Silencing p62 reduces ox-LDL-induced M1 polarization and inflammation in macrophages by inhibiting mTOR/NF-κB signaling pathways

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Abstract
Macrophages can change their phenotypes according to the changes in the microenvironment, and thus have various functions, that is, macrophages polarization. Macrophage phenotype is associated with the progression of atherosclerotic plaques. Studies have shown a large accumulation of p62 protein in atherosclerotic plaques. Whether the accumulation of p62 protein affects the level of macrophage polarization and inflammation and its mechanism is not clear. The p62 levels of macrophages treated with ox-LDL were detected by western blotting and qRT-PCR. Several polarizing markers and cytokines associated with atherosclerosis were detected by western blotting, ELISA, qRT-PCR, and flow cytometry to assess macrophage phenotype. The effect of p62 on the treatment of macrophage polarization by ox-LDL was studied by silencing p62 by gene silencing technique. The activity of mTOR and NF-κB signaling pathways was evaluated by detecting p-mTOR and intranuclear p65 levels in western blotting to explore the mechanism of p62. Rapamycin inhibited mTOR to demonstrate its role in activating the NF-κB signaling pathway and in ox-LDL therapy of p62 induced M1 polarization in macrophages. ox-LDL induced a significant increase in p62 and an increase in M1 markers and inflammatory cytokines. After p62 silencing, M1 markers and inflammatory cytokines decreased significantly, while M2 markers and anti-inflammatory cytokines increased significantly. Silencing p62 inhibited p-mTOR and p65 nuclear translocation. Rapamycin inhibited ox-LDL-induced p65 nuclear translocation and M1 markers, and increased M2 markers. p62 protein accumulation in ox-LDL treatment macrophages induces M1 polarization and inflammatory cytokines through the mTOR/NF-κB pathway.

Keywords
P62, mTOR, NF-κB, macrophages, polarization, atherosclerosis

Introduction
Atherosclerosis (AS) is a chronic inflammatory disease of the arteries characterized by intravascular lipid deposition and plaque formation. Macrophages are important components of plaque and participate in the development of AS. From the infiltration of macrophages in the early plaque to the accumulation of lipid phagocytosis to the formation of foam cells, the status of macrophages determines the
outcome of plaque.\(^2\) Macrophages in AS plaque show different polarization states when stimulated by microenvironment factors. The polarization of macrophages implies different gene and protein expression patterns. Macrophages, as the key mediator of inflammation, affect the progression of AS. In recent years, targeted macrophage polarization has been recognized as a viable method to prevent the development of AS.\(^3\) The polarization phenotype of macrophages can be divided into M1 phenotype and M2 phenotype. M1 phenotype can promote inflammation, while M2 phenotype can inhibit inflammation and prevent AS progression. M1 macrophages accumulate in unstable plaque and necrotic core, and M2 macrophages accumulate in peripheral plaque and stable plaque tissue.\(^4\)

P62 is a multifunctional scaffold protein, not only as a ligand of ubiquitin protein, but also as a substrate for autophagy degradation.\(^5,6\) In physiological conditions, basal autophagy maintains low levels of p62.\(^7\) However, studies have found that large amounts of oxidized low density lipoprotein (ox-LDL) in atherosclerotic plaques block autophagy, leading to p62 accumulation.\(^8,9\) It remains to be seen whether the accumulation of the p62 protein affects macrophage polarization and worsens AS. The aim of this study was to investigate the effect of ox-LDL in plaque on macrophage phenotype, and the role and mechanism of p62 in macrophage phenotype, providing basis for prevention and treatment of AS.

Materials and methods

Reagents and antibodies

DMEM Basal Medium and fetal calf serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Phorbol myristate acetate (PMA), oil red O and rapamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ox-LDL was obtained from Yiyuan Biotechnologies (Guangzhou, China). All the antibodies were purchased from Abcam (Cambridge, England).

Animal models

We used SPF ApoE\(^{-/-}\) mice (Weitonglihua Laboratory Animal Technology Company, Beijing, China). All mice were divided into two groups: The control group was fed with a normal diet, while the AS group was fed with a high-cholesterol diet and received perivascular carotid collar placement (PCCP) to build AS models. Mice in AS group underwent carotid artery perivascular neck ring implantation in the third week and were fed a high-cholesterol diet for another 7 weeks after the operation to induce unstable plaque formation quickly. Operation method: First, we fixed the mice then anesthetized the animals by intraperitoneal injection of sodium pentobarbital. Secondly, the common carotid artery is exposed and then separated from the surrounding connective tissue. Next, we spread it on the left and right sides of the common carotid artery with a collar with an inner diameter of 0.3 mm then put it on the common carotid artery and fixed its edges by placing three dissolvable sutures. Finally, the surgical incision was closed and the animal was returned to the cage for recovery. All experimental procedures follow the spirit of the Declaration of Helsinki on Animal Management and comply with the Guidelines for the Care and Use of Laboratory Animals.

Cell culture

THP-1 monocytes (Shanghai Zhong Qiao Xin Zhou Biotechnology Co, Ltd, China) were cultured in the medium at 5% CO\(_2\) and 37\(^\circ\)C. The medium was changed every 48 h. Before experiments, the cells were transferred to 6-well/96 well plates with 2% FBS RPMI1640. Then we used PMA at a final concentration of 100 ng/mL for 48 h to induce macrophages. Macrophages treated with ox-LDL at different concentrations for 24 h to induce foam cells, and the foam degree was detected by using oil red O. Ox-LDL+rapamycin group was added 1 mM rapamycin 2 h before ox-LDL treatment to inhibit mTOR.

Histological and immunohistochemical assays

The mice’s carotid artery was surgically removed. HE staining of carotid tissue to detect plaque formation. Detection of P62 protein levels in tissues using IHC and Western Blot. Carotid artery samples were cut into 5 \(\mu\)m for hematoxylin and eosin (HE) staining and IHC analysis. For IHC, paraffin sections were dewaxed to repair the antigen. The slices were blocked with 5% goat serum and then cultured together with primary antibodies of p62 (1: 250) for 1 h at 37\(^\circ\)C. The sections were washed with PBS 3 times and incubated with secondary antibody for 1 h at 37\(^\circ\)C. The staining slides were assessed with a light microscope at \(\times400\) magnification.

Serum lipids and inflammatory cytokines

At the end of the experiment, all mice fasted overnight and collected serum from their inner canthus. Determination of Serum Triglyceride (TG) and Total Cholesterol (TC) Using Beckman AU480 Biochemical Automatic Analyzer Based on Commercial Kits. Part of the sample was used in ELISA tests to detect levels of TNF-\(\alpha\), IL-1\(\beta\) and IL-10.
Transfection of small interfering RNA

We used X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland) transfection p62 siRNA (GenePharma, China) and control siRNA (GenePharma, China) to inhibit the expression of p62 according to the manufacturer’s instructions. After 24 h we detected whether gene silencing was successful and then conducted other experiments. siRNA-p62-1 is in the following order: GGAGGATCGAGTGTGAAT. siRNA-p62-2 is in the following order: GGCACAGGGAAAAGA.

qRT-PCR to detect gene expression

We used a high-purity total RNA rapid extraction kit/miRNA column extraction kit to extract RNA/miRNA from each group of cells and carotid artery tissue, and reversed transcription of RNA using ReverTra Ace qRT-PCR Master Mix with gDNA Remover kit (TOYOBO, Japan). Real-time quantitative transcription PCR (qRT-PCR) used the SYBR green method and Line Gene 9600 Plus RT-PCR detection system (Hangzhou Bori Company). The reaction mixture (20 μL final volume) included 10 μL THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan), 0.4 μL ROX reference dye, 0.6 μL forward/reverse primers (250 nM final concentration), 6.4 μL ddH2O and 2 μL 1/10 diluted cDNA. Primers were purchased from (GenePharma, China) and the sequences were listed in Table S1. The thermal cycle program consists of holding at 95°C for 1 min, then performing 15 s at 95°C and 40 cycles of 60 s at 60°C. After completing these cycles, melt curve data was collected. The relative gene expression was calculated by the 2^-ΔΔCt method, where Ct represents the cycle threshold.

Western blotting

The RIPA lysis buffer (Beyotime, China) containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) is used to process cells or tissues to produce cell extracts for protein imprinting. The extracted protein was denatured and then added to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), for electrophoresis separation and transfer to PVDF membrane. The membrane was sealed with 5% bovine serum albumin (BSA) warm bath for 1 h. After three washes, the membrane was incubated overnight at 4°C with a first antibody. The membrane coupled with horseradish peroxidase (HRP)-conjugated secondary antibody was incubated for 1 h after washing. The target protein was then observed with an enhanced chemical luminescent agent. The target protein content was quantified by ImageJ software. The dilution ratios of various antibodies are: p62/SQSTM (1: 1000), p65 (1: 1000), IkBα (1: 2000), iNOS (1: 1000), Arg1 (1: 1000), CD206/MRC1 (1: 1000), p-motor (1:1000), β-actin (1:1000), Lamin B1 (1:1000), goat anti-mouse second antibody (1: 2000), goat anti-rabbit second antibody (1: 2000).

Enzyme-linked immunosorbent assay (ELISA)

The cell culture solution or mouse serum were sucked out and placed in a centrifuge at 1000 g for 20 min. The supernatant was sucked and transferred to EP tubes. Used commercially available ELISA kits (Wuhan Elite Biotechnology Company, Ltd, China), the cell supernatant TNF-α, IL-1β, and IL-10 protein were detected according to the manufacturer’s instructions.

Oil red O staining

Evaluation of Macrophage Foaming Degree by Oil Red O Staining. The cells were washed 3 times with PBS and fixed with 4% parafomaldehyde for 5–10 min, then washed with PBS three times to remove residual paraformaldehyde solution. The prepared oil red O solution was added (2 mL to each well of a 6-well plate or 0.5 mL to each well of a 24-well plate) and incubated at 37°C for 15 min. The cells were washed three times with PBS, differentiated with 60% isopropanol for 30 s, and washed three times with PBS for microscope observation and photography. Discarded PBS from the plate and the dye were extracted with 100% isopropyl alcohol for 10 min. The OD value of the mixture was quantitatively analyzed at 520 nm.

Flow cytometry

First, collected macrophages and made a single cell suspension. Then cells were incubated with cell surface fluorescent antibodies CD86 (1:200) (add IgG antibody to the isotype control group) in the dark at 4°C for 30 min. After washing, the cells were permeabilized and incubated with intracellular fluorescent antibody CD206 (1:200) for 45 min. For each sample, at least 1 × 10^5 cells were analyzed using a BD FACS Calibur cytometer (Becton Dickinson, New Jersey, USA).

Statistical analysis

We used GraphPad Prism 8.0 to generate the diagram. Values of three independent experiments are presented as mean ± SD. In the case of normal distribution, the data were analyzed by t-test (comparison between two groups) or ANOVA (comparison between multiple groups). The difference of p < 0.05 was statistically significant.
Results
ox-LDL induces macrophage M1 polarization and increases inflammatory cytokines

Inflammation is an important factor of AS. Although the phenotype of macrophages, the main immune cells involved in inflammation in AS, has been proved to be a key determinant of AS, its mechanism needs further study. To assess whether ox-LDL affects macrophage polarization, we studied the effects of ox-LDL on several polarizing markers and several cytokines associated with AS. THP-1 macrophages were treated with ox-LDL at different concentrations. The degree of macrophage foaming increased with the increase of ox-LDL concentration (Figure 1(a)). We examined the expression of M1/M2 markers at different concentrations of ox-LDL treated macrophages at mRNA levels. The results showed that the levels of iNOS (M1 marker) and inflammatory cytokines IL-6, TNF-α were upregulated by ox-LDL. However, the mRNA expression of Arg1 and CD206 (M2 markers) was significantly downregulated by ox-LDL (Figure 1(b)). In addition, ELISA results showed that the secretion of IL-1β and TNF-α increased significantly with the increase of ox-LDL concentration, while the secretion of anti-inflammatory cytokine IL-10 decreased significantly (Figure 1(c)–(e)). Flow cytometry was used to detect CD86 (M1 marker) and CD206 (M2 marker). Compared with the control group, CD86^+ cells increased and CD206^+ cells decreased in the ox-LDL treatment group (Figure 1(f)). These results suggest that ox-LDL induces macrophage M1 polarization and promotes inflammatory levels. Inflammatory cytokines and M1 markers were most strongly induced by 100 μg/mL of ox-LDL. We used this concentration of ox-LDL in subsequent experiments.

To test whether the above effects of ox-LDL occur in vivo, we detected cytokines and polarizing markers in AS mice. ApoE^{-/-} mice were operated on with PCCP and fed a diet high in cholesterol to induce AS mice. HE staining showed significant plaque in carotid artery of AS mice compared with control group (Figure 2(a)). Serum tests on mice showed a significant increase in triglycerides (TG) and total cholesterol (TC) in the AS group (Figure 2(b) and (c)). Importantly, serum levels of inflammatory cytokines IL-1β and TNF-α were elevated in AS group, while levels of anti-inflammatory cytokines IL-10 were significantly decreased (Figure 2(d)–(f)). The detection of mRNA in plaque tissue of mice showed significant increase of inflammatory cytokines IL-6 and IL-10.
TNF-α in AS group (Figure 2(g) and (h)). The expression of iNOS was also increased, while the expression of Arg1 and CD206 in AS group was decreased (Figure 2(i)–(k)). These results suggest that ox-LDL induces macrophage M1 polarization and promotes inflammatory levels.

**p62 involved in ox-LDL-induced polarization of macrophages**

To investigate the role of p62 in ox-LDL-induced polarization of macrophages, we first measured p62 protein levels. Western blot results showed that the greater the concentration of ox-LDL treatment, the more p62 proteins in macrophages (Figure 3(a)). But qRT-PCR results showed no significant effect of ox-LDL treatment on p62 mRNA expression (Figure 3(b)). Consistent with previous studies, this suggests that the accumulation of p62 is due to decreased p62 degradation due to ox-LDL inhibition of autophagic flux. In addition, the results of western blot and IHC in vivo showed that the expression of p62 in carotid plaque of AS mice increased significantly compared with control mice (Figure 3(c) and (d)).

In subsequent experiments, we transfected the siRNA control sequence and p62 siRNA respectively to inhibit p62 protein levels. qRT-PCR results confirmed that siRNA-p62-1 successfully inhibited p62 mRNA expression, so subsequent experiments used siRNA-p62-1 to inhibit p62 expression (Figure 4(a)). Western blot results showed that p62 siRNA successfully inhibited p62 protein expression in macrophages treated with 100 μg/mL ox-LDL (Figure 4(b)). It is noteworthy that mRNA expression of inflammatory factors IL-6 and TNF-α decreased significantly after silencing p62, iNOS(M1 marker) decreased significantly, while Arg1 and CD206(M2 markers) increased significantly (Figure 4(c)). In addition, ELISA results showed a significant decrease in IL-1β and TNF-α secretion and a significant increase in IL-10 secretion in the ox-LDL+siRNA-p62 group (Figure 4(d)–(f)). Western blot also showed a significant decrease in iNOS proteins and an increase in Arg1 and CD206 proteins after p62 inhibition by siRNA-p62 (Figure 4(g)). These results suggest that down-regulation of p62 inhibits ox-LDL-induced macrophage inflammation and M1 polarization, increases anti-inflammatory cytokines and M2 polarization.
mTOR/NF-κB pathway participates in p62 induced polarization of M1

In recent years, it has been found that mTOR enhances the inflammatory response of AS and participates in the pathogenesis of AS by activating key factors such as NF-κB. To understand the mechanism of siRNA-p62 inhibiting ox-LDL-induced macrophage inflammatory response and M1 polarization, we studied siRNA-p62 on mTOR/NF-κB signaling pathway associated with AS inflammation. As shown in Figure 5(a), ox-LDL induced a significant increase in p-mTOR levels, a decrease in IκBα, and a significant increase in p65 nuclear translocation in macrophages compared with controls. Compared with ox-LDL+siRNA-control, ox-LDL+siRNA-p62 significantly decreased p-mTOR level and p65 nuclear translocation, increased IκBα level (Figure 5(a)). These results suggest that ox-LDL activates mTOR and NK-κB, while p62 silences inhibit their activation.

NF-κB is an important signal pathway for M1 polarization. To further determine the important role of mTOR in the NF-κB signaling pathway and macrophage polarization under our experimental conditions, we studied the effects of mTOR on IκBα levels, p65 nuclear translocation, and polarizing markers. As shown in Figure 5(b), ox-LDL induces a decrease in IκBα and a nuclear translocation of p65, which means that the NF-κB signal pathway is activated. After inhibition of mTOR with rapamycin, the decrease of IκBα and the nuclear translocation of p65 were reversed (Figure 5(b)). In addition, the increase of iNOS induced by ox-LDLs and the decrease of Arg1 and CD206 were reversed by rapamycin in the western blot assay of polarized markers (Figure 5(b)). Inhibition of mTOR activity reduces M1 polarization by inhibiting the ox-LDL-induced NF-κB signaling pathway.

Discussion

The formation of AS is a long and chronic process. The major cause of cardiovascular disease is AS. Recent studies on the pathogenesis of AS have found that immune cells and inflammation play a key role in hyperlipidemia. Macrophage is the most important immune cell in AS plaque, its function is closely related to the occurrence and development of AS. It is generally believed that macrophages can be divided into two subtypes, M1 subtype representing immune response and M2 subtype representing tolerance. M1 macrophages predominate in progressive lesions, while M2 macrophages are enriched in regressing plaques. Consistent with this view, we demonstrated that mRNA levels of M1 markers were significantly increased and mRNA levels of M2 markers were significantly decreased in carotid plaques of AS mice, suggesting that M1 macrophages may increase with plaque...

Figure 3. Increased content of p62 in Arterial plaque and ox-LDL treated macrophages. (a) Western blotting was used to analysis the p62 protein in macrophages. (b) Detection of p62 mRNA in macrophages by qRT-PCR. (c) The results of IHC showed that the expression of p62 in carotid artery of AS group increased significantly. The scale is 10 μm. (d) The expression of p62 in control group and AS group using western blot. Choose one pair at a time for each group and repeat three times. **p < 0.01, ***p < 0.001 vs. Control.
progression. Detection of the entire tissue without focusing on macrophages within the plaque, and a small sample size may not be sufficient for statistical power were limitations of this study. However, this study confirmed the effect of ox-LDL on Macrophage polarization in vitro. Ly6Chi cells, commonly known as “inflammatory monocytes,” are thought to become M1 macrophages at most inflammatory sites. Previous studies have shown that the transformation of Ly6Chi monocytes into M2 macrophages is required during AS regression.15,16 These results suggest that the polarization of macrophages from M1 phenotype to M2 phenotype can promote the stabilization and regression of AS. In view of the reversibility of M1 and M2 macrophages, macrophages with high plasticity are potential therapeutic targets. Promoting the polarization of macrophages into atherosclerotic protective phenotypes is beneficial to the treatment of AS.14,17 In this study, M1 markers increased and M2 markers decreased significantly in mouse AS plaque, suggesting that M1 is the main macrophage in AS plaque.

The formation of foam cells is the first step in the development of AS. Previous studies have shown that ox-LDL plays a key role in macrophage polarization in AS plaques. ox-LDL binds to LMP10 on the surface of macrophages, activates the NF-κB signaling pathway, makes the cells secrete inflammatory factors such as IL-6 and IL-8, and finally induces macrophages to transfer to M1.18 Meanwhile, it has been found that ox-LDL activates eosinophils in AS plaques and regulates the transformation of macrophages from M2 to M1 through CD36 receptors.19 In addition, Francisco found that ox-LDL inhibits M2 and increases M1 by regulating the secretion of related cytokines.20 The role of ox-LDL in promoting M1 polarization was also demonstrated in this study. In order to test the effect of ox-LDL on macrophage polarization, we treated THP-1 macrophages with ox-LDL and detected several polarizing markers and cytokines associated with AS. The results showed that CD86, iNOS, TNF-α, and IL-10 expression increased after ox-LDL treatment, suggesting that ox-LDL could promote macrophage polarization to M1 and inflammatory cytokine levels. Lower levels of CD206, Arg1 and IL-10 suggest that ox-LDL inhibits the polarization and anti-inflammatory cytokines of macrophages toward M2.
Studies have shown that when macrophages in AS plaques absorb high concentrations of ox-LDL, autophagy is blocked and p62, the substrate for autophagy, accumulates in large quantities. Consistent with these findings, we found a significant increase in p62 protein levels in macrophages and arterial plaques treated with ox-LDL, with no significant change in p62 mRNA levels. This suggests that the high aggregation of p62 which as autophagy substrate is most likely due to autophagy degradation disorders. The relationship between p62 and macrophage polarization has not been reported. We found that the levels of p62 protein and M1 polarization in macrophages increased synchronously with the increase of ox-LDL concentration. We therefore speculate that p62 accumulation may be associated with ox-LDL-induced M1 polarization of macrophages. Our results suggest that the accumulation of p62 protein is involved in ox-LDL-induced polarization of M1 by detecting markers of macrophage polarization and associated cytokines after silencing of p62.

mTOR complex participates in the initiation and progression of AS through lipid metabolism and immune response in vivo. Inhibition of mTOR pathway plays a protective role in coronary artery disease. In animal models, the administration of rapamycin reduces inflammation, inhibits progression and enhances the stability of atherosclerotic plaques by inhibiting mTOR. It has been suggested that mTOR is involved in macrophage polarization, but the effect of mTOR on macrophage phenotype is not consistent in different studies. For example, a recent study showed that HSPA12B secretion of tumor-associated endothelial cells induces M2 polarization of macrophages by activating PI3K/Akt/mTOR signaling pathway, while another study showed that activation of mTOR signaling pathways mediated M1 polarization in exposed macrophages after PM2.5 exposure. This suggests that mTOR...
may have different effects on the activation of macrophages in different disease settings. NF-κB is a classical signal pathway of M1 polarization in macrophages.\textsuperscript{26} Our study showed that mTOR and NF-κB were activated in macrophages treated with ox-LDL, and that silencing p62 significantly inhibited their activity. In addition, inhibition of mTOR with rapamycin can effectively inhibit the activity of NF-κB and reduce M1 polarization. These results suggest that the mTOR/NF-κB pathway plays an important role in the activation of M1 polarization in macrophages treated with ox-LDL by p62. Therefore, inhibiting the mTOR/NF-κB pathway by lowering the cumulative p62 level in plaque may be a mechanism for reducing inflammatory M1 macrophages in plaque.

However, this study has some limitations. Here, we focused on macrophages in vitro. Further studies should investigate the effect of p62 on macrophages in atherosclerotic plaques in vivo. In addition, Xiaofei Liang found that silencing p62 promoted ABCA1 and ABCG1-mediated cholesterol efflux in macrophages incubated with ox-LDL.\textsuperscript{27} Further research is needed to investigate whether this effect is associated with p62 regulating macrophage polarization.

**Conclusions**

Our results suggest that ox-LDL induces M1 polarization in macrophages and that the p62/mTOR/NF-κB signaling pathway is involved in this effect. Inhibition of p62 inhibits ox-LDL induced M1 polarization of macrophages, which is related to the inhibition of mTOR signal pathway. Our study highlights that p62 and mTOR are key molecules in the transition of macrophages from M1 to M2 in atherosclerotic plaques, which will help uncover new directions in the treatment of AS.

**Author’s contributions**

WY and YHL designed the study. GMS analyzed the data. WY performed the experiments. WY and YHL wrote the manuscript. All authors read and approved the final manuscript. WY, GMS and YHL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Ethics approval and consent to participate**

The present study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Harbin Medical University (No.2019047). We confirm that non-retrospective ethical approval obtained for the animal experiments conducted in this study, and animals were anesthetized and sacrificed using acceptable methods.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**Supplemental Material**

Supplemental material for this article is available online.

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