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Original Article

Atorvastatin up-regulates TRIB3 independent of ATF4-CHOP pathway in atherosclerotic patients

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Abstract: Background: Macrophage apoptosis triggered by endoplasmic reticulum (ER) stress contributes much to atherosclerosis, especially plaque vulnerability. Activating transcription factor 4 (ATF4)-CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP)-Tribbles 3 (TRIB3) pathway is closely related to the ER stress. This study aimed to investigate the effect of atorvastatin on the ATF4-CHOP-TRIB3 pathway. Methods: Forty-seven patients were randomized into 80-mg and 20-mg atorvastatin group. Follow-up was performed at weeks 6 and 12, and complete blood chemistry, lipid assay and detection of 5 target genes (tumor protein 53, ATF4, C/EBP, CHOP and TRIB3) in monocytes/macrophages were conducted. Furthermore, the interaction between dosage and duration of therapy was evaluated. Results: After 12-week therapy, patients in both groups experienced significant reductions in ATF4 (P=0.038) and C/EBP (P=0.003) expressions. Tumor protein 53 (P=0.015) and TRIB3 (P=0.045) expressions increased markedly in 80-mg atorvastatin group. However, there was no significant difference in CHOP expression at three time-points and between atorvastatin groups. Moreover, there was no interaction between dosage and duration of therapy. Conclusions: Atorvastatin has an effect on ER stress through ATF4-CHOP pathway. Atorvastatin at a high dose is more likely to increase TRIB3 expression, but this warrants further investigation.

Keywords: Atherosclerosis, atorvastatin, monocyte, tribbles 3

Introduction

Atherothrombotic vascular diseases, such as myocardial infarction and stroke, are the leading causes of death in the industrialized world [1]. The immediate cause of these diseases is acute occlusive thrombosis in medium-sized arteries feeding critical organs. Thrombosis is triggered by the rupture or erosion of a minority of atherosclerotic plaques that have advanced to a particular stage of "vulnerability" [2]. In early-through mid-stage atherogenesis, the most prominent macrophage process is foam cell formation, which involves the ingestion and metabolism of lipoprotein-derived cholesterol. Specifically, the lipoprotein-cholesterol is trafficked from late endosomes to the endoplasmic reticulum (ER), where the enzyme acyl-CoA/cholesterol acyl transferase (ACAT) esterifies the cholesterol to cholesteryl fatty acyl esters (CEs) [3]. In advanced lesions, macrophage apoptosis increases, and the functional consequences appear to be markedly different from those in earlier lesions [4]. In particular, increasing evidence suggests that advanced lesional macrophage apoptosis is associated with the development of a key feature of so-called "vulnerable plaques": plaque necrosis [5].

Macrophage apoptosis is a critical process in the formation of necrotic cores in vulnerable atherosclerotic plaques [5]. Plaque necrosis, in turn, is thought to promote plaque disruption and arterial thrombosis, which are the proximate causes of acute cardiovascular events. *In-vitro* and *in-vivo* findings have shown that the apoptosis of smooth muscle cells and macrophages in advanced atheroma may be triggered by endoplasmic reticulum (ER) stress [6]. Furthermore, increased ER stress may occur in unstable plaques [3]. Therefore, ER stress-induced apoptosis may contribute to the plaque vulnerability [7]. It is now recognized that activating transcription factor 4 (ATF4)-CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) pathway is pivotal for the ER

stress [8, 9]. Moreover, TRIB3 has recently been reported as a novel ER stress-inducible gene, may be induced via the ATF4-CHOP pathway and is implicated in cell apoptosis [10, 11].

ER stress contributes to atherosclerosis, especially plaque vulnerability [7, 12]. However, there are no effective strategies for the inhibition of this vicious process and amelioration of ER stress-related diseases. Recently, Chen et al. [13] found that fluvastatin could protect macrophages from ER stress-induced cell death, which provides new insights into the protection of statins on ER stress. It has been confirmed that statins can exert short term as well as long term cardiovascular protective effects via their pleiotropic effects, besides their lipid lowering effect [14, 15]. However, whether statins may stabilize atherosclerotic plaques by suppressing ER stress and whether statins exert an effect on TRIB3 remain uncertain. To study the genes mentioned in the circulating macrophages would help to clarify the impact of ER stress as well as statins on macrophages in vulnerable atherosclerotic plaques.

Subjects and methods

Subjects

Forty-seven eligible patients were recruited into present study. The age of these patients ranged from 50 years to 61 years, and they had a history of myocardial infarction. The exclusion criteria were as follows: exposure to cholesterol-modulating drugs and supplements within prior 6 weeks, diagnosed diabetes or fasting glucose > 126 mg/dl (7.0 mmol/L), hepatic disease (alanine aminotransferase [ALT] or aspartate aminotransferase [AST] > 1.5 × upper limit of normal), triglycerides > 400 mg/dl (4.5 mmol/L), and familial hypercholesterolemia. Written informed consent was obtained from all the subjects before study, and all the procedures followed the tenets of the Declaration of Helsinki. The study was approved by the Institutional Review Board of the Second Hospital of Shandong University.

Study design

At screening, a questionnaire survey, fasting blood collection, and electrocardiography were conducted. Forty-seven eligible patients were randomly assigned to either 20-mg atorvastatin group or 80-mg atorvastatin group in which patients were treated with oral atorvastatin 20 or 80 mg daily. Follow-up was administered at weeks 6 and 12. At each follow up, fasting blood was collected, adverse events were recorded, and treatment compliance was evaluated. The expressions of 5 target genes in the monocyte/macrophages of blood were detected by Western blot assay.

Laboratory measurements

Blood was collected into EDTA containing tubes and plasma was separated immediately by centrifugation at 2500 × g for 10 min. Glucose was measured with the glucose oxidase method. Total cholesterol and total triglyceride were determined with enzymatic methods. High-density lipoprotein cholesterol (HDL-C) was measured in the supernatant after precipitation of apo B-containing lipoproteins with phosphotungstic acid and magnesium chloride. Low-density lipoprotein cholesterol (LDL-C) was calculated with the Friedewald formula. LDL-C was not calculated for individuals with triglyceride > 4.5 mmol/L.

Peripheral blood mononuclear cells (PBMCs) were isolated as previously reported [16], and then incubated with anti-CD14 monoclonal antibody. Monocytes/CD14 positive cells were collected and used for RNA purification.

Total protein was extracted from monocytes/CD14 positive cells, and protein concentration was determined with the Bradford method. Lysates were kept on ice for 30 min, and then centrifuged at 15,000 × g for 15 min at 4°C . The supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Primary antibodies used were as follows: TRIB3, tumor protein 53 (Tp53), activating transcription factor 4 (ATF4), CCAAT/enhancer binding protein (C/EBP), and C/EBP homologous protein (CHOP, Cell signaling). Secondary antibody was HRP-labeled antibody (Thermo Scientific Pierce). Visualization was done with enhanced chemifluorescence using a Pierce ECL Western blotting Substrate (Thermo Scientific Pierce).

Statistical analysis

Data with normal distribution are presented as mean ± standard deviation (SD) or means ± standard error (SEM). Repeated-measures

Table 1. Baseline characteristics of included patients

	Atorvastatin 20 mg (n=23)	Atorvastatin 80 mg (n=24)
Age (yr)	58.7±6.2	55.8±6.0
Sex (male/female)	14/9	15/9
SBP	162.4±25.6	154.0±17.2
DBP	91.4±17.0	94.8±14.1
TC	6.00±0.85	6.02±0.73
TG	2.28±0.86	2.66±0.82
HDL-C	1.13±0.21	1.11±0.30
LDL-C	3.82±1.24	4.18±0.63
FBG	4.70±0.52	4.89±0.61

Notes: Data with normal distribution are expressed as means ± SD. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FBG, fasting blood glucose.

Table 2. Lipid profiles of included patients after treatments

	Atorvastatin 20 mg (n=23)	Atorvastatin 80 mg (n=24)
TC	3.98±0.41	3.57±0.46*
TG	1.52±0.40	1.63±0.67
HDL-C	1.25±0.17	1.32±0.17
LDL-C	2.50±0.57	1.93±0.30*

Notes: Data with normal distribution are means ± SD. *P < 0.005 vs atorvastatin 20-mg group. Abbreviations: TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

analysis of variance (ANOVA) was used to compare the effect of atorvastatin at different doses on monocyte/macrophages. A 2-factor repeated-measure ANOVA was used to compare the temporal responses with significant interactions further explored. A two-sided P value < 0.05 was considered statistically significant whether obtained from a one-tailed or two-tailed test of significance. Statistical analysis was performed with SPSS version 13.0 (SPSS Inc., Chicago, IL).

Results

Baseline characteristics

The baseline characteristics of included patients are shown in **Table 1**. There were no significant differences in the baseline characteristics between two groups. During the study, none withdrew, and adverse events were not observed. The characteristics after treatments are shown in **Table 2**. Significant differences were found in the total cholesterol and LDL-C.

Influence of atorvastatin on monocyte-macrophages

Tp53: There were no significant differences in baseline Tp53 expression. After 12 weeks, patients in 80-mg atorvastatin group experienced an increase in Tp53 expression whereas Tp53 expression remained unchanged in 20-mg atorvastatin group. Furthermore, there was significant difference between two groups in Tp53 expression (P=0.015). However no statistically significant differences were noted among the three time-points. In addition, there was no interaction between time and dosage in both groups (**Table 3**).

ATF4: At baseline, there was no significant difference in ATF4 expression between two groups. After 12-week therapy, patients in both groups showed a decrease in ATF4 expression without significant differences between the atorvastatin 20 mg and 80 mg groups. However, significant difference was noted in ATF4 expression across three time-points (P=0.038). There was no interaction between time and dosage in both groups (**Table 3**).

C/EBP: There was no significant difference in baseline C/EBP expression between two groups. After 12-week therapy, patients in both groups showed a decrease in C/EBP expression without significant differences between the atorvastatin 20 mg and 80 mg groups. However, significant difference was noted across three time-points (P=0.003). There was no interaction between time and dosage in both groups (**Table 3**).

CHOP: There was no significant difference in baseline CHOP expression between two groups. After 12-week therapy, CHOP expression remained unchanged in both groups. Furthermore, no significant difference was noted across three time-points and between two groups. There was no interaction between time and dosage (**Table 3**).

TRIB3: There was no significant difference in baseline TRIB3 expression. After 12-week ther-

Table 3. Effects of atorvastatin on the expressions of different genes in monocytes/macrophages

	Atorvastatin 80 mg (n=24)			Atorvastatin 20 mg (n=23)			*P*		
	Baseline	Week 6	Week 12	Baseline	Week 6	Week 12	Time	Dosage	Interactions
Tp53	0.27±0.05	0.25±0.03	0.35±0.06	0.20±0.05	0.17±0.03	0.21±0.06	0.358	0.015	0.707
ATF4	0.82±0.04	0.84±0.04	0.74±0.03	0.80±0.04	0.85±0.04	0.78±0.03	0.038	0.737	0.643
CHOP	0.60±0.05	0.84±0.17	0.62±0.11	0.53±0.05	0.60±0.16	0.65±0.11	0.350	0.374	0.451
C/EBP	0.80±0.04	0.72±0.03	0.63±0.04	0.79±0.03	0.77±0.03	0.73±0.04	0.003	0.088	0.244
TRIB3	0.80±0.05	0.74±0.05	0.86±0.08	0.72±0.05	0.70±0.05	0.74±0.08	0.358	0.045	0.717

Notes: Data are means ± SEM. Abbreviations: Tp53, tumor protein 53; ATF4, activating transcription factor 4; C/EBP, CCAAT/enhancer binding protein; CHOP, C/EBP homologous protein; TRIB3, Tribbles 3.

apy, patients in atorvastatin 80 mg group experienced an increase in TRIB3 expression whereas TRIB3 expression remained unchanged in atorvastatin 20 mg group. Furthermore, there was significant difference between two groups in TRIB3 expression (P=0.045). However no significant difference was noted across three time-points, and there was no interaction between time and dosage (**Table 3**).

Associations of target gene expression with blood lipids

The LDL-C had no correlations with the expressions of Tp53 (r=0.192, P=0.195), ATF4 (r=0.085, P=0.569), CHOP (r=-0.172, P=0.249), TRIB3 (r=0.0.001, P=0.993), and C/EBP (r=0.139, P=0.352). Furthermore, the TC, TG and HDL-C had no correlations with the expressions of these target genes.

Discussion

In the present study, the effect of atorvastatin on ER stress was investigated by focusing on the ATF4-CHOP pathway and TRIB3, which may provide a better understanding of protective effect of atorvastatin on atherosclerosis.

In the TRIB3 promoter, the CCAAT-like box may be involved in the response to ER stress. By binding to the CCAAT-like box, CHOP/C/EBP promotes the transcriptional activity of TRIB3 [17]. In the present study, atorvastatin was administered to inhibit the expression of C/EBP. However, TRIB3 expression increased after treatment with atorvastatin at a high dose. It seems that the TRIB3 expression is independent of C/EBP. Previous study has showed that the ATF4-CHOP pathway is crucial for this expression [10].

CHOP, a member of the C/EBP family, can bind to TRIB3 promoter and activate TRIB3 through hetero dimerization with endogenous proteins such as C/EBP and ATF4. However, overexpressed CHOP may not activate TRIB3 due to a shortage of dimerization partners. Although C/EBP and ATF4 are both partners of CHOP, CHOP can only cooperate with ATF4 to induce TRIB3 expression at early stage and the maintenance of TRIB3 expression at late stage, whereas C/EBP is critical for the downstream signal transduction following CHOP induction during ER stress [10]. In the present study, the CHOP expression remained unchanged, suggesting that atorvastatin has no effect on CHOP expression. Hence, decreased ATF4 and C/EBP exposed unchanged CHOP to the insufficiency of dimerization partners. Not being induced by ATF4-CHOP pathway, the TRIB3 expression might be up-regulated by atorvastatin.

Over-expression of TRIB3 will selectively inhibit the transcriptional activity of CHOP [10]. That is, TRIB3 interacts with CHOP but does not promote CHOP degradation or interfere with the dimerization of CHOP [10]. However, in our study, increased TRIB3 was concomitant with unchanged CHOP and decreased ATF4, indicating that TRIB3 is out of the control of ATF4-CHOP pathway. Furthermore, TRIB3 functions as a negative regulator of ATF4 by promoting the degradation of ATF4 [18].

In the present study, our results showed that atorvastatin at a high dose resulted in a higher TRIB3 expression, and long-term therapy with atorvastatin reduced the expressions of C/EBP and ATF4 regardless of dosage. As described above, TRIB3 may regulate its own expression via repressing both CHOP and ATF4 [10]. Moreover, blocking of ATF4-CHOP pathway will reduce the TRIB3 expression. However, atorvastatin might interrupt the interaction between TRIB3 and ATF4-CHOP pathway. Enhancement of TRIB3 expression independent of ATF4-

CHOP pathway implies that atorvastatin may increase the TRIB3 expression though ATF4-CHOP pathway is suppressed. Therefore, long-term therapy with atorvastatin may ameliorate ER stress via interfering with downstream molecules including ATF4 and C/EBP. Unfortunately, atorvastatin at a high concentration would result in a higher TRIB3 expression, which was independent of the alteration of lipid profiles.

Up-regulated TRIB3 expression may increase the apoptosis of monocytes and reduce the Akt phosphorylation [19]. In the present study, the expression of Tp53, a regulator of the cycle of cell division by signaling cells to undergo apoptosis and keeping them from growing and dividing, also increased with the increase in atorvastatin dose. Therefore, it is compatible that the apoptosis of monocytes increases with the increase in atorvastatin dose.

However, the increased TRIB3 expression will cause insulin resistance in monocytes [18], which has a profound implication for glucose homeostasis. Although decreased C/EBP would promote the release of insulin [20] and thereby improve glucose homeostasis, yet incremental TRIB3 would interfere with the effects of insulin on glucose metabolism by binding to Akt and interfering with Akt phosphorylation [18]. It appears that atorvastatin may cause hyperglycaemia. Pimpinella et al. found the difference in blood glucose between controls and atorvastatin treated patients in the Collaborative Atorvastatin Diabetes Study (CARDS) could indicate more rapid deterioration of glycaemic control in patients treated with atorvastatin than among controls [21]. However, in the present study, the increased TRIB3 expression was not correlated with blood glucose.

Although atorvastatin interferes with the feedback loop of TRIB3 and ATF4-CHOP pathway, leading to an increase in TRIB3 expression, yet atorvastatin did suppress ER stress. Even though the increased TRIB3 expression may cause hyperglycaemia via inducing insulin resistance, a very large adverse effect on glycaemic control would be needed for the benefits of atorvastatin treatment to be outweighed. Furthermore, in fact glycaemic control is monitored and treated as part of routine clinical management.

Limitations

In the present study, peripheral blood mononuclear cells were investigated, but they can not be regarded as monocyte/macrophages in the atherosclerotic plaques, though viewed as a window of them. Even though our results suggest such therapy may lead to monocyte apoptosis, its clinical significance remains unclear. In order not to bring unnecessary confusion, the accumulating clinical data and experience are still the most important.

Conclusion

In summary, atorvastatin may protect against ER stress through interfering with ATF4-CHOP pathway. However, the expression of TRIB3 increases with the increase in the dose of atorvastatin that may increase the apoptosis of monocytes, which warrants further investigation. In clinical practice, In clinical practice, atorvastatin at a low dose is required for the long term therapy which not only ameliorates ER stress, reduces the apoptosis of foam cells in the atherosclerotic plaques, but facilitates glycaemic control.

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Disclosure of conflict of interest

None.

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