Effects of Simvastatin on Endoplasmic Reticulum Stress-Mediated Apoptosis in Atherosclerotic Calcification

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Abstract
Objective: The effectiveness of statins in reducing atherosclerotic calcification remains controversial. The aim of this study was to confirm that simvastatin reduces atherosclerotic calcification and stabilizes plaque by restricting endoplasmic reticulum stress (ERS)-mediated apoptosis.

Methods: Twenty-four 8-week-old male apolipoprotein E (ApoE)−/− mice (C57BL/6J genetic background) were selected and randomly divided into model (n = 12) and simvastatin (n = 12) groups. Twelve male C57BL/6J mice were selected as control group (n = 12). The mice were adaptively fed for 2 weeks and were put on a high-fat diet thereafter. After 9 weeks, they were treated with simvastatin (20 mg/kg) or phosphate-buffered saline daily for 8 weeks. Aortic sinus samples were obtained from ApoE−/− and C57BL/6J mice for hematoxylin and eosin, von Kossa, alizarin Red S, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, and immunohistochemical staining after in vivo treatment with simvastatin. In addition, mouse vascular smooth muscle cells were analyzed after exposure to simvastatin in vitro.

Results: Administration of simvastatin in vivo drastically attenuated the atherosclerosis, calcification, and apoptosis, and decreased the serum levels of triglycerides, total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol. The expression levels of glucose-regulated protein, 78 kDa (GRP78), C/EBP homologous protein (CHOP), and caspase 12 (CASP12) in the aortic sinus decreased in the simvastatin group compared with the model group.

In vitro, simvastatin or simvastatin plus ERS inhibitor (taurine) attenuated calcification and apoptosis, and reduced the expression of ERS-related proteins GRP78, CHOP, and CASP12.

Conclusion: Treatment with simvastatin suppressed atherosclerotic calcification. This effect may be mediated through the inhibition of ERS-related apoptosis.

Keywords: Endoplasmic reticulum stress; Statins; Atherosclerotic calcification; Apoptosis

Introduction
Vascular calcification, classified into medial and intimal calcification, is a common complication of chronic kidney disease, atherosclerosis, diabetes, etc. Medial calcification is typically associated with chronic kidney disease and diabetes, while intimal calcification is mainly related to atherosclerosis.[1] The degree of atherosclerotic calcification is directly correlated with the burden of atherosclerotic plaque and incidence of cardiovascular events.[2,3]

In recent years, researchers have found that vascular calcification is an active, preventable, and invertible biological process,
which is similar to the formation of bone and cartilage.\cite{4-7}

Cell apoptosis is considered an initiating mechanism of vascular calcification. The apoptosis body resembling matrix vesicle can actively absorb and gather calcium and phosphate, generate amorphous calcium phosphate, and further transform to hydroxyapatite. Apoptosis is an cell death process controlled by genes and mainly divided into endogenous pathways (mitochondrial pathway), exogenous pathways (death receptor pathway), and apoptotic pathways induced by endoplasmic reticulum stress (ERS).\cite{8}

The endoplasmic reticulum (ER) plays important roles in protein synthesis, modification and processing, folding, assembly, and the transportation of nascent peptide chains.\cite{9} Disruption of ER homeostasis leads to initiation of an adaptive process termed the unfolded protein response. The accumulation of unfolded proteins in the ER causes ERS. Upon ERS, cells mainly elicit 2 responses leading to either cellular survival or apoptosis. High-intensity or prolonged ERS impairs the restoration of homeostasis, resulting in the induction of apoptosis by ER-related molecules.\cite{10} ERS-mediated apoptosis occurs via 3 primary pathways, namely the inositol-requiring enzyme 1/ apoptosis signal regulating kinase 1c/JUN N-terminal kinase (IRE1/ASK1/JNK) pathway, caspase 12 (CASP12) kinase pathway, and C/EBP homologous protein/DNA damage-inducible gene 153 (CHOP/GADD153) pathway.\cite{11} Recent studies have discovered that cell apoptosis mediated by ERS participates in the development of atherosclerosis and vascular calcification.\cite{12-15}

The statins 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors are mainly used to control coronary heart disease and stroke by reducing the plasma levels of low-density lipoprotein cholesterol (LDL-C). Recent studies have shown that statins, which play an essential role in preventing cardiovascular events, exert multiple effects besides reducing serum lipids; these findings have extended the clinical application of these agents.\cite{16,17} Indeed, the detailed mechanism of statins involved in stabilizing atherosclerotic plaque remains unclear. Moreover, the ability of statins to inhibit atherosclerotic calcification remains controversial. Thus far, there are no unified final conclusions drawn regarding the potential reduction of atherosclerotic calcification and stabilization of the plaque by restricting ERS-mediated apoptosis. Based on this hypothesis, the present study was conducted to investigate the effect of simvastatin (one of the most widely used statins) on atherosclerotic calcification, as well as the potential mechanism involved in this process. The purpose of this research was to provide a new target for the treatment of clinical atherosclerotic calcification.

**Materials and methods**

**Animal experiments**

Animal studies complied with the Animal Management Rule of the Ministry of Health, People’s Republic of China (documentation 55, 2001) and were approved by the Institutional Animal Care and Use Committee of Academy of Military Medical Sciences (IACUC-DWZX-2020-602). All animals were obtained from the Animal Center, Health Science Center, Peking University, Beijing, China. The experiment included 24 male apolipoprotein from the Animal Center, Health Science Center, Peking University, Care and Use Committee of Academy of Military Medical Sciences (catalog number CRL-2797). They were incubated in 90% Dulbecco’s modified Eagle's medium supplemented with 10% fetal bovine serum medium and a 1% antibiotic-antimycotic mixture in an atmosphere of 95% air and 5% CO₂ at 37°C in plastic flasks. For calcification, VSMCs were cultured in calcifying media containing 2.5 mmol/L Ca²⁺ (0.7 mmol/L CaCl₂) was added to Dulbecco’s modified Eagle’s medium containing 1.8 mmol/L CaCl₂) and 5 mmol/L β-glycerophosphate for 21 days. Subsequently, the calcified VSMCs were treated with simvastatin (1 mmol/L), taurine (TAU) (5 mmol/L), or simvastatin (1 mmol/L) plus TAU (5 mmol/L) for 72 hours.\cite{18}

**Cell culture and treatment**

Vascular smooth muscle cells (VSMCs) were obtained from the American Type Culture Collection (Manassas, Virginia, USA; catalog number CRL-2797). They were incubated in 90% Dulbecco’s modified Eagle's medium supplemented with 10% fetal bovine serum medium and a 1% antibiotic-antimycotic mixture in an atmosphere of 95% air and 5% CO₂ at 37°C in plastic flasks. For calcification, VSMCs were cultured in calcifying media containing 2.5 mmol/L Ca²⁺ (0.7 mmol/L CaCl₂) was added to Dulbecco’s modified Eagle’s medium containing 1.8 mmol/L CaCl₂) and 5 mmol/L β-glycerophosphate for 21 days. Subsequently, the calcified VSMCs were treated with simvastatin (1 mmol/L), taurine (TAU) (5 mmol/L), or simvastatin (1 mmol/L) plus TAU (5 mmol/L) for 72 hours.\cite{19}

**Measurement of lipid levels in serum**

The serum levels of cholesterol and triglycerides (TG) were measured using an automated biochemical analyzer (Mindray, Shenzhen, China). Serum samples were extracted from blood drawn at the time of mouse sacrifice, following an overnight fast.

**Analysis of atherosclerotic lesions**

The mice were anesthetized and euthanized at the end of the experiment. The heart and aorta were removed and placed in 4% paraformaldehyde for 24 hours. Serial paraffin-embedded sections (thickness: 4 μm) from the root of the aorta and at the level of the aortic valves in the aortic sinus were produced and stained with hematoxylin and eosin (HE). Quantification and analysis of the atherosclerotic lesion areas were performed by a trained observer blinded to the allocation of the experimental mice.

**Von Kossa staining**

After deparaffinization, the sections were immersed in 2% silver nitrate for 60 minutes under an ultraviolet light, followed by immersion in 5% sodium thiosulfate for 2 minutes and 0.1% nuclear fast red for 1 minute. Subsequently, the sections were observed and photographed using an Olympus BX 53 microscope (Olympus Optical, Tokyo, Japan) for further analysis of atherosclerotic calcification.

**Alizarin Red S staining**

Alizarin Red S staining was used to identify calcium. VSMCs cultured in 12-well plates were washed 3 times with PBS (250 μL), fixed with 95% ethanol for 15 minutes, and exposed to Alizarin Red S solution. The cultures were washed again with distilled water and observed using the Olympus BX 53 microscope.

**Alkaline phosphatase (ALP) activity assay**

ALP activity was measured using a p-nitrophenyl substrate supplied with an ALP Assay Kit (Beihuakangtai Clinical Reagent Co., Beijing, China). The results were normalized according to the total protein concentration.

**Flow cytometry**

VSMCs cultured in 12-well plates were washed 3 times with PBS after centrifugation, mixed in binding buffer (100 μL), Annexin V-fluorescein isothiocyanate (10 μL), and propidium iodide (5 μL) at room temperature in the dark for 30 minutes. Next, PBS (400 μL) was added and the rate of apoptosis was determined through flow cytometry.
**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining**

Apoptotic cells in the aortic sinus were detected using TUNEL staining according to the instructions provided by the manufacturer (Roche Applied Science, Indianapolis, Indiana, USA). For the quantitative analysis of cell apoptosis, TUNEL-positive cells were counted from 10 randomly selected fields in each section.

**Immunohistochemical staining**

Sections were incubated in 0.3% hydrogen peroxide for 10 minutes at room temperature to block endogenous peroxidase and incubated with 2% bovine serum albumin in PBS for 30 minutes at room temperature. Next, the sections were incubated with primary antibodies that included glucose-regulated protein, 78 kDa (GRP78) antibody (1:300; Abcam, Cambridge, UK), CHOP antibody (1:300; Abcam), and CASP12 antibody (1:300; Abcam) for 2 hours at room temperature. This was followed by incubation with goat anti-rabbit immunoglobulin M (1:500; ZSGB-BIO, Beijing, China) secondary antibody for 10 minutes at room temperature. This was followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase; Abcam, Cambridge, United Kingdom) for 40 minutes. The expression of ERS-related proteins. Equal amounts of proteins extracted from VSMCs were subjected to sodium dodecyl sulfate-polyacryl-amide gel electrophoresis. Subsequently, the proteins were transferred to polyvinylidene fluoride membranes for blocking with nonspecific proteins using 5% nonfat dried milk for 1 hour. Thereafter, the membrane was probed with the primary antibodies anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; 1:10,000; Ruierkang-BIO, Tianjing, China), anti-GRP78 (1:500), anti-CASP12 (1:500), and anti-CHOP (1:1000) at 4 °C overnight. Next, they were incubated with secondary antibody (anti-goat or anti-rabbit IgG conjugated to horseradish peroxidase; Abcam, Cambridge, United Kingdom) for 40 minutes. The expression of GRP78, CASP12, and CHOP was evaluated by the manufacturer (Roche Applied Science, Indianapolis, Indiana, USA) image software and compared with the expression of GAPDH.

**Western blotting analysis**

Western blotting analysis was used to determine the expression of ERS-related proteins. Equal amounts of proteins extracted from VSMCs were subjected to sodium dodecyl sulfate-polyacryl-amide gel electrophoresis. Subsequently, the proteins were transferred to polyvinylidene fluoride membranes for blocking with nonspecific proteins using 5% nonfat dried milk for 1 hour. Thereafter, the membrane was probed with the primary antibodies anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; 1:10,000; Ruierkang-BIO, Tianjing, China), anti-GRP78 (1:500), anti-CASP12 (1:500), and anti-CHOP (1:1000) at 4 °C overnight. Next, they were incubated with secondary antibody (anti-goat or anti-rabbit IgG conjugated to horseradish peroxidase; Abcam, Cambridge, United Kingdom) for 40 minutes. The expression of GRP78, CASP12, and CHOP was evaluated by the manufacturer (Roche Applied Science, Indianapolis, Indiana, USA) image software and compared with the expression of GAPDH.

**Statistical analysis**

Statistical analysis was performed using the SPSS 13.0 software (SPSS Inc., Chicago, Illinois, USA). Data were expressed as the mean ± standard error of the mean (SME). One-way analysis of variance was employed for multiple data comparisons. For all comparisons, *P* values <0.05 denoted statistically significant differences.

### Results

**Effects of simvastatin on body weight and serum lipid levels in ApoE−/− mice**

The body weight of mice in the 3 groups increased gradually with time. By the end of the experiment, the mean body weight increased to (38.2±3.1) g, (31.0±1.6) g, and (30.5±2.6) g in the control, model, and simvastatin groups, respectively. As shown in Table 1, the levels of lipid parameters in serum, namely TG, total cholesterol (TC), LDL-C, and high-density lipoprotein cholesterol (HDL-C), were significantly higher in the model group versus the control and simvastatin groups (P<0.01). Interestingly, although the levels of HDL-C decreased in the simvastatin group, the ratio of HDL-C/LDL-C tended to increase after the administration of simvastatin (0.167±0.005 vs. 0.154±0.003, *P = 0.09*).

**Simvastatin reduced atherosclerotic lesions and atherosclerotic calcification in the aortic sinus**

HE staining of the aortic sinus did not reveal obvious atherosclerotic lesions in the control group. The mean plaque area of the aortic sinus in the simvastatin group was significantly smaller than that observed for the model group (*P<0.05*) [Figure 1A and 1C]. Atherosclerotic calcification in the aortic sinus was stained black/brown via von Kossa staining. There was no obvious calcium deposit observed in the control group. A large number of calcium deposits were discovered in the model group [Figure 1B]; however, this number was smaller in the simvastatin group. The percentages of calcification areas for the simvastatin and model groups exhibited statistically significant differences (2.33%±0.73% vs. 10.87%±2.41%, respectively; *P<0.05*) [Figure 1D].

**Simvastatin and ERS inhibitor reduced ALP activity and calcification in vitro**

We detected differences in calcified nodules and ALP activity between the groups. On day 21 after incubation of VSMCs with calcifying media in vitro, positive calcified nodules (Alizarin Red S staining) were detected and ALP activity was increased. Subsequently, we investigated the effects of simvastatin or TAU (ERS inhibitor) on ALP activity and calcification of VSMCs in vitro. The area of calcified nodules and ALP activity were reduced after treatment with simvastatin, TAU, or simvastatin plus TAU [Figure 2A and 2B]. Interestingly, the most significant decreases in calcified nodules and ALP activity were observed after treatment with simvastatin plus TAU.

**Simvastatin or TAU inhibited apoptosis in the aortic sinus or VSMCs in vitro**

Apoptosis was quantified to investigate the mechanisms underlying the suppression of atherosclerotic calcification by simvastatin. In vivo, abundant brown nuclei were found in all

### Table 1: Comparison of lipid levels (mmol/L) in the serum between the 3 groups (*n = 12*), mean ± SME.

| Groups     | TG       | TC       | LDL-C    | HDL-C    |
|------------|----------|----------|----------|----------|
| Control    | 0.32±0.03| 1.39±0.06| 1.00±0.09| 0.31±0.04|
| Model      | 1.43±0.05*| 2.42±1.39*| 19.77±1.19*| 3.05±0.10*|
| Simvastatin| 0.85±0.09†| 8.57±6.15†| 9.17±1.60†| 1.52±0.19†|

* † P < 0.01, versus the control group; 
  † P < 0.01, versus the model group.

HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TC: Total cholesterol; TG: Triglycerides.
groups [Figure 3A]. Compared with the control group, the model group showed a significantly increased rate of apoptosis (11.85% ± 3.62% vs. 30.83% ± 4.02%, respectively; \( P < 0.05 \)); of note, this rate was significantly lower in the simvastatin group (19.67% ± 3.20%, \( P < 0.05 \)) [Figure 3B]. In vitro, the rates of apoptosis in VSMCs following different interventions were compared using flow cytometry. Similarly, compared with the control group, the rate of apoptosis in calcified VSMCs was significantly increased. Notably, this rate was significantly decreased after treatment with simvastatin, TAU, or simvastatin plus TAU. Interestingly, the most significant decrease in apoptosis was noted in the group treated with simvastatin plus TAU [Figure 3C and 3D].

**Simvastatin alleviated ERS in the aortic sinus or VSMCs in vitro**

We evaluated the expression of ERS-related proteins to explore the potential reasons responsible for the suppressive effects of simvastatin on apoptosis in the aortic sinus in vivo. The expression of the ERS chaperone GRP78 was significantly upregulated by approximately 58% in the model group versus the control.
However, this effect was reversed by simvastatin ($P < 0.05$). As key molecules of ERS-associated apoptosis, caspase 12 and CHOP showed statistically significant increases in the model group versus the control group. In the presence of simvastatin, the expression levels of CASP12 and CHOP were markedly decreased (both $P < 0.05$) [Figure 4A-D]. In vitro, the expression levels of ERS-related proteins following different interventions were compared using western blotting. Similarly, the expression of ERS-associated proteins GRP78, CASP12, and CHOP was significantly downregulated after treatment with simvastatin, TAU, or simvastatin plus TAU [Figure 4E and 4F]. These findings indicated that simvastatin may restrain apoptosis by alleviating ERS in the aortic sinus or VSMCs in vitro.

**Discussion**

Atherosclerotic calcification, which has been considered a vulnerable plaque diagnostic criterion by the American Heart Association, is characteristic of atherosclerosis and associated with cardiovascular events. Therefore, studying atherosclerotic calcification lesions is important for the development of strategies to stabilize plaques and reduce the incidence of cardiovascular events. It has been reported that statins play an important role in preventing cardiovascular events. Nevertheless, it remains unknown whether they can stabilize plaque by reducing atherosclerotic calcification mediated by ERS-related apoptosis. In the present study, we showed that ApoE−/− mice displayed distinctly increased atherosclerosis, calcification, and apoptosis, as well as expression levels of GRP78, CHOP, and CASP12 in aortic sinus tissues; these elevations were reversed by the administration of simvastatin. Similarly, we also found that calcification of VSMCs was reduced by suppression of ERS-related apoptosis in vitro. In brief, simvastatin suppressed atherosclerotic calcification, and this effect may be mediated through the inhibition of ERS-related apoptosis in atherosclerotic plaques.
Figure 3: Comparison of apoptosis in the aortic sinus between the control, model, and simvastatin groups. (A) Representative aortic sinus sections stained using a TUNEL kit. The bar represents 20 μm. (B) The rate of apoptosis in the aortic sinus was determined ($n = 12$). $^* P < 0.05$, versus the control group; $^† P < 0.05$, versus the model group. (C) The rate of apoptosis in representative calcified VSMCs was determined using flow cytometry ($n = 6$). (D) Flow cytometry showing the rate of apoptosis in VSMCs. $^* P < 0.05$, versus the calcification group. Cal: Calcium; Con: Control; Sim: Simvastatin; TAU: Taurine; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; VSMCs: Vascular smooth muscle cells.
Recent studies have shown that statins prevent coronary heart disease through multiple effects, including anti-inflammatory actions, antioxidative properties, and improvement in endothelial dysfunction. \(^{[24]}\) The influence of statins on stabilizing plaque and lowering the risk of mortality caused by cardiovascular diseases has been widely accepted. In this experiment, we established an atherosclerotic calcification model using ApoE\(^{-}\) mice fed with a high-fat diet. The blood lipid parameters of mice (TG, TC, LDL-C, and HDL-C) were significantly higher in the model group versus the control group. Moreover, the plaque area and calcification were significantly greater in the model group versus the control group. These findings revealed that ApoE\(^{-}\) mice could be used to duplicate an atherosclerotic calcification model. We found that the atherosclerotic plaque

Figure 4: Expression of ERS-related proteins. The expression of GRP78 (A), CHOP (B), and CASP12 (C) proteins in the aortic sinus was determined using immunohistochemistry. The bar represents 20 μm. (D) Quantitative analysis of GRP78, CHOP, and CASP12 protein levels using optical densitometry. \(n = 12\) mice per condition. \(^{*}P<0.01, \text{versus the control group; } ^{†}P<0.05, \text{versus the model group.}\) Expression of ERS-related proteins. (E and F) The expression of GRP78, CHOP, and CASP12 proteins in representative calcified VSMCs was determined using western blotting. Cal: Calcium; CASP12: Caspase 12; Con: Control; ERS: Endoplasmic reticulum stress; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GRP78: Glucose-regulated protein, 78kDa; Sim: Simvastatin; TAU: Taurine; VSMCs: Vascular smooth muscle cells.
was obviously smaller in the simvastatin group versus the model group, suggesting that simvastatin could significantly restrain atherosclerotic plaque. This finding is consistent with the results of previous studies.\textsuperscript{2,3,6}

Nonetheless, the ability of statins to inhibit calcification in the plaque remains controversial. Evidence from a study conducted in LDL receptor−/− mice suggested that the progression of artery calcification was suppressed through inhibition of the inflammation mediators tumor necrosis factor-α (TNF-α) and TNF receptor 1 (TNFR1).\textsuperscript{2,7} Li et al\textsuperscript{2,8} demonstrated that atorvastatin alleviated calcification in both rat arteries and VSMCs. Shavelle et al\textsuperscript{2,9} showed that statins inhibited coronary artery and valve calcifications in patients with hyperlipidemia. However, Emmanuel et al\textsuperscript{10} illustrated that treatment with lovastatin resulted in the generation and development of vascular calcification by upregulating the expression of bone morphogenetic protein 2 (BMP2) in VSMCs. Healy et al\textsuperscript{11} found that statins facilitated the calcification process by upregulating the expression of bone morphogenetic protein 2 (BMP2) in VSMCs. Healy et al\textsuperscript{11} found that statins facilitated the calcification process by upregulating the expression of bone morphogenetic protein 2 (BMP2) in VSMCs.

In vitro, we found that calcification, apoptosis, and the expression of ERS-related proteins in VSMCs were significantly alleviated after treatment with simvastatin, TAU, or simvastatin plus TAU. Our in vivo experiment revealed that both simvastatin and TAU can inhibit ERS-induced apoptosis and further reduce calcification. These findings suggested that the combination of simvastatin with TAU exerted a synergistic effect. Thus, we concluded that inhibition of the ERS-related apoptosis may be a mechanism involved in the alleviation of atherosclerotic calcification by simvastatin. Nevertheless, the mechanisms of vascular calcification and other mechanisms involved in the reduction of atherosclerotic calcification by simvastatin were not elucidated in the present study. Hence, further investigation is warranted for the development of therapeutic strategies in this setting.

**Author contributions**

Jianhua Li, Libo Zhao, Zhe Zhou, Lin Liu, Xiaojun, and Weihao Xu collected and analyzed the data. Jianhua Li, Libo Zhao, and Zhe Zhou drafted the manuscript. Li Fan, Vijaysinh, and Shengqi Wang designed this study. All the authors contributed to the design of this research study and reviewed the manuscript.

**Conflicts of interest**

None.

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[21] Yan, and Shengqi Wang designed this study. All the authors contributed to the design of this research study and reviewed the manuscript.

**Conclusion**

Our study demonstrates that treatment with simvastatin suppress atherosclerotic calcification. Inhibition of the ERS-related apoptosis may be a mechanism in the alleviation of atherosclerotic calcification by simvastatin.

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[36x611]protein 2 (BMP2) in VSMCs. Healy et al\textsuperscript{11} found that statins facilitates the calcification process by upregulating the expression of bone morphogenetic protein 2 (BMP2) in VSMCs. Healy et al\textsuperscript{11} found that statins facilitates the calcification process by upregulating the expression of bone morphogenetic protein 2 (BMP2) in VSMCs. Healy et al\textsuperscript{11} found that statins facilitates the calcification process by upregulating the expression of bone morphogenetic protein 2 (BMP2) in VSMCs.
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