atorvastatin could antagonize homocysteine (Hcy)-induced endothelial dysfunction by increasing the viability of endothelial progenitor cells, and suppressing the apoptosis of endothelial cells (12,13). To further define the underlying mechanisms by which atorvastatin inhibits HHCy-induced injury, the present study aimed to test the hypothesis that the plaque stabilizing effects of atorvastatin are partly attributed to inhibition of ER stress. Initially, we aimed to confirm whether atorvastatin was able to suppress ER stress activation in the vessel walls of hyperhomocysteinemic apolipoprotein E-deficient (ApoE−/−) mice. Subsequently, the ability of atorvastatin to inhibit ER stress in macrophages was investigated.

Materials and methods

Animal experiments. The 6-week-old male ApoE−/− mice were obtained from Peking University Health Science Center (Beijing, China). ApoE−/− mice were chosen since they enabled the generation of a HHcy atherosclerotic model within 2 months, following the administration of 1 ml 2% (w/v) methionine (Sigma-Aldrich, St. Louis, MO, USA) per day by gastric gavage. The mice were housed in a temperature-controlled environment with a 12-h dark-light cycle and ad libitum access to food and water. The mice were divided into three groups: The methionine group, ApoE−/− mice were administered methionine (n=15); the atorvastatin group, ApoE−/− mice were administered atorvastatin (5 mg·kg−1·d−1; National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) suspended in 1 ml 2% (w/v) methionine (Sigma-Aldrich). The mice were administered atorvastatin (5 mg·kg−1·d−1) at 12 h to mimic the administration of atorvastatin at 12 h a day by gastric gavage. The sections were analyzed using an Axiosplan 2 imaging microscopy system (Carl Zeiss GmbH, Jena, Germany).

Histological analysis. The aortic roots were carefully dissected and cleaned of adherent connective tissue under a dissecting microscope. The aortic roots were then embedded in paraffin and were serially sectioned at 4 µm. For quantification of atherosclerotic lesions, the sections were collected and stained with hematoxylin and eosin (H&E) and Masson's trichrome (Sigma-Aldrich). Images of staining were captured and quantified using an Axiosplan 2 imaging microscope system (Carl Zeiss GmbH, Jena, Germany).

Immunohistochemical (IHC) analysis. For IHC analysis, the paraffin-embedded sections were deparaffinized by immersion in xylene, followed by a series of alcohol treatments. Endogenous peroxidase activity was quenched by immersing the slides in 0.3% hydrogen peroxide in methanol for 15 min. The sections were rinsed three times in PBS (5 min/wash) and were blocked with 5% normal serum (Cell Signaling Technology, Inc., Danvers, MA, USA). The sections were then incubated with primary antibodies (1:1,000) overnight at 4°C. Rabbit polyclonal phosphorylated (p)-protein kinase RNA-like endoplasmic reticulum kinase (PERK) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA; cat. no. sc-32577); rabbit monoclonal antibodies against glucose-regulated protein 78 (GRP78; cat. no. 3177) and p-eukaryotic initiation factor 2α (eIF2α; cat. no. 3398) were obtained from Cell Signaling Technology, Inc. Following rinsing in PBS with Tween 20, the sections were incubated with labeled polymer-horseradish peroxidase (HRP) goat anti-rabbit IgG (Cell Signaling Technology, Inc.; cat. no. 7074) at 37°C for 1 h, and then incubated with diamonobenzidine chromogen. Following the final wash, the sections were counterstained with hematoxylin. The intensity of IHC staining was measured using the Axiosplan 2 imaging analysis system (Carl Zeiss GmbH). The average value in each group was calculated.
from random observation of five high power microscopic views of entire sections.

**Cell culture.** The RAW264.7 murine macrophages were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Beyotime Institute of Biotechnology, Haimen, China). The cultures were maintained at 37˚C in a humidified incubator containing 5% CO₂ until subconfluent. Cells received treatments in serum-free medium. A total of 1 and 10 µmol/l atorvastatin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), in the presence or absence of thapsigargin (2 µmol/l; Sigma-Aldrich) was added 30 min prior to 500 µmol/l Hcy (Sigma-Aldrich) stimulation for 24 h.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** According to the manufacturer’s protocol, RNA was extracted from macrophages or homogenized aortic roots using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The quality of the isolated RNA was determined using agarose gel electrophoresis, and RNA concentration was determined by measuring optical density at 260 and 280 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). A reverse transcription kit (Takara Bio Inc., Otsu, Japan) was used for reverse transcription. The following primer sequences were designed (Invitrogen; Thermo Fisher Scientific, Inc.): TNF-α, forward 5’-TTCTATGCCCCAGACCTCA-3’, reverse 5’-ACTTGTGGTTTTCCTACGACG-3’; MMP-9, forward 5’-GTCCCCTATACCTCCGACG-3’, reverse 5’-ATTGCAAGATGGTGCTGGCCG-3’; and β-actin, forward 5’-ATGGTTGGGAATGGTGCAGA-3’ and reverse 5’-GTACGGCACTTTCCCTCCT-3’. RT-qPCR was performed using SYBR® Premix Ex Taq™ (Perfect Real Time) kit (Takara Bio Inc., Otsu, Japan) and an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR was performed using an initial step of denaturation at 95˚C for 30 sec, 40 cycles of amplification, denaturation at 95˚C for 5 sec and annealing at 60˚C for 30 sec. All reactions were performed in a 20-µl volume in triplicate. The relative amounts of mRNA in untreated and treated cells were compared using the comparative cycle quantification (2ΔΔCq) method (16), with β-actin mRNA as the internal standard.

**Western blot analysis.** The cells were collected and radioimmunoprecipitation assay lysis buffer containing protease inhibitors (Beyotime Institute of Biotechnology) was used for extraction of total cellular protein. Protein concentrations were determined using a protein assay kit (Pierce Protein Biology; Thermo Fisher Scientific, Inc.). After boiling for 5 min, equal quantities of the denatured protein sample (40 µg) were separated by 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then protein was transferred electrophoretically to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The resulting membranes were blocked...
using 5% non-fat milk in Tris-buffered saline containing Tween (10 mmol/1 Tris-HCl, pH 7.4; 150 mmol/1 NaCl; 0.05% Tween-20) for 1 h, and hybridized with primary antibodies (antibody against p-PERK, 1:400 dilution; cat. no. sc-32577; Santa Cruz Biotechnology, Inc.; antibodies against GRP78 and p-eIF2α; cat. nos. 3177 and 3398; Cell Signaling Technology, Inc.; 1:1,000 dilution) overnight at 4˚C. Following incubation with the appropriate HRP-conjugated goat anti-rabbit IgG secondary antibody (1:1,000 dilution; cat. no. 7074; Cell Signaling Technology, Inc.), the immune complexes were detected using an enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Chalfont, UK). Staining was quantified by scanning densitometry (Microtek Scanwizard 5; Informer Technologies, Inc., Walnut, CA, USA) with β-actin used as an internal standard.

Statistical analysis. The obtained data are presented as the mean ± standard deviation. One-way analysis of variance was used to statistically analyze the data, using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 (two-tailed) was considered to indicate a statistically significant difference.

Results

Effects of atorvastatin on plasma Hcy levels in ApoE−/− mice. The methionine group were administered 1 ml 2% (w/v) methionine, and the atorvastatin group were administered atorvastatin (5 mg/kg·d⁻¹) suspended in 1 ml 2% methionine daily for 2 months. After 2 months, plasma Hcy levels in the methionine group were significantly increased, as compared with in the control ApoE−/− mice (32.8±3.0 vs. 5.3±1.2 µmol/l, P<0.01). In addition, plasma Hcy levels were upregulated in the atorvastatin group (25.0±3.9 µmol/l) compared with the control group; however, there was no statistical difference between the atorvastatin and methionine groups (Fig. 1).

Atorvastatin inhibits vulnerable plaque formation in the aortic roots of HHcy mice. The present study analyzed large necrotic core area and collagen content, in order to investigate plaque stability. Paraffin sections from the aortic roots of ApoE−/− mice were stained with H&E to assess lesion growth and histological morphology (Fig. 2A). To determine whether atorvastatin was able to reduce necrotic core formation, both the number and the relative size of necrotic cores was analyzed. Treatment with atorvastatin resulted in fewer necrotic cores, as compared with the methionine group; 30.83±7.91 vs. 44.2±13.12% of the sections covering the entire lesion that contained a necrotic core (P<0.05). Furthermore, the relative size of the necrotic cores decreased from 26.42±8.23 to 19.41±7.05% of the plaque surface area (P<0.05). Collagen is the main stabilizing component of plaques. Masson's trichrome staining detected a 45% increase in the relative amount of collagen in the plaques following atorvastatin treatment, as compared with that in the methionine group (17.75±5.25 vs. 12.21±4.28%, P<0.05) (Fig. 2B).

Atorvastatin reduces TNF-α and MMP-9 mRNA expression in the aortic roots of HHcy mice. To explore the effects of atorvastatin on the HHCy-induced inflammatory response in aortic roots of ApoE−/− mice, TNF-α and MMP-9 mRNA expression levels were detected by RT-qPCR. TNF-α and MMP-9 expression levels were significantly upregulated in the methionine
group, as compared with the control group, whereas treatment with atorvastatin significantly decreased the expression levels in atherosclerotic lesions (Fig. 3).

**Atorvastatin prevents ER stress activation in aortic lesions of HHcy ApoE<sup>−/−</sup> mice.** To further determine the mechanisms underlying statin-induced plaque stabilization, the present study investigated whether atorvastatin affected HHcy-induced ER stress in aortic root lesions (Fig. 4). The IHC analysis detected a significant decrease in p-PERK immunostaining in the atorvastatin group, as compared with in the methionine group. Furthermore, p-eIF2α and GRP78 immunostaining were significantly downregulated following treatment with atorvastatin, as compared with following methionine treatment only. These results indicate that atorvastatin may inhibit ER stress activation in aortic lesions of HHcy mice. The plaque stabilizing effects of atorvastatin may be due to ER stress inhibition in HHcy mice.

**Atorvastatin inhibits Hcy-induced ER stress in macrophages.** To determine the effects of atorvastatin on Hcy-induced ER stress in macrophages, various concentrations of atorvastatin (1 and 10 µmol/l) were added 30 min prior to 500 µmol/l Hcy stimulation. As shown in Fig. 5, 500 µmol/l Hcy induced ER stress activation in macrophages, as determined by the increased phosphorylation of PERK and its substrate, eIF2α, and the increased expression of the chaperone GRP78. Compared with the Hcy group, Hcy-induced ER stress was inhibited in response to 10 µmol/l atorvastatin treatment. Furthermore, thapsigargin, an ER stress inducer, attenuated the inhibitory effects of atorvastatin against Hcy-induced ER stress.

**Atorvastatin suppresses the Hcy-induced inflammatory response in macrophages.** The present study also evaluated the effects of atorvastatin on Hcy-induced TNF-α and MMP-9 mRNA expression. As shown in Fig. 6, the relative mRNA expression levels of TNF-α were significantly upregulated in response to Hcy stimulation. However, treatment with 10 µmol/l atorvastatin could evidently inhibit Hcy-induced TNF-α mRNA expression. MMP-9 expression was also significantly upregulated in the Hcy group, as compared with in the control group, whereas treatment with 10 µmol/l atorvastatin significantly decreased its expression. Furthermore, thapsigargin attenuated the inhibitory effects of atorvastatin against Hcy-induced TNF-α and MMP-9 upregulation. These results strongly suggest that ER stress pathways may participate in the reaction, and atorvastatin inhibits the Hcy-induced inflammatory response via suppressing ER stress in macrophages.

**Discussion**

The present study demonstrated that HHcy promoted the development of atherosclerotic plaques and plaque instability. Atorvastatin was able to downregulate Hcy-induced ER stress activation in atherosclerotic lesions and macrophages, which may suppress inflammatory responses and improve the stability of atherosclerotic plaques.

Alterations in atherosclerotic plaque composition can impact plaque stability, and determine the clinical course of atherosclerosis. Previous studies have suggested that the inflammatory cytokines have an important role...
in the disruption of vascular function, and the resultant
development of vascular diseases (17,18). In addition,
epidemiological studies have reported that TNF-α is mark-
edly elevated in the plasma and arteries of human patients
with vascular complications (19,20). Elevated circulating
levels of matrix biomarkers are a key feature of atheroscle-
rotic plaque development, vascular remodeling and plaque
rupture. MMP-9, which contributes to the formation of
unstable plaques, has been suggested as an atherosclerotic
inflammatory marker (21). In the present study, methionine
treatment enabled the generation of a HHcy atherosclerotic
ApoE−/− mouse model. Atherosclerotic plaques in the aortic
arteries of the mice appeared to possess several key histo-
logical features of unstable plaques, including large necrotic
cores, reduced collagen content and increased inflammatory
cytokine infiltration.

The pathophysiological environment in HHcy mice was
able to activate prolonged ER stress in the arterial wall. The
present study demonstrated that the expression of p-ERTK,
p-eIF2α and GRP78 were predominantly situated within the
atherosclerotic lesions in HHcy ApoE−/− mice. Previous studies
have revealed that ER stress is a cross-point that links cellular
processes with numerous risk factors that exist in all stages of
atherosclerosis, and is believed to have a critical role in
endothelial dysfunction (22,23), activation of inflammatory
reactions (24) and foam cell formation (25). Activation of
ER stress is associated with the severity and clinical compli-
cations of atherosclerosis in humans. In a previous study,
directional coronary atherectomy specimens demonstrated that
ruptured plaques exhibited a markedly increased expression
of the ER chaperones GRP78 and CCAAT-enhancer-binding
protein homologous protein (CHOP) (26). These findings
further supported the hypothesis that vulnerability of artery
plaques in HHcy mice may be partially attributed to ER stress
activation.

Therapeutic interventions that reduce ER stress may be
considered promising strategies to reduce Hcy-induced
vascular injury and consequent plaque instability. In the
present study, administration of atorvastatin to ApoE−/− treated
with methionine resulted in a decreased number and size of
necrotic cores, and increased collagen content in the plaques,
both compared with the plaques in the methionine group.
Downregulation of TNF-α and MMP-9 in the lesions of the
atorvastatin-treated mice further substantiated these findings.
Atorvastatin is widely used in the treatment and prevention
of atherosclerosis. Atorvastatin has previously been shown to
downregulate GRP78, caspase-12 and CHOP expression in
myocardial cells (27), and attenuate myocardial ischemia
reperfusion injury via inhibiting ER stress-related apop-
tosis (28). Atorvastatin may function as a pharmacological
inhibitor of ER stress. In the present study, treatment with
atorvastatin significantly suppressed ER stress activation and
reduced plaque vulnerability, thus indicating that atorvastatin
may maintain ER homeostasis and prevent HHcy-induced
atherosclerotic lesion progression. However, atorvastatin did
not reduce plasma Hcy levels in ApoE−/− mice, thus indicating
that the plaque stabilizing effects of atorvastatin against
Hcy injury may not depend on the lowering of Hcy levels.
Inhibition of ER stress may serve as an important role in
the plaque stabilizing effects of atorvastatin.

The present study also demonstrated that atorvas-
tatin could suppress Hcy-induced ER stress activation in
macrophages. Macrophage infiltration plays a crucial role
throughout the entire process of atherogenesis and plaque
rupture. Cytokines secreted by macrophages, including
MMPs and TNF-α, can attenuate plaque stability and prompt
rupture of atherosclerotic plaques. In the present study, treat-
ment with Hcy increased MMP-9 and TNF-α expression in
murine macrophages; however, Hcy-induced MMP-9 and
TNF-α mRNA expression was markedly attenuated by
atorvastatin. Thapsigargin attenuated the protective effects
of atorvastatin against Hcy-induced ER stress and MMP-9
and TNF-α production, thus suggesting that ER stress path-
ways have predominant roles in Hcy-induced inflammation
in macrophages. Atorvastatin may inhibit the Hcy-induced
inflammatory response via suppressing ER stress in macro-
phages.

In conclusion, the results of the present study provide a
novel insight into the protective effects of atorvastatin against
Hcy-induced vascular injury. Hcy markedly promoted the
development of atherosclerotic plaques and plaque instability.
Atorvastatin was used to antagonize Hcy-induced injury in
HHcy ApoE−/− mice and in macrophages, and it was shown to
target ER molecules and inhibit the development of
atherosclerotic lesions. Atorvastatin may therefore attenuate
the progression of atherosclerotic lesions and plaque vulner-
bility by regulating ER stress activation, which may provide a
novel interpretation of its pleiotropic effects. However,
further studies are required to fully understand the relation-
ship between atorvastatin and ER stress activation in the
treatment of atherosclerosis.

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References

1. Silvestre-Roig C, de Winther MP, Weber C, Daemen MJ,
Lutgens E and Soehnlein O: Atherosclerotic plaque destabili-
zation: Mechanisms, models, and therapeutic strategies. Circ

2. Hopkins PN: Molecular biology of atherosclerosis. Physiol

3. Gautier T, Huby T, Witztum JL, Ouzilleau B, Miller ER,
Saint-Charles F, Aucouturier P, Chapman MJ and Lesnik P:
Macrophage apoptosis exerts divergent effects on ather-
genesis as a function of lesion stage. Circulation 119:
1795-1804, 2009.

4. Tabas I: The role of endoplasmic reticulum stress in the pro-

5. Yao S, Miao C, Tian H, Sang H, Yang N, Jiao P, Han J,
Zong C and Qin S: Endoplasmic reticulum stress promotes
macrophage-derived foam cell formation by up-regulating
distribution of differentiation 36 (CD36) expression. J Biol

6. Gao J, Ishigaki Y, Yamada T, Kondo K, Yamaguchi S, Imai J,
of endoplasmic stress protein C/EBP homologous protein in
arteriosclerosis acceleration with augmented biological stress

7. McAlpine CS, Bowes AJ, Khan MI, Shi Y and Werstuck GH:
Endoplasmic reticulum stress and glycogen synthase
kinase-3β activation in apolipoprotein E-deficient mouse
models of accelerated atherosclerosis. Arterioscler Thromb


