Phosphorylation of CHOP (C/EBP Homologous Protein) by the AMP-Activated Protein Kinase Alpha 1 in Macrophages Promotes CHOP Degradation and Reduces Injury-Induced Neointimal Disruption In Vivo

Xiaoyan Dai, Ye Ding, Zhaoyu Liu, Wencheng Zhang, Ming-Hui Zou

Rationale: Elevated levels of CHOP (C/EBP homologous protein), a member of the C/EBP transcription factor family, in advanced atherosclerotic plaques is reported to be associated with atherosclerotic plaque rupture in humans. However, the molecular mechanism by which CHOP accumulation occurs is poorly defined.

Objective: The aim of this study was to investigate if (1) macrophage AMPK (AMP-activated protein kinase) regulates cellular CHOP accumulation and (2) whole-body Ampk deletion leads to neointimal disruption.

Methods and Results: In isolated or cultured macrophages, Ampkα1 deletion markedly increased apoptosis and CHOP, whereas pharmacological activation of AMPK dramatically reduced CHOP protein level via promoting CHOP degradation by proteasome. In addition, cotransfection of Chop-specific siRNA, but not control siRNA, markedly reduced apoptosis in macrophages transfected with Ampkα1-specific siRNA. Mechanistically, AMPKα1 was found to coimmunoprecipitate with CHOP and phosphorylate CHOP at serine 30. Furthermore, serine 30 phosphorylation of CHOP triggered its ubiquitination and proteasomal degradation. In a mouse model of plaque stability, deletion of Ampkα1 but not Ampkα2 promoted injury-induced neointimal disruption. This was paralleled by increased CHOP expression and apoptosis in vivo. Finally, transfection of Chop-specific siRNA but not control siRNA reduced both CHOP level and injury-induced neointimal disruption in vivo.

Conclusions: Our results indicate that AMPKα1 mediates CHOP ubiquitination and proteasomal degradation in macrophages by promoting the phosphorylation of CHOP at serine 30. We conclude that AMPKα1 might be a valid therapeutic target in preventing atherosclerotic vulnerable plaque formation. (Circ Res. 2016;119:1089-1100. DOI: 10.1161/CIRCRESAHA.116.309463.)

Key Words: AMPK, apoptosis, CHOP, degradation, macrophage, neointima, ubiquitination

Rupture of advanced atherosclerotic plaques and subsequent partial or complete thrombotic artery occlusion are the main pathological mechanisms for acute myocardial infarction and stroke. Increased necrosis, inflammation, matrix degradation, cellular apoptosis, and reduced fibrous caps are the well-established morphological and pathological characteristics of vulnerable atherosclerotic plaques. Pathologically, lipid-overloaded macrophages, a major cellular component of advanced atherosclerotic plaques, make plaques vulnerable to rupture because cellular debris from necrotic or apoptotic macrophages triggers inflammation and matrix proteinase activation. Thus, higher levels of apoptotic macrophages within advanced plaques may promote plaque vulnerability.

The CHOP (C/EBP homologous protein) is a member of the C/EBP transcription factor family and is ubiquitously expressed. The expression of CHOP is induced under stress conditions, such as DNA damage, growth arrest, and nutrient deprivation. CHOP is reported to play a vital role in regulating macrophage apoptosis in advanced atherosclerosis. Myoishi et al demonstrated that elevated levels of both endoplasmic reticulum stress markers and apoptotic cells were observed within ruptured plaques when compared with the stable fibrous plaques. Mechanistically, CHOP-dependent pathway is activated in unstable plaques, suggesting that CHOP-mediated apoptosis may be involved in triggering coronary artery plaque rupture. Recently, a noteworthy clinical study reported that apoptosis and CHOP elevate in advanced rupture-prone plaques within human carotid arteries. In mice, genetic deletion of CHOP leads to reduced macrophage apoptosis both in vitro and in vivo, which contributes to improved atherosclerotic plaque stability. Taken together, it has been suggested that CHOP-mediated macrophage apoptosis is a
central contributor of instability of atherosclerotic plaque. However, how CHOP is regulated in atherosclerotic plaques is poorly studied.

AMPK (AMP-activated protein kinase) is a well-characterized regulator of cellular energy status. AMPKα1 is the predominant isoform of AMPK in vascular cells, including endothelial cells, vascular smooth cells, and monocytes/macrophages.18 However, both AMPKα1 and AMPKα2 have been demonstrated to regulate vascular cell function.17–21 Genetic deletion of Ampkα2 promotes atherosclerosis in vivo, likely via aberrant endoplasmic reticulum stress.22 Whether or not dysfunctional AMPK promotes atherosclerotic plaque vulnerability remains unknown. The aim of the present study was to test whether AMPK reduces atherosclerotic plaque vulnerability by promoting CHOP ubiquitination and degradation. Here, we report that phosphorylation of CHOP at serine 30 (Ser30) by AMPKα1 is the predominant AMPK isoform in human 

Methods

Murine Ligation and Cuff-Triggered Neointimal Disruption Model

A well-characterized murine model was used to assay neointimal formation and disruption, as described previously.15,23,24 Briefly, 9-week-old male Apoe−/−, Apoe−/−/Ampkα1−/−, and Apoe−/−/Ampkα2−/− mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine-HCl and 10 mg/kg xylazine-HCl, and their right common carotid artery was ligated proximal to the bifurcation. Four weeks after ligation, a polyethylene cuff (427410; BD Biosciences, San Jose, CA) was placed around the ligated right common carotid artery. At 4 days after cuff placement, the right common artery was collected, and the intracuff lesions were characterized by Western blot analysis.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AICAR</td>
<td>5-aminooimidazole-4-carboxamide ribonucleoside</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>BMDMs</td>
<td>bone marrow-derived macrophages</td>
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<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling</td>
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<td>WT</td>
<td>wild-type</td>
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Statistical Analysis

Quantitative values are expressed as the mean±SEM and represent data from at least 3 independent experiments. The difference between 2 groups was analyzed by Student’s t test. The difference among >2 groups was analyzed by 1-way analysis of variance, followed by Newman–Keuls multiple comparison test, and comparisons of different parameters between each group were made by 2-way ANOVA analysis followed by Bonferroni posttests. For incidence of injury-induced neointimal disruption, groups were compared with Chi-square test (3 groups) or Fisher exact test (2 groups). P values of <0.05 were considered statistically significant.

An expanded Materials and Methods are available in the Online Data Supplement.

Results

Ampkα1 Deletion Promotes Macrophage Apoptosis

Lipid-overloaded macrophages are a major cellular component of advanced atherosclerotic plaque. Overwhelming evidence suggests that atherosclerotic plaques become vulnerable to rupture when apoptotic macrophages trigger a local inflammatory response and matrix proteinase activation.5–9 To test whether AMPKα1 modulates macrophage apoptosis, we first detected the effect of genetic deletion of Ampkα1 on apoptosis in macrophages. As shown in Figure 1A, the number of terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL)–positive cells in Ampkα1−/− bone marrow–derived macrophages (BMDMs) was significantly higher compared with wild-type (WT). In contrast, the number of TUNEL–positive BMDMs from Ampkα2−/− mice did not vary from WT mice. Consistent with this observation, the levels of both cleaved caspase 3 and poly(ADP-ribose) polymerase, 2 well-characterized markers for apoptotic cells, were significantly higher in the BMDMs isolated from Ampkα1−/− mice (Figure 1B) compared with either WT or Ampkα2−/− mice. Taken together, our results indicate that the deletion of Ampkα1 but not Ampkα2 promotes macrophage apoptosis.

Ampkα1 Iversely Regulates CHOP Protein Levels in Macrophages

It has been reported that CHOP-induced macrophage apoptosis promotes atherosclerotic plaque rupture.13 Given that AMPKα1 is the predominant AMPKα isoform in human and mouse macrophages,18 we set out to determine whether AMPKα1 regulates apoptosis by altering the levels of CHOP in macrophages. To test the regulatory role of AMPKα1 in CHOP-induced macrophage apoptosis, mouse macrophage–like cell line RAW264.7 cells were transfected with scramble, Ampkα1–specific, or CHOP–specific siRNA. As shown in Online Figure I, transfection of Ampkα1 siRNA markedly increased the levels of CHOP and cleaved caspase 3. Scramble siRNA had no effect. CHOP siRNA reversed the increased cleaved caspase 3 level by Ampkα1 siRNA (Online Figure I). Therefore, we demonstrated that elevated caspase 3 cleavage in Ampkα1 siRNA-treated cells is CHOP-dependent by co-transfecting CHOP siRNA with Ampkα1 siRNA. In contrast, the transfection of CHOP siRNA without co-transfection with Ampkα1 siRNA did not alter cleaved caspase 3 levels compared with
those transfected with scramble siRNA. Taken together, these results indicate that CHOP is required for Amphk1 deficiency-induced apoptosis.

Next, we determined whether Amphkα deletion alters CHOP in BMDMs isolated from WT, Amphkα1−/−, and Amphkα2−/− mice. Figure 2A showed the deletion of Amphkα1 but not Amphkα2 significantly increased CHOP protein levels in macrophages. Consistent with this, BMDMs from Amphkα1−/− mice but not WT or Amphkα2−/− mice exhibited higher mRNA levels of well-known CHOP target genes, such as Wars, Sars, Trib3, and Ero1α, suggesting that Amphkα1 deletion increases CHOP activity in macrophages (Online Figure II). Furthermore, we demonstrated that activation of AMPK with either AICAR (5-aminoimidazole-4-carboxamide ribonucleoside; Figure 2B) or A769662 (Figure 2C) lowered CHOP levels in macrophages in a time-dependent manner. Conversely, inhibiting AMPK activity with compound C led to an upregulation of CHOP levels in a time-dependent manner (Figure 2D). Overall, our results support the hypothesis that AMPKα1 is an inverse regulator of CHOP.

To assess whether increased CHOP protein levels resulting from AMPK inhibition are because of an increase in Chop mRNA levels, we conducted quantitative real-time reverse transcriptase polymerase chain reaction to determine Chop mRNA in BMDMs isolated from WT, Amphkα1−/− and Amphkα2−/− mice. As shown in Online Figure III, similar levels

Figure 1. Amphkα1 deficiency promotes apoptosis in macrophages. A. Bone marrow–derived macrophages (BMDMs) from WT, Amphkα1−/−, and Amphkα2−/− mice were stained using a TUNEL kit. Nuclei were stained with DAPI (blue). Left, Representative immunofluorescence photomicrographs of TUNEL assays in BMDMs. Scale bar, 50 μm. Right, the percentage of TUNEL-positive (green) BMDMs. n=6. B, Immunoblots of BMDMs isolated from WT, Amphkα1−/−, and Amphkα2−/− mice. The protein-specific antibodies are indicated. n=5. Results are mean±SEM. *P<0.05 vs WT. Amphk indicates AMP-activated protein kinase; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; and WT, wild-type.

Figure 2. AMPKα1 downregulates CHOP protein levels in macrophages. A. Immunoblots of CHOP in WT, Amphkα1−/−, and Amphkα2−/− BMDMs. n=5. B, C, and D, Immunoblots of CHOP in RAW264.7 cells treated with AICAR (1 mmol/L), A769662 (100 μmol/L), or Compound C (10 μmol/L) for indicated time. n=5. Results are mean±SEM. *P<0.05 vs WT or control. AICAR indicates 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; BMDMs, bone marrow–derived macrophages; CHOP, C/EBP homologous protein; and WT, wild-type.
of Chop mRNA existed in all 3 genotypes. Next, we examined whether AMPK activation with AICAR altered the half-life \( t_{1/2} \) of Chop mRNA. To test this, RAW264.7 cells were incubated with actinomycin D and treated with or without AICAR for the indicated time. As depicted in Online Figure IV, AICAR did not accelerate Chop mRNA degradation in RAW264.7 cells. These data indicate that AMPK does not directly affect Chop mRNA at both the transcriptional and post-transcriptional levels.

### AMPK Decreases CHOP Protein Stability

To explore how AMPK activation decreases CHOP protein levels, cycloheximide-pretreated macrophages were exposed to AICAR, and the steady-state levels of CHOP were measured by Western blot analysis. As shown in Figure 3A, AMPK activation by AICAR significantly increased the degradation of CHOP. Transfection of Ampkα1-specific siRNA but not scramble siRNA prevented CHOP degradation (Figure 3B). Consistent with these results, genetic deletion of Ampkα1 in BMDMs inhibited CHOP degradation when compared with WT BMDMs (Figure 3C), indicating that AMPKα1 is an important regulator of CHOP protein stability in macrophages.

### AMPKα1 Phosphorylates CHOP at Ser30

CHOP has been reported to be phosphorylated by p38 MAP kinase and casein kinase 2.27,28 AMPK is a well-known threonine/serine protein kinase. Therefore, we hypothesized that AMPKα1 phosphorylates CHOP to control its stability. Computer alignment studies indicated that Ser30 within the substrate motif (Figure 4A).29 As shown in Figure 4B, AMPKα1 coimmunoprecipitates with CHOP in macrophages. Furthermore, we observed costaining of AMPKα1 and CHOP in BMDMs (Figure 4C). Finally, an in vitro kinase assay showed that both endogenous immunoprecipitated AMPKα1 and exogenous recombinant protein AMPKα1 phosphorylates CHOP (p-CHOP) at Ser30 (Figure 4D and 4E).

We next established if AMPK-dependent Ser30 phosphorylation of CHOP occurred in intact cells. Macrophages were exposed to either AICAR or A769662 at the times indicated. As depicted in Figure 4F and 4G, AMPK activator AICAR or A769662 markedly increased p-CHOP at Ser30 at 8 hours. Further, prolonged incubation \( \leq 24 \) hours increased levels of p-CHOP at Ser30. Conversely, inhibition of AMPK with compound C, a potent AMPK inhibitor, time-dependently lowered the levels of p-CHOP at Ser30 (Figure 4H). In line with these results, BMDMs isolated from Ampkα1−/− mice exhibited significantly lower levels of p-CHOP at Ser30 compared with those isolated from WT or Ampkα2−/− mice (Figure 4I). Furthermore, A769662 increased p-CHOP (Ser30) in WT BMDMs but not Ampkα1−/− BMDMs, suggesting that AMPKα1 is required for A769662-mediated CHOP phosphorylation at Ser30 (Figure 4J).

### CHOP Phosphorylation at Ser30 Is Required for AICAR-Induced Downregulation of CHOP

To determine whether phosphorylation of CHOP at Ser30 is involved in the AICAR-induced downregulation of CHOP, we generated a phosphorylation-deficient Chop mutant (Ser30 is replaced by alanine, S30A) and a phosphomimetic Chop mutant (Ser30 is replaced by glutamic acid, S30E). RAW264.7 cells were transfected with full-length WT Chop (WT), phosphorylation-deficient Chop (S30A), or phosphomimetic Chop (S30E). Forty-eight hours after the transfection, cells were treated with or without AICAR for 12 hours.

![Figure 3. AMPKα1 decreases CHOP protein stability. A. RAW264.7 cells were pretreated with cycloheximide (CHX; 5 μg/mL) for 30 minutes before being treated with or without AICAR (1 mmol/L) for the indicated time, followed by Western blot analysis to detect CHOP. n=5. B. RAW264.7 cells were transfected with scramble or Ampkα1 siRNA for 48 h and then treated with CHX (5 μg/mL) for the indicated time, followed by Western blot analysis to detect CHOP. n=5. C. BMDMs isolated from WT and Ampkα1−/− mice were treated with CHX (5 μg/mL) for the indicated time, followed by Western blot analysis to detect CHOP. n=5. Results are mean±SEM. *P<0.05 vs control, scramble siRNA, or WT. AICAR indicates 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; CHOP, C/EBP homologous protein; and WT, wild-type.](http://circres.ahajournals.org/doi/abs/10.1161/CIRCRESAHA.116.310567)
Figure 4. Phosphorylation of CHOP Ser30 by AMPKα1 is required for AICAR-induced CHOP downregulation. A, Alignment of peptide sequences flanking human and mouse CHOP Ser30. B, Interaction of AMPKα1 and CHOP. C, Co-staining of AMPKα1 (red) and CHOP (green) in BMDMs. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. D, AMPKα1 was immunoprecipitated (IP) from lysed RAW264.7 cells with a specific antibody. Human recombinant GST-CHOP (100 ng) and immunoprecipitated AMPKα1 were incubated in a kinase reaction buffer in the presence of AMP at 37°C for 30 minutes. Immunoblotting for phosphorylation of CHOP at Ser30 was shown. E, Human recombinant GST-CHOP (500 ng) and human recombinant AMPKα1 (500 ng) were incubated in the kinase reaction buffer in the presence of AMP at 37°C for 30 minutes. Immunoblotting for phosphorylation of CHOP at Ser30 was shown. F–H, Immunoblots of p-CHOP (Ser30) in RAW264.7 cells treated with AICAR (1 mmol/L), A769662 (100 μmol/L), or Compound C (10 μmol/L) for indicated time. n=5. I, Immunoblots of p-CHOP (Ser30) in WT, Ampkα1−/−, and Ampkα2−/− BMDMs. n=5. J, WT and Ampkα1−/− BMDMs were treated with 100 μmol/L A769662 for 12 h. p-CHOP (Ser30) was determined by Western blot. K and L, RAW264.7 cells were transfected with full-length WT DDK-Chop (WT) or the indicated site-directed mutants, DDK-Chop S30A or DDK-Chop S30E, for 48 h and then treated with or without AICAR for 12 h, followed by Western blot analysis to detect DDK. n=5. Results are mean±SEM. *P<0.05 vs control or WT. #P<0.05 vs as indicated. AICAR indicates 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; BMDMs, bone marrow-derived macrophages; CHOP, C/EBP homologous protein; DDK, DYKDDDDK tag; GST-CHOP, recombinant protein CHOP with GST-tag at N-terminal; NS, not significant; and p-CHOP, phosphorylated CHOP.
The Chop-S30A mutant alone did not alter CHOP protein levels (Figure 4K). As expected, AICAR reduced CHOP levels in WT-transfected macrophages, whereas AICAR did not alter CHOP protein levels in macrophages transfected with Chop-S30A (Figure 4K). Conversely, mutation of the phosphomimetic Chop-S30E variant markedly lowered the levels of CHOP compared with Chop in WT-transfected macrophages (Figure 4L). AICAR treatment did not cause further reduction of CHOP in cells transfected with the Chop-S30E mutant (Figure 4L). Taken together, these data indicate that phosphorylation of CHOP at Ser30 is required for AICAR-induced degradation of CHOP.

AMPK Increases CHOP Ubiquitination

Three major protein degradation pathways exist in mammalian cells: the proteasome, the lysosome, and the autophagosome. Cellular inhibitor of apoptosis protein-1 is reported to promote CHOP degradation through the proteasome pathway in β cells.29 We next determined which pathway is involved in AMPK-mediated CHOP degradation in macrophages. To assess which pathway mediates CHOP degradation in macrophages, cells were treated with either the proteasome inhibitor MG-132, the lysosome inhibitor chloroquine, or the autophagy inhibitor 3-MA. As shown in Figure 5A, neither chloroquine nor 3-MA altered CHOP levels. However, MG-132 led
to significantly increased CHOP expression in macrophages, indicating a role for proteasome degradation. Consistent with this, MG-132 abated the AICAR-induced reduction of CHOP (Figure 5B). Taken together, these results support the hypothesis that CHOP degradation is mediated by the proteasome in macrophages.

We next determined whether AMPK activation promotes CHOP ubiquitination. Macrophages were pretreated with MG-132 for 30 minutes before AICAR treatment. AICAR promoted CHOP ubiquitination with or without MG-132 (Figure 5C). In contrast, the levels of ubiquitinated CHOP in BMDMs of *Ampkα1* mice were markedly lower than their counterparts from WT mice (Figure 5D). Collectively, these data indicate that AMPK activation enhances CHOP ubiquitination.

### Phosphorylation of CHOP at Ser30 Positively Regulates CHOP Ubiquitination

We assayed if AMPK-mediated CHOP phosphorylation at Ser30 is required for CHOP ubiquitination. RAW264.7 cells were transfected with either full-length WT *Chop* (WT) or *Chop* mutants (*Chop*-S30A and *Chop*-S30E). Compared with WT *Chop*, phosphomimetic *Chop*-S30E showed elevated ubiquitination, whereas the phosphorylation-deficient *Chop*-S30A mutant exhibited reduced ubiquitination (Figure 5E). These data suggest that CHOP phosphorylation at Ser30 is important for CHOP ubiquitination and subsequent degradation.

To further examine the key role of Ser30 phosphorylation on CHOP stability, cycloheximide pulse-chase experiments were done with both the S30E and S30A mutants of *Chop* in RAW264.7 cells. As shown in Figure 5F, the half-life of *Chop*-S30A (>8 hours) was twice as long as that of the *Chop*-S30E. These data further confirm that CHOP Ser30 phosphorylation leads to CHOP ubiquitination and degradation.

### Ampkα1 Deletion Aggravates Injury-Induced Neointimal Disruption in *Apoe*<sup>−/−</sup> Mice

Given that CHOP plays an important role in promoting atherosclerotic instability<sup>15</sup> and our above mentioned results showed AMPKα1 downregulates CHOP level in vitro, we reasoned that AMPKα1 activation could reduce injury-induced neointimal disruption by lowering CHOP in vivo. To test the effects of AMPK-CHOP pathway in plaque stability, we generated the right carotid artery ligation plus cuff placement model, a well-characterized model for studying injury-induced neointima stability.<sup>15,23,24</sup> Four weeks after the ligation of the right carotid arteries of 9-week-old male *Apoe*<sup>−/−</sup>, *Apoe*<sup>−/−*/Ampkα1*<sup>−/−</sup>, and *Apoe*<sup>−/−*/Ampkα2*<sup>−/−</sup> mice, cuffs were placed in the ligated right carotid arteries for 4 days before mice were euthanized for further analysis. As shown in Online Figure V, serum triglycerides and total cholesterol were comparable among the 3 groups. After ligation and cuff placement, the right carotid arteries of most of the mice in all groups developed neointimal plaque, and mean lesion areas were similar between the groups (Table), suggesting that macrophage AMPKα1 deletion did not impact the sizes of injury-induced neointimal lesions.

Next, we assayed the roles of macrophage AMPKα1 in maintaining neointimal plaque stability. It is commonly believed that in neointimal lesions, both collagen and vascular smooth muscle cells (VSMCs) promote stabilization of the neointima, whereas macrophage destabilizes the plaques.<sup>31,32</sup> To this end, we measured the contents of collagen, VSMC, and macrophages in neointimal plaques. As depicted in Figure 6A, collagen contents, measured by picrosirius red staining, were significantly lower in the neointima of *Apoe*<sup>−/−*/Ampkα1*<sup>−/−</sup> mice than those in either the *Apoe*<sup>−/−</sup> or *Apoe*<sup>−/−*/Ampkα2*<sup>−/−</sup> mice. In addition, VSMC contents showed no difference among the 3 groups (Online Figure VI). On the contrary, the macrophage infiltration in neointimal lesion, detected by anti-CD68 immunofluorescence staining, was higher in *Apoe*<sup>−/−*/Ampkα1*<sup>−/−</sup> mice than that detected in either the *Apoe*<sup>−/−</sup> or *Apoe*<sup>−/−*/Ampkα2*<sup>−/−</sup> mice (Figure 6B). Taken together, these results indicate that AMPKα1 deletion promotes instability of the neointimal lesions.

It was important to assess if macrophage AMPKα1 deletion promoted neointimal disruptions in vivo. Disrupted neointima is defined as neointima with mural or occlusive thrombus.<sup>34</sup> Although incidence of neointimal disruption was similar between *Apoe*<sup>−/−</sup> and *Apoe*<sup>−/−*/Ampkα2*<sup>−/−</sup> mice (54.2% versus 52.6%, respectively; Table), the incidence of disrupted neointima was significantly increased in the *Apoe*<sup>−/−*/Ampkα1*<sup>−/−</sup> mice compared with *Apoe*<sup>−/−</sup> mice (54.2% versus 85.7%, respectively; Table), suggesting that AMPKα1 plays an essential and specific role in neointimal stability but not in neointimal lesion size. Taken together, deletion of Ampkα1 but not Ampkα2 promotes the injury-induced neointimal disruption in *Apoe*<sup>−/−</sup> mice.

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### Table. Characterization of Injury-Induced Neointimal Plaques in *Apoe*<sup>−/−</sup>, *Apoe*<sup>−/−*/Ampkα1*<sup>−/−</sup>, and *Apoe*<sup>−/−*/Ampkα2*<sup>−/−</sup> mice

<table>
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<tr>
<th>Mouse Genotype (Total Number)</th>
<th>Neointimal Plaque</th>
<th>Mean Plaque Area (×10&lt;sup&gt;3&lt;/sup&gt; μm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Neointimal Disruption With Thrombus</th>
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<td></td>
<td>Mural</td>
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<td><em>Apoe</em>&lt;sup&gt;−/−&lt;/sup&gt; (n=24)</td>
<td>21</td>
<td>15.74±1.66</td>
<td>6 (25.0%)</td>
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<td><em>Apoe</em>&lt;sup&gt;−/−<em>/Ampkα1</em>&lt;sup&gt;−/−&lt;/sup&gt; (n=21)</td>
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<td>17.18±2.43</td>
<td>4 (19.0%)</td>
</tr>
<tr>
<td><em>Apoe</em>&lt;sup&gt;−/−<em>/Ampkα2</em>&lt;sup&gt;−/−&lt;/sup&gt; (n=19)</td>
<td>15</td>
<td>16.34±1.69</td>
<td>4 (21.1%)</td>
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Neointimal disruption are defined as plaques that show disruptions in the neointima with mural or occlusive thrombus. Data represented as the mean±SEM. Ampk indicates AMP-activated protein kinase; and Apoe, apolipoprotein E.

*P<0.05 vs *Apoe*<sup>−/−</sup> or *Ampkα2*<sup>−/−</sup> mice.
Ampkα deletion aggravates injury-induced neointimal disruption and promotes apoptosis in injury-induced neointimal lesion. Male 9-week-old Apoe−/−, Apoe−/−/Ampka1−/−, and Apoe−/−/Ampka2−/− mice were treated as described in the Methods. A, Left, Picrosirius red staining viewed by polarized light. Scale bar, 100 μm. Right, Quantitative analysis of collagen content. n=6. B, Representative immunofluorescence staining of frozen cross sections of the intracuff carotid artery with a CD68 (red) antibody in Apoe−/−, Apoe−/−/Ampka1−/−, and Apoe−/−/Ampka2−/− mice. Sections were counterstained with DAPI (blue). Scale bar, 100 μm. The percentage of TUNEL-positive (green) cells is shown on the right. n=5. C, Representative immunofluorescence photomicrograph of the TUNEL assay. Nuclei were stained with DAPI (blue). Scale bar, 100 μm. The percentage of TUNEL-positive (green) cells is shown on the right. n=5. D, Representative immunohistochemical staining of frozen cross sections of the intracuff carotid artery with a cleaved caspase 3 antibody in Apoe−/−, Apoe−/−/Ampka1−/−, and Apoe−/−/Ampka2−/− mice. Sections were counterstained with hematoxylin. Scale bar, 50 μm. The percentage of cleaved caspase 3–positive staining is shown on the right. n=5. E, Lumen (L), neointima (NI), and media (M) in injured vessels were labeled. Results are mean±SEM. *P<0.05 vs Apoe−/− mice. AMPK indicates AMP-activated protein kinase; Apoe, apolipoprotein E; and TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.
CHOP expression was significantly increased in the injury-induced neointimal lesion of Apoe−/−/Ampkα1−/− mice compared with that in Apoe−/− or Apoe−/−/Ampkα2−/− mice (Figure 7A). In line with this, the presence of p-CHOP (Ser30) antibody in Apoe−/−, Apoe−/−/Ampkα1−/−, and Apoe−/−/Ampkα2−/− mice. Sections were counterstained with hematoxylin. Scale bar, 50 μm. Quantification of the CHOP-positive staining is shown on the right. n=5. B, Representative immunohistochemical staining of frozen cross sections of the intracuff carotid artery with p-CHOP (Ser30) antibody in Apoe−/−, Apoe−/−/Ampkα1−/−, and Apoe−/−/Ampkα2−/− mice. Sections were counterstained with hematoxylin. Scale bar, 50 μm. Quantification of the p-CHOP (Ser30)-positive staining is shown on the right. n=5. Lumen (L), neointima (NI), and media (M) in injured vessels were labeled. C, Representative Western blots of scramble siRNA and Chop siRNA knockdown of CHOP protein in carotid arteries 7 days after delivery. n=6. Both scramble siRNA and Chop siRNA were perivascularly applied to ligated arteries for 7 days. D, A bar graph shows the incidence of neointimal disruption (n=18 in the group of Apoe−/− mice treated with scramble siRNA, n=18 in the group of Apoe−/− mice treated with Chop siRNA). E, Proposed scheme for accelerated injury–induced neointimal disruption in Apoe−/−/Ampkα1−/− mice. Ampkα1 deletion decreases CHOP phosphorylation, which leads to reduced CHOP ubiquitination/CHOP degradation. As a result, CHOP levels increase, which subsequently triggers increased macrophage apoptosis and accelerates injury-induced neointimal disruption. AMPK indicates AMP-activated protein kinase; Apoe, apolipoprotein E; CHOP, C/EBP homologous protein; and p-CHOP, phosphorylated CHOP.
abated CHOP protein expression in the ligated and cuffed carotid arteries (Figure 7C). However, compared with scramble siRNA-treated carotid arteries, the Chop siRNA markedly decreased the incidence of neointimal disruption in Apoe−/− mice (Figure 7D; Online Table III). Compared with scramble siRNA-treated carotid arteries, the transfection of Chop-specific siRNA in treated-Apoe−/− mice markedly increased collagen contents and suppressed macrophage accumulation and apoptosis (Online Figures XIA, XIB, and XII). As expected, in vivo knockdown of Chop by siRNA had no effect on VSMC contents (Online Figure XIC). Taken together, these results indicate that Chop induces carotid artery neointimal disruption of Apoe−/− mice.

**Discussion**

In this study, we have demonstrated for the first time that AMPK activation promotes CHOP degradation via ubiquitination and that genetic deletion of Ampkα1 causes CHOP accumulation. This results in aberrant macrophage apoptosis and consequent injury-induced neointimal disruption in vivo. Mechanistically, AMPKα1 binds with CHOP and phosphorylates CHOP at Ser30. This phosphorylation triggers CHOP ubiquitination and consequent degradation by the proteasome. The principal findings of this study are as follows: (1) AMPKα1 inhibition increases macrophage apoptosis; (2) AMPKα1 inhibition-induced macrophage apoptosis is CHOP-mediated; (3) AMPK activation reduces and AMPK inhibition increases CHOP protein levels in macrophages; (4) AMPKα1 phosphorylates CHOP at Ser30; (5) CHOP phosphorylation at Ser30 initiates CHOP ubiquitination and subsequent CHOP degradation in the proteasome; (6) genetic inhibition of Ampkα1 promotes in vivo injury-induced neointimal disruption in mice via increase in CHOP accumulation; and (7) local inhibition of Chop by siRNA decreases the incidence of neointimal disruption in Apoe−/− mice. Thus, we conclude that AMPKα1 activation might prevent injury-induced neointimal disruption by promoting proteasomal degradation of CHOP in macrophages (Figure 7E).

The most important finding of this study is that we have demonstrated for the first time that AMPKα1 activation suppresses injury-induced neointima disruption by suppressing CHOP-dependent macrophage apoptosis. Compared with either Apoe−/− or Apoe−/−/Ampkα2−/− mice, the incidence of neointimal disruption with thrombus was significantly increased in Apoe−/−/Ampkα1−/− mice. Macrophage apoptosis in the advanced lesion is one of the most important inducers of atherosclerotic plaque vulnerability.33,34 Kolodgie et al reported an increased number of apoptotic macrophages at sites of plaque disruption compared with those in intact fibrous caps.8 We were able to show that macrophage infiltration and macrophage apoptosis in injured carotid arteries were significantly higher in Apoe−/−/Ampkα1−/− mice compared with those in Apoe−/− or Apoe−/−/Ampkα2−/− mice and that apoptosis-suppressing effects of AMPK in macrophages is important in maintaining the stability of injury-induced neointimas. These results are consistent with a published clinical study in which lesion macrophages are the primary apoptotic cell type involved in generating rupture-prone plaques of the human carotid artery.14

A key finding of this study is that AMPK promotes CHOP protein degradation by enhancing CHOP ubiquitination. First, we found that AMPK activation accelerated CHOP protein decay. Furthermore, this AMPK-accelerated CHOP degradation was abolished with Ampkα1 knockdown or genetic deletion. In addition, we found that AMPKα1 phosphorylated CHOP Ser30. CHOP degradation is mediated by the proteasome pathway,30 and we confirmed this in macrophages. Next, we addressed the question whether CHOP phosphorylation at Ser30 promoted its ubiquitination and then degradation. The phosphomimetic CHOP-S30E showed elevated levels of ubiquitination compared with WT CHOP, while phosphorylation-deficient mutant CHOP-S30A showed reduced ubiquitination compared with WT CHOP. This suggests that phosphorylation of CHOP at Ser30 positively regulates CHOP ubiquitination. Furthermore, CHOP expression was significantly higher in atherosclerotic plaques isolated from Apoe−/−/Ampkα1−/− mice compared to those isolated from Apoe−/− or Apoe−/−/Ampkα2−/− mice. Transfection of Chop-specific siRNA markedly decreased the incidence of neointimal disruption in Apoe−/− mice. This result strongly suggests that Ser30 phosphorylation of CHOP by AMPKα1 promotes CHOP ubiquitination and degradation. Importantly, CHOP accumulation promotes the disruption of the injury-induced carotid neointima in Apoe−/− mice.

AMPK might also be important in maintaining atherosclerotic plaque stability. Although mouse atherosclerotic plaques are, in general, stable and there is no commonly accepted mouse models to study atherosclerotic plaque stability, the atherosclerotic plaques in brachiocephalic arteries are considered as a well-characterized site for studying plaque stability.35,36 Indeed, we found that the aortic lesions of brachiocephalic arteries of Apoe−/−/Ampkα1−/− mice exhibited more characteristic features, including decreased collagen levels, increased necrotic cores, and cleaved caspase 3 when compared with those from Apoe−/− mice. In the same model, CHOP expression was significantly increased in the atherosclerotic plaques of brachiocephalic arteries of Apoe−/−/Ampkα1−/− mice compared with those in Apoe−/− mice. Conversely, the levels of p-CHOP (Ser30) in the atherosclerotic plaques of Apoe−/−/Ampkα1−/− mice were less than those in the plaques of the Apoe−/− mice, suggesting a negative association between CHOP and p-CHOP (Ser30) in vivo in Western diet–fed atherosclerotic mice. Overall, these results strongly support that Ampkα1 deficiency exacerbates atherosclerotic plaque vulnerability.

Atherosclerotic plaque rupture is a common pathogenic mechanism for coronary heart diseases and stroke. Our results strongly support the hypothesis that AMPK activation is a novel target in preventing atherosclerotic plaque rupture. Besides its beneficial effects on atherosclerotic plaque stability, AMPK activation inhibits the initiation and progression of atherosclerosis in vivo32,37,38 via its protective effects in endothelial cells39 and VSMCs.40 Consistently, metformin, a well-characterized AMPK activator, has been demonstrated to effectively reduce adverse cardiovascular events and mortality in patients with type II diabetes mellitus.41

In summary, our study has demonstrated that Ampkα1 deficiency increases injury-induced neointimal disruption in Apoe−/− mice, and AMPKα1 phosphorylates CHOP at Ser30
to promote CHOP ubiquitination and degradation. Our results indicate that AMPK may be a novel therapeutic target for promoting atherosclerotic plaque stability and the prevention of acute coronary heart disease and stroke.

**Sources of Funding**

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**Disclosures**

None.

**References**


24. Schrijvers DM, De Meyer GR, Herman AG, Martinet W. Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque


**Novelty and Significance**

**What Is Known?**

- *Chop* deletion leads to reduced macrophage apoptosis and inhibits plaque rupture in mice.
- AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) reduces *CHOP* (C/EBP homologous protein) protein levels in several cell types.

**What New Information Does This Article Contribute?**

- *Ampkα1* deficiency promotes macrophage apoptosis, *CHOP* accumulation, and injury-induced neointimal disruption.
- Inhibition of *CHOP* reduces *Ampkα1* knockdown-elevated macrophage apoptosis.
- Phosphorylation of *CHOP* at Ser30 by AMPKα1 promotes *CHOP* ubiquitination and degradation in macrophages.

We show that *Ampkα1* deficiency aggravates injury-induced neointimal disruption in *Apoe−/−* mice. In macrophages, AMPKα1 phosphorylates *CHOP* at Ser30 to promote its ubiquitination and degradation. Our results imply that AMPK may be a novel therapeutic target for promoting atherosclerotic plaque stability and the prevention of acute coronary heart disease and stroke.
Injury-Induced Neointimal Disruption In Vivo
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http://circres.ahajournals.org/content/119/10/1089

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/09/20/CIRCRESAHA.116.309463.DC1
Phosphorylation of C/EBP homologous protein (CHOP) by the AMP-activated protein kinase alpha 1 in macrophages promotes CHOP degradation and reduces injury-induced neointimal disruption in vivo

Xiaoyan Dai¹, Ye Ding¹, Zhaoyu Liu¹, Wencheng Zhang², Ming-Hui Zou¹*

¹Center for Molecular and Translational Medicine, Georgia State University, Atlanta, GA, 30303, USA
²The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Health; the State and Shandong Province Joint Key Laboratory of Translational Cardiovascular Medicine, Qilu Hospital of Shandong University, Jinan 250012, China

SUPPLEMENTAL MATERIAL

Online materials and methods supplement

Reagents
Antibodies to cleaved caspase-3 (9664) and cleaved PARP (9544) were from Cell Signaling Technology (Beverly, MA). Antibodies to CHOP (sc-7351), ubiquitin (sc-8017), and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-CHOP/DDIT3 (TA312978) and DDK (TA50011-1) were from Origene (Rockville, MD). Antibody to CD68 (ab31630) was purchased from Abcam (Cambridge, MA). Mouse M-CSF recombinant protein (14-8983-80) was from eBioscience (San Diego, CA). EnVision®+ Dual Link System-HRP (DAB+) was from DakoCytomation (Carpinteria, CA). CHOP/DDIT3 (Human) recombinant protein (H00001649-P01) was from Novus Biologicals (Littleton, CO). Polyethylene cuff was from BD Biosciences (San Jose, CA). Human AMPKα1 recombinant protein were kindly provided by Dr. Dietbert Neumann (Maastricht University, Netherlands). In Situ Cell Death Detection kit (11 684 795 910) was obtained from Roche Applied Science (Indianapolis, IN). Anti-α-smooth muscle actin (A 5691) and cycloheximide (CHX) was from Sigma-Aldrich (St. Louis, MO). 3-MA, MG-132, AICAR, and Compound C were purchased from EMD Millipore (Billerica, MA).

Animals
ApoE−/− (C57BL/6 background) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). AMPKα1 and AMPKα2 homozygous knockout mice (AMPKα1−/− or AMPKα2−/−) were generated as previously described. To breed ApoE−/−/AMPKα1−/− and ApoE−/−/AMPKα2−/− mice, AMPKα1−/− or AMPKα2−/− mice were backcrossed into a C57BL/6 genetic background for at least ten generations before crossing them with ApoE−/− mice. ApoE−/−/AMPKα−/− mice were used as controls. The mice were housed in a controlled environment (20 ± 2 °C, 12-h/12-h light/dark cycle) and had free access to water and standard chow diet. All experiments were conducted in accordance with the Animal Care and Use Committee of the University of Oklahoma Health Sciences Center.

Cell culture
RAW264.7 cells were from ATCC (Manassas, VA). Cells were cultured at 1.0×10⁶ per ml Dulbecco's Modified Eagle's Medium with 10% FBS under 95% air and 5% carbon dioxide (CO₂) at 37.0 °C.

Generation of BMDMs
Primary bone marrow-derived macrophages (BMDMs) were prepared by flushing the bone marrow of the femur and tibia of mice. Cells were cultured with complete RPMI-1640 medium supplemented with mouse M-CSF recombinant protein (50 ng/ml, eBioscience) for 5-7 days.²

**Apoptosis analysis**
Terminal deoxynucleotidyl transferase-mediated nick-end labeling assay (TUNEL) for BMDMs and mouse carotid artery fresh-froze sections were carried out according to a commercially available kit from Roche Applied Science (Indianapolis, Ind). Nuclei were stained with DAPI. OLYMPUS BX51 or Zeiss 710 fluorescent microscope was applied. The percentage of TUNEL-positive nuclei was calculated.

**Western blot analysis**
Cell lysates or tissue homogenates were subjected to Western blot analysis, as described previously.³ The protein content was assayed by BCA protein assay reagent (Pierce, USA). 50 µg protein was loaded to SDS-PAGE and then transferred to PVDF membrane. Membrane was incubated with a 1:1000 dilution of primary antibody, followed by a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by ECL (GE Healthcare). The intensity (area × density) of the individual bands on Western blots was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). The background was subtracted from the calculated area. We used control as 100%.

**Transfection of siRNA into RAW264.7 cells**
*In vitro*, transient transfection of CHOP siRNA (sc-35438) and AMPKα1 siRNA (sc29674) was carried out according to Santa Cruz's protocol. Briefly, the siRNAs were dissolved in siRNA buffer to prepare a 10 µmol/L stock solution. RAW264.7 cells grown in 6 well plates were transfected with siRNA in transfection medium (Gibco) containing liposomal transfection reagent (Lipofectamine RNAiMax; Invitrogen). For each transfection, 100 µl transfection medium containing 4 µl siRNA stock solution was gently mixed with 100 µl transfection medium containing 4 µl transfection reagent. After a 30-min incubation at room temperature, siRNA-lipid complexes were added to the cells in 800 µl transfection medium, and cells were incubated with this mixture for 6 h at 37 °C. The transfection medium was then replaced with normal medium, and cells were cultured for 48 h.

**Half-life of CHOP protein degradation determination**
Cells were incubated with a protein translation inhibitor cycloheximide (CHX, 5 µg/ml) to block de novo protein synthesis and treated with or without AICAR (1 mmol/L) for indicated time. Western blot analysis and quantification of CHOP/DDK level were performed.

**RNA extraction, reverse transcriptase-polymerase chain reaction (RT-PCR), and quantitative PCR (qPCR)**
Cell total RNA was extracted using the TRI Reagent® Solution (AM9738; Life technologies) and 1 µg of RNA was reverse-transcribed into cDNA using iScript cDNA Synthesis Kits (Bio-Rad, Hercules, CA). PCR amplification was performed using the SYBR PCR mix (Bio-Rad, Hercules, CA). Real-time primer sequences are shown in Online Table I.

**Immunoprecipitation**
RAW264.7 cells were collected in RIPA lysis buffer (sc-24948; Santa Cruz). Supernatants were immunoprecipitated with 5 µg of anti-AMPKα overnight and then incubated with protein A sepharose
CL-4B (95016-992; VWR) for another 3 h at 4 °C. CHOP was detected as described for western blot analyses.

**In vitro kinase assays.**

*In vitro* kinase assays were done as described previously.4

**DNA mutagenesis.**

Myc-DDK-tagged ORF clone of CHOP (MR201400) was purchased from Origene Company (Rockville, MD). Ser30 of CHOP was mutated to Alanine or Glutamic acid by using the QuikChange kit (Stratagene), according to the manufacturer's instructions.5, 6 Oligonucleotides used for point mutation are listed in Online Table II. All mutations were confirmed by DNA sequencing. Plasmid DNA was extracted on a large scale using Qiagen's EndoFree plasmid maxikit (12362) and transfected into RAW264.7 cells by using the X-tremeGENE HP DNA Transfection Reagent (06 366 236 001; Roche), according to the instructions provided by the manufacturer. After transfection, cells underwent 24-h incubation before receiving any additional treatments. Cells transfected with the expression WT DDK-CHOP served as controls.

**Tissue collection, processing, and lesion analysis**

Anesthetized mice were perfused through the left ventricle with PBS under physiological pressure. The carotid arteries in the cuff were removed and embedded in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan), snap-frozen in liquid nitrogen, and stored at -80°C until use. Intracuff lesions were evaluated by analysis of three serial cross-sections (6 μm thickness) that were obtained at an interval of 0.3 mm. For morphometric analysis, the sections were stained with hematoxylin-eosin (H&E), and ruptured plaque is defined as a lesion of neointimal disruption with mural or occlusive thrombus.

**Immunohistochemistry staining**

Consecutive frozen sections were incubated in endogenous peroxidase and protein block buffer (DAKO), and then with primary antibodies overnight at 4°C. Slides were rinsed with washing buffer and incubated with labeled polymer-horseradish peroxidase-anti-mouse/anti-rabbit antibodies followed by DAB+ chromogen detection (DAKO). Immunohistochemical staining for α-smooth muscle actin was performed with detection by Permanent Red (DAKO). After final washes, sections were counterstained with hematoxylin. All positive staining was confirmed by ensuring that no staining occurred under the same conditions with the use of non-immune rabbit or mouse control IgG. Sections were mounted and visualized using an OLYMPUS BX51 microscope.

**Picrosirius red staining**

Sections were stained with picrosirius red staining to determine the collagen content. The images were viewed by normal or polarized light.

**Measurement of serum cholesterol and triglyceride levels**

Mice underwent overnight fast before blood samples were collected. Serum cholesterol and triglyceride levels were measured enzymatically, using Infinity reagents from Thermo DMA, according to the manufacturer’s instructions.

**References**


Online Table I. Specific primers for quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
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<tbody>
<tr>
<td>Chop</td>
<td>5'-CCACCACACCTGAAAGCAGAA-3'</td>
<td>5'-AGGTGAAAGGCAGGGACTCA-3'</td>
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<tr>
<td>18s</td>
<td>5'-GTCTGTGATGCCCCCCCTAGATG-3'</td>
<td>5'-AGCTTTATGACCCGCACTTAC-3'</td>
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Online Table II. Primers for site-directed mutagenesis of CHOP.

<table>
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<tr>
<th>Amino acid position</th>
<th>Point mutation</th>
<th>Primers for mutations (Forward/Reverse)</th>
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<tr>
<td>30</td>
<td>Serine to alanine (S30A)</td>
<td>5'-tctgcaggagttcctgctcagatgaaattgg-3'</td>
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<tr>
<td></td>
<td></td>
<td>5'-ccaattttcatctgaggccagacctcctgaga-3'</td>
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<tr>
<td>30</td>
<td>Serine to glutamic acid (S30E)</td>
<td>5'-ggatctgcaggagtgcttgcatgaaattgggggca-3'</td>
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<tr>
<td></td>
<td></td>
<td>5'-tgcccccaattttcatctgagtccagacctcctgagatcc-3'</td>
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Online Table III. Characterization of injury-induced neointimal plaques in scramble siRNA or Chop siRNA-administrated Apoe−/− mice.

<table>
<thead>
<tr>
<th>Apoe−/− mice (total number)</th>
<th>Neointimal plaque</th>
<th>Mean plaque area (×10³ mm²)</th>
<th>Neointimal disruption with thrombus</th>
</tr>
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<tbody>
<tr>
<td>Scramble siRNA (n=18)</td>
<td>16</td>
<td>16.83 ± 1.55</td>
<td>5 (27.8%) 8 (44.4%) 13 (72.2%)</td>
</tr>
<tr>
<td>Chop siRNA (n=18)</td>
<td>15</td>
<td>15.57 ± 1.44</td>
<td>3 (16.6%) 3 (16.7%)* 6 (33.3%)*</td>
</tr>
</tbody>
</table>

Neointimal disruption are defined as plaques that show disruptions in the neointima with mural or occlusive thrombus. Data represented as the mean ± SEM. *P < 0.05 vs scramble siRNA Apoe−/− mice.
Online Figure I. Immunoblots of lysed RAW264.7 cells transfected with scramble siRNA, Ampkα1 siRNA and/or Chop siRNA for 48 h. n = 4. The antibodies against targeted proteins are indicated. Data are presented as means ± SEM. *P < 0.05 vs as indicated. #P < 0.05 vs as indicated.
Online Figure II. Ampkα1 deletion upregulates CHOP target gene expression in bone marrow-derived macrophages (BMDMs). Gene expression was measured by qRT-PCR using 18S as a negative control. n = 5. Data are presented as means ± SEM. *P < 0.05 vs WT.
**Online Figure III.** Ampkα1 deletion has no effect on Chop gene expression in BMDMs. Gene expression was measured by qRT-PCR using 18s as a negative control. n = 5. Data are presented as means ± SEM.
Online Figure IV. AICAR does not accelerate Chop mRNA degradation in RAW264.7 cells. RAW264.7 cells were incubated with actinomycin D (Act-D, 5 μg/mL) and treated with or without AICAR (1 mmol/L) for the indicated time. Chop mRNA levels were analyzed by qRT-PCR using 18S as a negative control. n = 3. Data are presented as means ± SEM.
Online Figure V. Plasma lipid levels in mice. n = 7-9. Data are presented as means ± SEM.
Online Figure VI. Vascular smooth muscle cell (VSMC) contents in neointimal lesion of the three groups. Representative immunohistochemical staining of frozen cross sections of the intracuff carotid artery with α-smooth muscle actin antibody in Apoe−/−, Apoe−/−/Ampkα1−/−, and Apoe−/−/Ampkα2−/− mice. Sections were counterstained with hematoxylin. Lumen (L), neointima (N), and media (M) in injured vessels were labeled. Scale bar, 50 μm. Quantification of the α-actin-positive staining is shown below. n = 5.
Online Figure VII. *Ampkα1* deletion promotes atherosclerotic plaque instability in brachiocephalic artery. *ApoE*−/− and *ApoE*−/−/*Ampkα1*−/− mice were fed Western diet for 10 weeks. The brachiocephalic arteries of the mice were isolated, sectioned and stained. **A.** Left, representative Masson’s trichrome staining of brachiocephalic artery cross sections in *ApoE*−/− and *ApoE*−/−/*Ampkα1*−/− mice. Sections were counterstained with hematoxylin. Scale bar, 200 μm. Right, quantitative analysis of collagen content (n = 9-11 per group). **B.** Left, representative cross sections of the brachiocephalic artery with HE staining in *ApoE*−/− and *ApoE*−/−/*Ampkα1*−/− mice. Red circles indicate necrotic core area. Scale bar, 200 μm. Right, quantification of necrotic core area (n = 8 per group). Results are mean ± SEM. *P < 0.05 vs *ApoE*−/− mice.
Online Figure VIII. Co-staining of CD68 (red) and TUNEL (green) in ruptured carotid artery plaque. Lumen (L), neointima (NI), and media (M) in injured vessels were labeled. Scale bar, 50 μm.
Online Figure IX. *Ampkα1* deletion promotes apoptosis in atherosclerotic plaques of brachiocephalic artery. Representative immunofluorescence staining of frozen cross sections of the brachiocephalic artery with a cleaved caspase 3 antibody (red) in *Apoε*<sup>−/−</sup> and *Apoε*<sup>−/−/Ampkα1</sup><sup>−/−</sup> mice. Nuclei were stained with DAPI (blue). Scale bar, 200 μm.
Online Figure X. In vivo level of CHOP and p-CHOP in the atherosclerotic plaques of Apoe<sup>+</sup> and Apoe<sup>+</sup>/Ampkα1<sup>+</sup> mice fed Western diet for 10 weeks. A. Representative immunofluorescence staining of frozen cross sections of the brachiocephalic artery with a CHOP-specific antibody (red) of Apoe<sup>+</sup> and Apoe<sup>+</sup>/Ampkα1<sup>+</sup> mice. B. Representative immunofluorescence staining of frozen cross sections of the brachiocephalic artery with p-CHOP (Ser30) antibody (green) of Apoe<sup>+</sup> and Apoe<sup>+</sup>/Ampkα1<sup>+</sup> mice. Nuclei were stained with DAPI (blue). Scale bar, 200 μm.
Online Figure XI. In vivo knockdown of Chop by siRNA increases collagen content, decreases macrophage infiltration, and has no effect on VSMC contents in ApoE<sup>−/−</sup> mic. A. Left, picrosirius red staining viewed by normal or polarized light. Scale bar, 100 μm. Right, quantitative analysis of collagen content. n = 5. B. Representative immunofluorescence staining of frozen cross sections of the intracuff carotid artery with CD68 (red) antibody in scramble or Chop siRNA-treated Apoe<sup>−/−</sup> mice. Nuclei were stained with DAPI (blue). Scale bar, 100 μm. Quantification of the CD68-positive staining is shown on the below. n = 5. C. Representative immunohistochemical staining of frozen cross sections of the intracuff carotid artery with α-smooth muscle actin antibody in scramble or Chop siRNA-treated Apoe<sup>−/−</sup> mice. Lumen (L), neointima (NI), and media (M) in injured vessels were labeled. Scale bar, 50 μm. Quantification of the α-actin-positive staining is shown on the below. n = 5. Results are mean ± SEM. *P < 0.05 vs scramble siRNA Apoe<sup>−/−</sup> mice.
Online Figure XII. *In vivo* knockdown of *Chop* by siRNA inhibits apoptosis in *Apoe<sup>−/−</sup>* mice. Representative immunofluorescence photomicrograph of the TUNEL (green) assay. Nuclei were stained with DAPI (blue). Lumen (L), neointima (NI), and media (M) in injured vessels were labeled. Scale bar, 100 μm. The percentage of TUNEL-positive (green) cells is shown on the right. n = 5. Results are mean ± SEM. *P < 0.05 vs scramble siRNA *Apoe<sup>−/−</sup>* mice.