AMP-Activated Protein Kinase Alpha 2 Deletion Induces VSMC Phenotypic Switching and Reduces Features of Atherosclerotic Plaque Stability

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Rationale: AMP-activated protein kinase (AMPK) has been reported to play a protective role in atherosclerosis. However, whether AMPKα2 controls atherosclerotic plaque stability remains unknown.

Objective: The aim of this study was to evaluate the impact of AMPKα2 deletion on atherosclerotic plaque stability in advanced atherosclerosis at the brachiocephalic arteries and to elucidate the underlying mechanisms.

Methods and Results: Features of atherosclerotic plaque stability and the markers for contractile or synthetic vascular smooth muscle cell (VSMC) phenotypes were monitored in the brachiocephalic arteries from ApoE/−/AMPKα2−/− mice or VSMC-specific AMPKα2+/− mice in an ApoE−/− background (ApoE−/−/AMPKα2+/−) fed Western diet for 10 weeks. We identified that ApoE−/−/AMPKα2−/− mice and ApoE−/−/AMPKα2+/− mice exhibited similar unstable plaque features, aggravated VSMC phenotypic switching, and significant upregulation of Kruppel-like factor 4 (KLF4) in the plaques located in the brachiocephalic arteries compared with those found in ApoE−/− and ApoE−/−/AMPKα2−/− control mice. Pravastatin, an AMPK activator, suppressed VSMC phenotypic switching and alleviated features of atherosclerotic plaque instability in ApoE−/−/AMPKα2−/− mice, but not in ApoE−/−/AMPKα2+/− mice. VSMC isolated from AMPKα2−/− mice displayed a significant reduction of contractile proteins (smooth muscle actin-α, calponin, and SM-MHC [smooth muscle-mysion heavy chain]) in parallel with increased detection of synthetic proteins (vimentin and osteopontin) and KLF4, as observed in vivo. KLF4-specific siRNA abolished AMPKα2 deletion–induced VSMC phenotypic switching. Furthermore, pharmacological or genetic inhibition of nuclear factor-κB significantly decreased KLF4 upregulation in VSMC from AMPKα2−/− mice. Finally, we found that AMPKα2 deletion markedly promoted the binding of nuclear factor-κBp65 to KLF4 promoter.

Conclusions: This study demonstrated that AMPKα2 deletion induces VSMC phenotypic switching and promotes features of atherosclerotic plaque instability in nuclear factor-κB–KLF4–dependent manner. (Circ Res. 2016;119:718-730. DOI: 10.1161/CIRCRESAHA.116.308689.)

Key Words: AMP-activated protein kinases ■ atherosclerosis

Atherosclerosis, characterized by the accumulation of lipids and inflammatory cells in the large arteries, is one of the most common causes of morbidity and mortality in developed and developing countries.1 2 Atherosclerotic plaque rupture–induced thrombosis or obstruction of coronary artery is the most important cause for the sudden and unpredictable onset of acute coronary syndromes.3 Vulnerable plaques have thin fibrous caps and contain reduced collagen contents. Vascular smooth muscle cells (VSMCs), which are able to form and maintain the fibrous cap and synthesize collagen,4 play a pivotal role in enhancing plaque stability in advanced lesions. Most studies of the role of VSMC on atherosclerotic plaque stability are focused on VSMC apoptosis.5 6 It has been shown that apoptosis of VSMC induces features of plaque vulnerability in atherosclerosis.7 However, recently, Shankman et al8 reported that the contribution of VSMC to atherosclerotic plaques has been greatly underestimated, and Kruppel-like factor 4 (KLF4)–dependent transitions in SMC phenotype are critical in lesion pathogenesis. Using VSMC-specific KLF4 knockout mice, they identified that KLF4 deletion increases multiple indices of plaque stability, suggesting that therapeutic approaches aimed at reducing KLF4 may be a viable means...
of treating advanced atherosclerosis. But how KLF4 itself is regulated in atherosclerosis remains unclear. Over the years, pathogenic factors causing atherosclerotic plaque instability have been a subject of intensive investigation.

AMPK-activated protein kinase (AMPK) is a serine/threonine kinase composed of α, β, and γ subunits.9,10 The α subunit, which controls catalytic activity, has 2 isoforms, α1 and α2, that are differentially expressed in various cell types.11,12 All 3 major cell types in the vasculature (endothelial cells, VSMC, and monocytes/macrophages) express AMPKα. The major isoform in these vasculature cells is AMPKα1, whereas AMPKα2 is the minor isoform. Despite being the minor isoform, AMPKα2 plays an important role in cardiovascular diseases. Recent studies indicate that AMPK not only functions as an intracellular energy sensor and regulator13,14 but also plays critical roles in the pathogenesis of several cardiovascular diseases.15,16 For example, genetic deletion of AMPKα2 in endothelial cells accelerates atherosclerosis by promoting NAD(P)H oxidase, reactive oxygen species (ROS), endothelial dysfunction, and endoplasmic reticulum stress.17,18 In addition, AMPKα2 deletion in VSMC promotes neointimal formation by enhancing VSMC migration and proliferation.19,20 But the contributions of VSMC-derived AMPKα2 in atherosclerosis and plaque stability remain unknown.

By using loss-of-function approach (global AMPKα2−/− and VSMC-specific AMPKα2−/− mice) and activation of AMPKα2 by pravastatin, we aimed to determine the effect and molecular mechanisms of AMPKα2 on atherosclerosis and atherosclerotic plaque stability. Our results indicate that AMPKα2 deletion in VSMC promotes features of atherosclerotic plaque instability via upregulating KLF4 expression. Conversely, pravastatin suppressed VSMC phenotypic switching and enhanced plaque stability via AMPKα2 activation.

Methods
Animal Diet, Feeding Schedule, and Preparation of Tissues
Male Apoe−/− and Apoe−/−AMPKα2−/− mice were fed a Western diet containing 21% milk fat and 0.15% cholesterol for 10 weeks starting at 8 weeks of age. Similarly, 8-week-old male Apoe−/−AMPKα2+/− and Apoe−/−AMPKα2−/− mice were placed on Western diet for the initial 6 weeks to establish aortic lesions. In the presence of Western diet, mice were treated with 50 mg/kg per day pravastatin for an additional 4 weeks. Saline solution was used as solvent control. Mice were euthanized, and blood was collected. Mice were then perfused via the left ventricle with 5-mL PBS followed by 10-mL 4% paraformaldehyde. Brachiocephalic arteries (BA) were carefully dissected and fixed overnight in 4% paraformaldehyde before embedding in optimum cutting temperature compound (OCT; BDH Laboratory Supplies). Details of materials and experimental procedures are in the Methods section in the Online Data Supplement.

Results
AMPKα2 Deletion Enhances Features of Atherosclerotic Plaque Instability
To examine the effects of AMPKα2 deletion on atherosclerotic plaque stability at the BA, we first analyzed the lesion sizes with oil-red-O staining. Consistent with our previous report,17 Apo e−/−AMPKα2−/− mice exhibited an elevation in atherosclerotic plaque size spanning >6 locations within the BA compared with those of Apoe−/− controls (Online Figure IA through ID).

The phenotypic characteristics of vulnerable plaques include increased intraplaque hemorrhage,21,22 presence of buried fibrous cap,23,24 presence of discontinuity in the fibrous cap,25 increased lipid-rich necrotic core size, decreased thickness of fibrous cap,26 decreased plaque collagen content,27 increased macrophage content,28,29 and increased matrix metalloproteases (MMPs), all of which have been widely used as indicators of plaque instability. To test whether AMPKα2 deletion influence the features of plaque stability, the aforementioned parameters were detected within the BA, a widely used artery for studying plaque stability or vulnerability in terms of an advanced atherosclerotic lesion in a mouse model. Intraplaque hemorrhage (Figure 1A, black arrow), defined as the presence of erythrocytes within the plaque, contributes independently to plaque instability as they promote oxidative stress and cholesterol accumulation.30 was significantly increased in Apoe−/−AMPKα2−/− mice relative to Apoe−/− mice (Figure 1B); In addition, buried fibrous caps that may represent old plaque ruptures that have healed31,32 dramatically increased in Apoe−/−AMPKα2−/− mice compared with that of Apoe−/− mice (Figure 1A, black arrowhead and 1C). The presence of fibrous cap discontinuity, which also called acute plaque rupture, defined as a visible breach in the cap,33 may directly reflect plaque rupture and was found increased in Apoe−/−AMPKα2−/− mice (Figure 1D). Furthermore, plaque necrosis, which contributes to inflammation, thrombosis, physical stress on the fibrous cap, and plaque breakdown,33 was analyzed, and the result showed that the necrotic core size in Apoe−/−AMPKα2−/− mice was significantly increased relative to Apoe−/− mice (Figure 1E and 1F). Fibrous cap area, which is widely used as an indirect indicator of plaque stability, was markedly decreased in Apoe−/−AMPKα2−/− mice relative to controls, consistent with features of unstable plaques in humans (Figure 1G and 1H). Also, plaque collagen content, which plays an important structural role in stabilizing plaques,14 was decreased in Apoe−/−AMPKα2−/− mice relative to control mice (Figure 1I and 1J). In addition, macrophage content was increased within plaques from Apoe−/−AMPKα2−/− mice relative to controls, which is also consistent with increased plaque instability (Figure 1K and 1L). Finally, the expression of MMP2, which is a key factor in promoting the vulnerability of an atherosclerotic plaque, was significantly increased in plaque-enriched areas of the BA in Apoe−/−AMPKα2−/− mice relative to control mice (Online Figure II). Taken together, these results demonstrate that AMPKα2 deletion promotes features of an unstable plaque phenotype in advanced atherosclerosis.
AMPKα2 Deletion Induces VSMC Phenotypic Switching In Vivo

VSMC phenotypic switching, mostly defined by a decreased expression of contractile genes and an increase in synthetic genes, plays a pivotal role in enhancing atherosclerotic plaque instability in advanced lesions. To examine the effect of AMPKα2 on regulating VSMC phenotypic switching in vivo, we evaluated changes in expression of molecular markers for contractile and synthetic VSMC phenotypes in the BA from $\text{Apoe}^+/-$ and $\text{Apoe}^+/-\text{AMPKα}2^{-/-}$ mice. As observed by immunohistochemistry and immunofluorescence imaging, smooth muscle actin (SMA)-α expression, which is a typical marker of contractile VSMC, in both the fibrous caps and total plaque, showed significant decrease in $\text{Apoe}^+/-\text{AMPKα}2^{-/-}$ mice relative to $\text{Apoe}^+/-$ controls (Figure 2A through 2C). Although vimentin, which is considered a marker of synthetic VSMC, was significantly increased in the $\text{Apoe}^+/-\text{AMPKα}2^{-/-}$ mice compared with the $\text{Apoe}^+/-$ controls (Figure 2D through 2F). In addition, the expression of SMA-α and vimentin in the media of BA (in which the major cell type is VSMC) showed similar trend with that in the plaque area. All these results demonstrate that AMPKα2 deletion promotes contractile VSMC switching to the synthetic phenotype, which might lead to plaque instability.

AMPKα2 Deletion Upregulates KLF4 Expression in Advanced Atherosclerotic Plaque in BA

KLF4 is a transcription factor of the KLF family and regulates differentiation, proliferation, and apoptosis.36 KLF4 is a well-known negative transcriptional factor for VSMC contractile proteins, which is reported to be upregulated in VSMC phenotype modulation and plaque instability.3 We reasoned that AMPKα2 deletion causes increased KLF4 resulting in VSMC phenotypic switching and plaque instability. To this end, we detected KLF4 expression in the plaques of BA from $\text{Apoe}^+/-$ and $\text{Apoe}^+/-\text{AMPKα}2^{-/-}$ mice. As shown in Figure 3A and 3B, KLF4 expression was significantly increased in both the plaque areas and media layer of the vessel (mainly VSMC) in $\text{Apoe}^+/-\text{AMPKα}2^{-/-}$ mice. Values represent the mean±SEM. *P<0.05 vs $\text{Apoe}^+/-$ mice. Scale bar, 100 μm.
KLF4 might be required for AMPK

AMPK in

Figure 3. AMP-activated protein kinase (AMPK) α2 deletion upregulates Kruppel-like factor 4 (KLF4) expression in advanced atherosclerotic plaque in the brachiocephalic arteries (BA). A. Immunofluorescence staining of KLF4 (red) in BA of Apoe−/− and Apoe−/−AMPKα2−/− mice. DAPI=blue staining of nucleus. Scale bar, 100 μm. B. Quantification of KLF4 expression in BA of Apoe−/− and Apoe−/−AMPKα2−/− mice. n=10 in each group. Values represent the mean±SEM. *P<0.05 vs Apoe−/− mice.
Pravastatin Treatment Alleviates Western Diet–Induced Plaque Instability in Apoe\(^{-/-}\)AMPK\(_{\alpha2^{-/-}}\) Mice but Not in Apoe\(^{-/-}\)AMPK\(_{\alpha2^{+/+}}\) Mice

Pravastatin, which is widely used for reducing the risk of cardiovascular disease, has been reported as an AMPK\(_{\alpha2}\) activator.\(^{37}\) To test whether pravastatin activates AMPK in VSMC, human aortic smooth muscle cells (HASMCs) were treated with pravastatin (0.01–50 \(\mu\)mol/L) for 24 hours. Consistent with a previous report in endothelial cells,\(^{37}\) pravastatin caused a dose-dependent increase in AMPK phosphorylation at Thr172 in HASMC (Figure 4G). In addition, the Thr172 phosphorylation/activity of AMPK in mouse aorta was observed after 4 weeks of pravastatin administration (Figure 4H). ROS has been reported as an upstream signal for AMPK activation.\(^{38,39}\) As shown in Online Figure VIIA through VIIE, exposure of HASMC to pravastatin increased ROS and the activation of AMPK by pravastatin was effectively blocked by either Tempol or mito-Tempol, 2 potent ROS scavengers, suggesting that AMPK activation by pravastatin is ROS mediated.

Pravastatin treatment had no effect on mice body weight (Online Table I) and total plasma cholesterol and triglyceride levels (Online Table II). We had earlier determined whether pravastatin’s protective effect on plaque stability was mediated by VSMC-derived AMPK\(_{\alpha2}\). As shown in Figure 4, pravastatin, administrated for 4 weeks, caused a 49% decrease of necrotic core size (Figure 4C), 17% increase of collagen (Figure 4D and 4E), and 10% increase of fibrous cap thickness (Figure 4F), in Apoe\(^{-/-}\)AMPK\(_{\alpha2^{-/-}}\) mice compared with control. There was a slight decrease in plaque size after treatment with pravastatin, suggesting that pravastatin had little effect on plaque regression. As expected, unlike the effects of pravastatin in Apoe\(^{-/-}\)AMPK\(_{\alpha2^{-/-}}\) mice, pravastatin treatment had no effect on plaque stability, including plaque size, necrotic core size, collagen content, and fibrous cap thickness, in Apoe\(^{-/-}\)AMPK\(_{\alpha2^{+/+}}\) mice (Figure 4A through 4F). These data support the notion that pravastatin enhances plaque stability in the BA via VSMC AMPK\(_{\alpha2}\) signaling.

VSMC AMPK\(_{\alpha2}\) Knockdown Eliminates the Effect of Pravastatin Treatment on VSMC Phenotypic Switching Signaling In Vivo

We further determined whether VSMC-specific AMPK\(_{\alpha2}\) knockdown could induce VSMC phenotypic switching in the BA. As shown in Figure 5A through 5C, Apoe\(^{-/-}\)AMPK\(_{\alpha2^{+/+}}\) mice fed with Western diet for 10 wk and treated with or without pravastatin for 4 wk.
Apoe\(^{-/-}\) mice exhibited significantly reduced SMA-\(\alpha\) content in the fibrous cap and total plaque area relative to Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice. Conversely, the expression of synthetic marker vimentin was markedly increased in the plaque of Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice compared with Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice (Figure 5D through 5F). Meanwhile, we found enhanced KLF4 staining within the BA of Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice, which is consistent with the AMPK\(\alpha2\) global knockout mice (Figure 3). As shown in Figure 5A through 5H, pravastatin treatment not only markedly downregulated KLF4 expression in BA but also significantly increased SMA-\(\alpha\) expression and decreased vimentin expression on the plaque cap and in the total plaque of Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice. However, pravastatin treatment had no effects on the expression of SMA-\(\alpha\), vimentin, and KLF4 in BA in Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice (Figure 5). In addition, the levels of SMA-\(\alpha\), vimentin, and KLF4 in the media of BA of these 4 group mice displayed similar trend with that in the plaque area (Figure 5). Finally, we observed weak costaining of KLF4 and CD68 in BA of the mice in these 4 groups (Online Figure VIIIA). Taken together, these results suggest that VSMC is the major cell origin of KLF4 and that pravastatin via its activation of VSMC AMPK\(\alpha2\) suppresses VSMC phenotypic switching.

**VSMC AMPK\(\alpha2\) Deficiency Does Not Promote VSMC-to-Macrophage Phenotypic Switching**

Shankman et al\(^8\) demonstrate that KLF4 promotes switching of VSMC-to-macrophage phenotype in atherosclerotic plaques. Thus, we reasoned that AMPK\(\alpha2\) deficiency may promote VSMC-to-macrophage phenotypic switching by upregulating KLF4. To address this issue, we conducted both in vivo and in cultured VSMC experiments. We first performed immunofluorescence staining of CD68 and SMA-\(\alpha\) in BA of Apoe\(^{-/-}\) AMPK \(\alpha2^{m/-}\) mice and Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice with or without

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**Figure 5.** Vascular smooth muscle cell (VSMC) AMP-activated protein kinase (AMPK) \(\alpha2\) knockdown eliminates the effect of pravastatin treatment on VSMC phenotypic switching signaling in vivo. **A**, Representative images of immunohistochemistry staining of smooth muscle actin (SMA-\(\alpha\) (dark pink) in the brachiocephalic arteries (BA) of Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) and Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice treated with or without pravastatin. Scale bar, 100 \(\mu\)m. **B** and **C**, Quantification of plaque SMA-\(\alpha\) coverage on the plaque cap (**B**) and total plaque SMA-\(\alpha\) content (**C**) in BA of Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) and Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice treated with or without pravastatin. **D**, Representative images of immunofluorescence (IF) staining of vimentin (red) in the BA of Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) and Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice treated with or without pravastatin. DAPI=blue staining of nucleus. Scale bar, 100 \(\mu\)m. **E** and **F**, Quantification of plaque vimentin coverage on the plaque cap (**E**) and total plaque vimentin content (**F**) in BA of Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) and Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice treated with or without pravastatin. **G**, Representative images of IF staining of Kruppel-like factor 4 (KLF4; red) in the BA of Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) and Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice treated with or without pravastatin. DAPI=blue staining of nucleus. Scale bar, 100 \(\mu\)m. **H**, Quantification of KLF4 expression in BA of Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) and Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice treated with or without pravastatin. n=10 in each group. Values represent the means±SEM. *\(<0.05 vs Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice without pravastatin treatment. #\(<0.05 vs Apoe\(^{-/-}\text{AMPK} \alpha2^{m/-}\) mice with pravastatin treatment.
pravastatin. As shown in Online Figure VIIIB, pravastatin reduced macrophage contents both in Apoe\(^{-}\)/AMPKa2\(^{+/+}\)mice and Apoe\(^{-}\)/AMPKa2\(^{−/−}\)mice, indicating that macrophage-lowering effects of pravastatin is AMPKa2 independent. Western diet reduced SMA-\(\alpha\) staining in mouse aortas from Apoe\(^{-}\) mice (data not shown). Interestingly, pravastatin attenuated the reduction of SMA-\(\alpha\) in Western diet–fed Apoe\(^{-}\) mice but not in Apoe\(^{-}\)/AMPKa2\(^{−/−}\)mice (Online Figure VIIIB), indicating AMPKa2 activation in VSMC is required for pravastatin’s effects on SMA-\(\alpha\).

We next examined macrophage markers in cultured VSMC under cholesterol loading. As depicted in Online Figure VIIIC, cholesterol loading caused a 2-fold increase of the mRNA levels of macrophage marker lgals3 in both wild-type (WT) and AMPK\(\alpha\)2–/– VSMC. In addition, under either basal- or cholesterol-treated conditions, the level of lgals3 in AMPK\(\alpha\)2–/– VSMC was lower than that of WT VSMC (Online Figure VIIIC), suggesting that the loss of AMPK\(\alpha\)2 in VSMC unlikely promotes its VSMC switching into macrophage under cultured conditions. Taken together, these results provide further evidence that AMPK\(\alpha\)2 deficiency has no direct effect on promoting VSMC-to-macrophage phenotypic switching.

AMPK\(\alpha\)2 Deficiency Induces VSMC Phenotypic Switching In Vitro

To elucidate the mechanism of AMPK\(\alpha\)2 on VSMC phenotypic switching, we isolated VSMC from the aorta of WT and AMPK\(\alpha\)2–/– mice and detected markers of contractile and synthetic VSMC phenotypes by real-time polymerase chain reaction and Western blotting. As shown in Figure 6A, SMA-\(\alpha\), calponin, and SM-MHC (smooth muscle-mysion heavy chain), which are 3 canonical contractile markers of VSMC, were significantly decreased in AMPK\(\alpha\)2–/– VSMC compared with those from WT. In contrast, the mRNA and protein levels of vimentin and osteopontin, 2 well-characterized markers for synthetic VSMC, were dramatically increased in AMPK\(\alpha\)2–/– VSMC (Figure 6A and 6B). Consistently, transfection of AMPK\(\alpha\)2-specific siRNA, but not of control siRNA, in HASMC not only significantly lowered the amount of contractile proteins (SMA-\(\alpha\), calponin, and SM-MHC) but also caused a marked upregulation of synthetic proteins (vimentin and osteopontin; Figure 6C and 6D). Furthermore, we analyzed the levels of extracellular matrix and MMP2 protein expression and activity in WT or AMPK\(\alpha\)2–/– VSMC. As we expected, both collagen I and collagen IV were increased in AMPK\(\alpha\)2–/– VSMC compared with those from WT. Consistently, MMP2 expression and MMP2 activity were markedly elevated in AMPK\(\alpha\)2–/– VSMC when compared with those in WT (Online Figure IXA). Consistently, transfection of AMPK\(\alpha\)2-specific siRNA, but not of control siRNA, in HASMC significantly increased collagen I and MMP2 (Online Figure 9B). Taken together, our results indicate that AMPK\(\alpha\)2 deletion triggers the switch of contractile VSMC to the synthetic phenotypes in vitro and in vivo.

AMPK\(\alpha\)2 Deficiency Upregulates KLF4 in VSMC

Next, we detected KLF4 protein expression in VSMC isolated from WT and AMPK\(\alpha\)2–/– mice. As shown in Figure 7A, KLF4 protein was significantly increased in VSMC from AMPK\(\alpha\)2–/– mice compared with their counterparts from WT. Furthermore, the mRNA levels of KLF4 in the VSMC from AMPK\(\alpha\)2–/– mice were 2.6-fold greater than those in VSMC from WT (Figure 7B). Similarly, siRNA knockdown of AMPK\(\alpha\)2 in HASMC significantly increased both the mRNA level and protein expression of KLF4 (Figure 7C and 7D). Taken together, these results indicate that genetic inhibition of AMPK\(\alpha\)2 in VSMC upregulated KLF4.

KLF4 Knockdown Ablates AMPK\(\alpha\)2 Deletion–Induced VSMC Phenotypic Switches in Cultured VSMC

To further investigate a causative role of KLF4 accentuation in AMPK\(\alpha\)2 deletion–induced VSMC phenotypic switching,
the mRNA and protein expression levels of the contractile and synthetic markers were monitored in VSMC transfected with either KLF4-specific siRNA or con siRNA. As expected, transfection of KLF4-specific siRNA, but not con siRNA, significantly suppressed KLF4 expression in VSMC (Figure 7E).

Silencing of KLF4 in AMPKα2−/− VSMC prevented the reduction of mRNA and protein levels for contractile markers such as SMA-α and calponin, as well as prevented upregulation of synthetic markers (Figure 7E and 7F).

To further confirm the involvement of KLF4 in AMPKα2 deletion–induced VSMC phenotypic switching, we concomitantly silenced both KLF4 and AMPKα2 in HASMC. As expected, siRNA-mediated KLF4 silencing rescued the attenuation of contractile markers caused by AMPKα2 deficiency (Figure 7G and 7H). Meanwhile, elevated expression of both protein and mRNA levels of synthetic markers mediated by AMPKα2 silence was blocked in HASMC transfected with KLF4-specific siRNA (Figure 7G and 7H). In summary, our results indicate that KLF4 is required for AMPKα2 deletion–induced VSMC phenotype switching in VSMC.

**AMPKα2 Deficiency Upregulates KLF4 Through Nuclear Factor-κB Signaling**

Next, we determined whether AMPKα2 deletion activates the nuclear factor (NF)-κB pathway in VSMC. First, we analyzed the protein expression of NF-κB pathway molecular

Figure 7. AMP-activated protein kinase (AMPK) α2 deficiency–induced vascular smooth muscle cell (VSMC) phenotype switching is in Kruppel-like factor 4 (KLF4)–dependent manner. A, Western blot analysis of KLF4 protein expression in wild-type (WT) and AMPKα2−/− mouse VSMC (n=5). *P<0.05 vs WT. B, Quantitative real-time polymerase chain reaction (PCR) analysis of KLF4 mRNA level in WT and AMPKα2−/− mouse VSMC (n=5). *P<0.05 vs WT. C, Western blot analysis of KLF4 protein expression in HASMC treated with con siRNA and AMPKα2 siRNA (n=5). *P<0.05 vs con siRNA. D, Quantitative real-time PCR analysis of KLF4 mRNA level in human aortic smooth muscle cells (HASMCs) treated with con siRNA and AMPKα2 siRNA (n=5). *P<0.05 vs con siRNA. E, Western blot analysis of protein expression of smooth muscle actin (SMA)-α, calponin, vimentin, and osteopontin in WT and AMPKα2−/− mouse VSMC treated with con siRNA and KLF4 siRNA for 48 h (n=5). *P<0.05 vs WT+con siRNA. #P<0.05 vs AMPKα2−/−+con siRNA. F, Quantitative real-time PCR analysis of mRNA level of SMA-α, calponin, vimentin, and osteopontin in WT and AMPKα2−/− mouse VSMC treated with con siRNA and KLF4 siRNA for 48 h (n=5). *P<0.05 vs WT+con siRNA. #P<0.05 vs AMPKα2−/−+con siRNA. G, Western blot analysis of protein expression of SMA-α, calponin, vimentin, and osteopontin in HASMC treated with con siRNA, AMPKα2 siRNA, and KLF4 siRNA for 48 h (n=5). *P<0.05 vs con siRNA. #P<0.05 vs AMPKα2 siRNA. H, Quantitative real-time PCR analysis of mRNA level of SMA-α, calponin, vimentin, and osteopontin in HASMC treated with con siRNA, AMPKα2 siRNA and KLF4 siRNA for 48 h (n=5). *P<0.05 vs con siRNA. #P<0.05 vs AMPKα2 siRNA.
markers in VSMC isolated from WT and AMPKa2−/− mice by using Western blots. As shown in Online Figure X, the expression of p-IκBα, pNF-κB p65, and NF-κB p65 in the nucleus were significantly increased in AMPKa2−/− VSMC relative to VSMC from WT. In addition, AMPKa2−/− VSMC showed decreased expression of IκBα indicating enhanced degradation of IκBα in VSMC. These data suggest that AMPKa2 deficiency in VSMC resulted in overactivation of NF-κB and consequent KLF4 upregulation. To further study the contributions of the NF-κB pathway in AMPKa2 deletion–mediated KLF4 upregulation, we used a synthetic NF-κB inhibitor that inhibits translocation of the NF-κB active complex into the nucleus, thereby pharmacologically blocking the NF-κB pathway in WT and AMPKa2−/− VSMC. Notably, the inhibition of NF-κB abolished AMPKa2 deficiency–induced upregulation of KLF4 protein and mRNA levels (Figure 8A and 8B). NF-κB p65 and AMPKa2 were concomitantly silenced in HASMC. As depicted in Figure 8C and 8D, silencing of NF-κB p65 ablated KLF4 upregulation in AMPKa2-silenced HASMC, suggesting that the NF-κB pathway plays a critical role in AMPKa2 deficiency–mediated KLF4 upregulation.

Considering that AMPKa2 deficiency upregulates both the mRNA level and protein expression of KLF4, we hypothesized that AMPKa2 deficiency may upregulate KLF4 through transcriptional activation. By using the Transcription Factor Database (http://www.gene-regulation.com), we found that KLF4’s upstream promoter contains a putative NF-κB p65-binding site (ttccagggaagtccct; at 2018 bp). To confirm the predicted site of the KLF4 promoter is required for increased binding site (tcccagggaagtccct; at 2018 bp). To confirm the transcriptional activation. By using the Transcription Factor Database (http://www.gene-regulation.com), we found that KLF4’s upstream promoter contains a putative NF-κB p65-binding site (ttccagggaagtccct; at 2018 bp). To confirm the predicted site of the KLF4 promoter is required for increased transcriptional regulation induced KLF4 upregulation. Notably, the inhibition of NF-κB abolished AMPKa2 deficiency–induced upregulation of KLF4 protein and mRNA levels (Figure 8A and 8B). NF-κB p65 and AMPKa2 were concomitantly silenced in HASMC. As depicted in Figure 8C and 8D, silencing of NF-κB p65 ablated KLF4 upregulation in AMPKa2-silenced HASMC, suggesting that the NF-κB pathway plays a critical role in AMPKa2 deficiency–mediated KLF4 upregulation.

To directly test whether the 2018 bp predicted site within the KLF4 promoter was responsible for transcriptional regulation by AMPKa2/NF-κB pathway, we performed a promoter assay (luciferase reporter assay) using a KLF4 promoter containing a mutation at 2018 bp. HASMC were simultaneously transfected with WT or KLF4 mutant 2018 bp promoter-reporter plasmid along with con siRNA and AMPKa2 siRNA. Promoter activity was analyzed by luciferase assay. As depicted in Figure 8F, AMPKa2 deficiency increased the activity of the 2600-bp promoter, however, had no effect on the activity of the 1583-bp promoter, implying that the regulatory element necessary for AMPKa2 deficiency–induced increase in KLF4 expression is located between 1583 and 2600 bp, which is consistent with our prediction.

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Finally, we determined the effect of AMPKa2 deficiency on binding activity of NF-κB p65 with the KLF4 promoter. Different primers were designed, and DNA chromatin immunoprecipitation assays were performed in AMPKa2−/− and WT VSMC. As expected, AMPKa2 deletion markedly promoted the binding of NF-κB p65 to the KLF4 promoter (Figure 8H). Taken together, AMPKa2 deletion via NF-κB transcriptional regulation induced KLF4 upregulation.

**Discussion**

The current study demonstrates for the first time that AMPKa2 plays a novel role as a powerful negative regulator of VSMC phenotypic switching. Genetic inactivation of AMPKa2 downregulated contractile proteins while upregulating synthetic proteins in the atherosclerotic plaques and in isolated VSMC. Mechanistically, this phenotype is attributable to NF-κB activation–induced transcriptional upregulation of KLF4 in VSMC. Consistently, genetic or pharmacological inhibition of either KLF4 or NF-κB ablated VSMC phenotypic switching in cultured VSMC. Furthermore, our study suggests that AMPKa2 deletion promotes the features of atherosclerotic plaque instability. VSMC-specific AMPKa2−/− mice exhibited aggravated VSMC phenotypic switching and obvious features of plaque instability. Importantly, pravastatin treatment significantly suppressed VSMC phenotypic switching and enhanced plaque stability in Apoe−/−AMPKa2−/− control mice but had no effect in VSMC-specific AMPKa2−/− mice. These results imply that AMPKa2 plays a protective role in regulating VSMC phenotypic switching and plaque stability.

The major finding of this study is that AMPKa2 deletion in VSMC enhances atherosclerotic plaque instability. Accumulating evidence indicate that AMPK acts as an important regulator in the pathogenesis of cardiovascular diseases. In our present study, we found that genetic inactivation of AMPKa2 promotes features of unstable plaques in advanced Western diet–induced atherosclerotic lesions within the BA. We further confirmed our findings using VSMC-specific AMPKa2−/− mice, which displayed similar features of plaque instability in the BA to that of global AMPKa2−/− mice. Those data indicate that VSMC-derived AMPKa2, even as a minor isoform, does play an important protective role in plaque stabilization.

Another major finding of this study is we demonstrate that AMPKa2 deletion promotes VSMC phenotypic switching in a NF-κB-KLF4–dependent manner. KLF4 has been reported to function as a critical regulator in atherosclerosis. However, the regulation of KLF4 is still not fully understood. Accumulated evidence suggests that KLF4 can be transcriptionally induced in macrophages, VSMC, and other cell types of the vessel in response to vascular injury. In addition, KLF4 activity may also be regulated by post-translational modifications including acetylation, phosphorylation, and sumoylation. Here, in the present study, we demonstrate that AMPKa2 deficiency activates the NF-κB pathway in VSMC, which is consistent with our previous studies in endothelial cells. Importantly, we report for the first time that NF-κB p65 binds with KLF4 promoter and transcriptionally regulates its expression, which provide a novel mechanism for KLF4 regulation in VSMC. Evidence demonstrates that many transcriptional regulatory pathways, including but not limited to serum response factor, myocardin, KLF4, and FoxO4 control VSMC switching from...
Figure 8. AMP-activated protein kinase (AMPK) α2 deletion upregulates Kruppel-like factor 4 (KLF4) through nuclear factor-κB (NF-κB) signaling. A, Western blot analysis of KLF4 expression in wild-type (WT) and AMPKα2−/− mouse VSMC treated with NF-κB control and NF-κB inhibitor (n=5). *P<0.05 vs WT VSMC treated with NF-κB control. #P<0.05 vs AMPKα2−/− VSMC treated with NF-κB control. B, Quantitative real-time polymerase chain reaction (PCR) analysis of mRNA level of KLF4 in WT and AMPKα2−/− mouse VSMC treated with NF-κB control and NF-κB inhibitor (n=5). *P<0.05 vs WT VSMC treated with NF-κB control. #P<0.05 vs AMPKα2−/− VSMC treated with NF-κB control. C, Western blot analysis of KLF4 expression in human aortic smooth muscle cells (HASMCs) treated with NF-κBp65 siRNA and AMPKα2 siRNA (n=5). *P<0.05 vs con siRNA. #P<0.05 vs AMPKα2 siRNA. D, Quantitative real-time PCR analysis of mRNA level of KLF4 in HASMCs treated with NF-κBp65 siRNA and AMPKα2 siRNA (n=5). *P<0.05 vs con siRNA. #P<0.05 vs AMPKα2 siRNA. E, The KLF4 promoter was analyzed using the Transcription Factor Database software, suggesting one binding site within the promoter. 2600 bp and 1583 bp KLF4 promoter luciferase constructs are shown. F, HASMC were transfected with 2600- and 1583-bp KLF4 promoter luciferase constructs and treated with con siRNA or AMPKα2 siRNA, and luciferase activity was measured after 24 h. Results of the luciferase reporter assay are presented as fold changes±SEM of the Firefly/Renilla luciferase activities (n=5). *P<0.05 vs HASMC transfected with 2600-bp KLF4 promoter luciferase construct and con siRNA. #P<0.05 vs HASMC transfected with 2600-bp KLF4 promoter luciferase construct and AMPKα2 siRNA. G, HASMC were transfected with either WT or the mutant KLF4 promoter-reporter and treated with con siRNA or AMPKα2 siRNA for 24 h to detect the luciferase activity (n=5). *P<0.05 vs HASMC transfected with WT 2600-bp KLF4 promoter reporter and con siRNA. #P<0.05 vs HASMC transfected with WT 2600-bp KLF4 promoter reporter and AMPKα2 siRNA. H, Chromatin immunoprecipitation assay for NF-κBp65 binding with KLF4 promoter in WT and AMPKα2−/− mouse VSMC.
expression and MMP2 activity were also markedly elevated in VSMC compared with those in WT. Meanwhile, MMP2 (Figure 3) expression and MMP2 activity were also markedly elevated in VSMC when compared with those in WT (Online Figure IXA). Because MPPs can degrade collagen, the levels of collagen in vascular tissues are determined by the rates of its de novo synthesis and MMP2 degradation. The MMPs-mediated collagen degradation seems to output the rates of its de novo synthesis and MMP2 degradation.

AMPK\(\alpha_2\) deficiency enhanced VSMC phenotypic switching via upregulating KLF4.

Our results provide a novel role of AMPK\(\alpha_2\) as a strong negative regulator in VSMC phenotypic switching. Consistent with the strong secretion character of synthetic VSMC, we observed significantly increased extracellular matrix such as collagen I and collagen IV in AMPK\(\alpha_2\)−/− VSMC compared with those in WT. Meanwhile, MMP2 expression and MMP2 activity were also markedly elevated in AMPK\(\alpha_2\)−/− VSMC when compared with those in WT (Online Figure IXA). Because MPPs can degrade collagen, the levels of collagen in vascular tissues are determined by the rates of its de novo synthesis and MMP2 degradation. The MPPs-mediated collagen degradation seems to output de novo collagen synthesis in AMPK\(\alpha_2\)−/− VSMC, as the levels of collagen contents in the atherosclerotic plaque of Apoe\(−/−\)AMPK\(\alpha_2\)−/− mice are lower than those in Apoe\(−/−\) mice (Figure 1I and J). VSMC deletion of AMPK\(\alpha_2\) promotes KLF-mediated VSMC phenotypic switching resulting in decrease of collagen and reduced plaque stability. Overall, our study supports the notion that selective AMPK\(\alpha_2\) activation in VSMC might be an effective therapy for treating unstable coronary heart diseases.

Accumulating studies have reported that statins can reduce the risk of acute coronary syndrome caused by plaque rupture; however, the mechanism is still unclear. It has been reported that pravastatin could increase plaque stability and inhibit thrombosis through both lipid-dependent and lipid-independent way. Recently, increasing evidence demonstrates that statins have potent anti-inflammatory effects that contribute to atherosclerotic plaque stabilization.\(^{49}\) In our study, 50-mg/kg per day pravastatin was used for the treatment of plaque instability. This dose was chosen according to the guideline of pravastatin sodium tablets, in which illustrates that 100-mg/kg per day dose produces drug exposures ≥2x the human dose of 80 mg based on area under the curve. In addition, several groups have verified that 50-mg/kg per day pravastatin is an appropriate dose for preventing cardiovascular disease and renal ischemia reperfusion injury in mouse model.\(^{50,51}\) We found that 4-week treatment with pravastatin alleviates Western diet–induced plaque instability in advanced atherosclerosis, which is consistent with previous studies reporting the beneficial effect of statins on plaque stability.\(^{27,32,52,53}\) Interestingly, pravastatin has no effect on serum cholesterol and triglyceride levels, which demonstrate that the protective effect of pravastatin on plaque stability in our study is lipid independent. Consistent with early reports that statin activated AMPK in mice endothelial cells,\(^{37}\) we found that pravastatin efficiently activated AMPK in VSMC and in aorta from Western diet–treated mice (Figure 4A and 4B). However, AMPK activation in endothelial cells reported by us\(^{37}\) and others\(^{38}\) seems to be unrelated to statin’s protective effects in plaque stability because the effect of pravastatin on plaque stability was abolished in VSMC-specific AMPK\(\alpha_2\)−/− mice. These data indicate that pravastatin enhances plaque stability via directly activating VSMC-derived AMPK\(\alpha_2\). These findings suggest that AMPK\(\alpha_2\), especially VSMC-derived AMPK\(\alpha_2\), is an attractive therapeutic target for enhancing atherosclerotic plaque stability in clinical practice. As would be expected, AMPK\(\alpha_2\) agonist, especially VSMC-specific AMPK\(\alpha_2\) agonist, may have direct beneficial effect on prevention of atherosclerotic plaque instability. This finding holds promise in leading to effective preventive and therapeutic strategies for vascular diseases.

Because rupture of the atherosclerotic plaque is hard to study directly in humans, it is important to use mouse model to understand how rupture occurs and explore novel therapeutic measures to prevent it from happening. The BA has been reported as the only site in high-fat diet–fed mouse that could display multiple features of plaque instability.\(^{32}\) However, this model only partially mimics the features of unstable plaque in humans, likely because of its lack of thrombosis formation in mice.\(^{23}\) In addition, the BA is very small and consequently difficult to process for histology. Until now, there has been no ideal mouse model of human plaque rupture; hence, we focused on BA for studying plaque vulnerability in terms of an advanced atherosclerotic lesion as a useful mouse model.

In conclusion, results of the present study provide evidence that AMPK\(\alpha_2\) plays a protective role in suppressing VSMC phenotypic switching and enhancing plaque stability in advanced atherosclerosis. This novel finding provides rationale for AMPK\(\alpha_2\) as a potential therapeutic target in preventing atherosclerotic plaque instability.

Sources of Funding

This study was supported by grants from the National Institutes of Heart, Lungs, and Blood and National Institute of Aging (HL079584, HL080499, HL089920, HL110488, and AG047776). This work is in part supported by the Georgia Research Alliance. Dr Zou is a Georgia Research Alliance Eminent Scholar in Molecular Medicine.

Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- Atherosclerotic plaque rupture leading to intraluminal obstructive thrombosis is the most important cause for acute coronary syndromes.
- The AMPK has been reported to play a protective role in atherosclerosis.
- Statins have been reported to increase plaque stability and reduce the risk of acute coronary syndrome.
- KLF4 is a well-known regulator for VSMC phenotypic switch. Deletion of the Klf4 gene increases plaque stability.

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

- AMPKα2 deficiency in VSMC enhances diet-induced atherosclerotic plaque instability in vivo.
- Administration of pravastatin prevents diet-induced VSMC phenotypic switch and atherosclerotic plaque instability via activation of AMPKα2 in vivo.
- AMPKα2 deficiency promotes VSMC phenotypic switch and atherosclerotic plaque instability via upregulating KLF4.
- AMPKα2 deletion activates NF-κB pathway, transcriptionally upregulates KLF4, leading to VSMC phenotypic switch, and atherosclerotic plaque instability.
AMP-Activated Protein Kinase Alpha 2 Deletion Induces VSMC Phenotypic Switching and Reduces Features of Atherosclerotic Plaque Stability
Ye Ding, Miao Zhang, Wencheng Zhang, Qiulun Lu, Zhejun Cai, Ping Song, Imoh Sunday Okon, Lei Xiao and Ming-Hui Zou

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SUPPLEMENTAL MATERIAL

AMP-activated protein kinase alpha 2 deletion induces VSMC phenotypic switching and reduces features of atherosclerotic plaque stability

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Online Supplemental Methods
Materials. Antibodies to KLF4 (4038) for western blotting, AMPKα (2532), AMPK phosphorylated at Thr172 (2535), Vimentin (5741), NF-κBp65 (8482), NF-κBp65 phosphorylated at Ser32 (3031), IκBα (4812), IκBα phosphorylated at Ser32 (2859) were purchased from Cell Signaling Technology. Antibodies to β-actin (sc-10731), AMPKα2 (sc-19129), MMP2 (sc-10736), and normal rabbit IgG (sc-2027) were obtained from Santa Cruz Biotechnology. Antibodies to SM-MHC (ab53219), Osteopontin (ab91655) and smooth muscle actin (SMA)-α (ab5694) for Immunofluorescence staining (IF) were from Abcam. Antibody to KLF4 (AF3158-SP) for IF staining was from R&D. All antibodies were used in a 1:1,000 dilution for western blotting and a 1:100 dilution for IHC. A goat anti-mouse IgG conjugated to Alexa 555 (A21422), a goat anti-Rabbit IgG conjugated to Alexa 488 (A11008), a goat anti-Rabbit IgG conjugated to Alexa 555 (A21428), a goat anti-Rat IgG conjugated to Alexa 647 (A21427) and Dihydroethidine (DHE) was purchased from Molecular Probes (Life Technologies). Pravastatin (2318) was obtained from Tocris Bioscience. The luciferase kit (T1033) was from Invitrogen and the ChIP kit (17-295) was from Upstate. Con siRNA (sc-37007) and siRNAs targeting mouse AMPKα2 (sc-38924), mouse KLF4 (sc-35479), human AMPKα2 (38923), human KLF4 (sc-35480), mouse NF-κBp65 (sc-29411), human NF-κBp65 (sc-29410) and NF-κB inhibitor (sc-3060), NF-κB control (sc-3061) were from Santa Cruz. The transfection reagents for siRNA (Lipofectamine RNAiMax, 13778150) and all primers were from Invitrogen. Nucleofector™ Kits (V4XP-1012) for HASMC was purchased from Lonza. Masson Trichrome Staining kit (HT15A-1KT) was from Sigma Aldrich. Picosirius Red Stain Kit (24901) was from Polysciences.

Animals. Male wild-type (WT) (C57BL6), AMPKα2−/− mice, APOE−/− mice, APOE−/−AMPKα2−/− mice, APOE−/−AMPKα2sm+/mice and APOE−/−AMPKα2sm−/− mice were used in this study. WT, APOE−/− mice and SM22cre mice were obtained from Jackson Laboratories. AMPKα2−/− mice were generated as previously described. AMPKα2−/− mice previously backcrossed to a C57BL6 background were crossed with APOE−/− mice of C57BL6 background to generate APOE−/−AMPKα2−/− mice. APOE−/− served as controls. AMPKα2fflox/fflox mice were provided by Dr. Benoit Viollet. APOE−/−AMPKα2sm−/− (VSMC-specific AMPKα2 knockout) mice and APOE−/−AMPKα2fflox/lyzMcre (macrophage-specific AMPKα2 knockout) mice were generated by crossing AMPKα2fflox/fflox mice with SM22Cre or lymCcre transgenic mice, then breeding into APOE−/− background. APOE−/−AMPKα2fflox/fflox mice served
as controls. The animal protocol was reviewed and approved by Georgia State University Institute Animal Care and Use Committee.

**Morphometric and Immunohistochemical analysis of brachiocephalic arteries (BA).** OCT-embedded BA were serially sectioned at 8-μm thickness from the distal end where it branches into the right subclavian and right carotid for 480 μm. For morphometric and immunohistochemical analysis, sections of each BA were stained at 80-μm intervals from 0 to 480 μm distal to the aortic arch. For example, 0, 80, 160, 240, 320, 400 μm, 6 different locations of the BA were used for each specific type of staining and analysis. For incidence evaluation, if we observed the phenomenon in any one of the six sections, it was counted as one. For other analysis, mean value of the six different sections was collected. H&E staining was performed for analysis of the incidence of intraplaque hemorrhage, buried fibrous cap, discontinuity in the fibrous cap and calculating necrotic core size. Oil red O staining was performed for analysis of plaque size in BA. Sirius Red staining was conducted for measuring the area of the fibrous cap. Masson Trichrome staining was performed for analysis of collagen content by measuring the blue staining area in the images. Immunohistochemical staining (IHC) for SMA-α was conducted with detection by Permanent Red. All other IHC staining was detected by DAB. Immunofluorescence staining (IF) was performed with antibodies specific to vimentin, KLF4, CD68, SMA-α and MMP2. Olympus fluorescence microscope was used for images collecting. Plaque size, necrotic core size and fibrous cap area were quantified by image J (NIH). Collagen content, areas of positive IHC staining and optical density of positive IF staining were analyzed using Image-Pro Plus 6.0 (Media Cybernetics), as described by^2^ 3. All the analyses were performed in a blinded fashion.

**Measurement of serum cholesterol and triglyceride levels.** Serum cholesterol and triglyceride levels were measured enzymatically, using Infinity reagents from Thermo DMA, according to the manufacturer’s instructions.

**Transfection of siRNA into mouse vascular smooth muscle cell (VSMC) and human aortic smooth muscle cell (HASMC).** Transient transfection of siRNA was carried out according to Santa Cruz’s protocol. Briefly, the siRNAs were dissolved in siRNA buffer (20 mM KCl; 6 mM HEPES, pH 7.5; 0.2 mM MgCl₂) to prepare a 10 μM stock solution. Mouse VSMC or HASMC grown in 6 well plates were transfected with siRNA in transfection medium (Gibcol) 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 1.0 ml 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.

**Detection of Reactive Oxygen Species.** Cell O₂⁻ levels were measured according to the DHE fluorescence/ High-performance liquidchromatography (HPLC) assay^4^ with minor modifications.

**Western blot analysis.** Cell lysates were subjected to Western blot analysis. For aorta, we collected the whole aorta, then removed the adventitia and endothelium, the media of the aortic wall homogenates were used for Western blot analysis. The protein content was measured by BCA protein assay reagent (Pierce, USA). 30 μg protein was loaded to SDS-PAGE and then transferred to membrane. Membrane was incubated with a 1:1000 dilution of primary antibody, followed by a 1:5000 dilution of horseradish peroxidase–conjugated secondary antibody. Protein bands were visualized by ECL (GE Healthcare).
MMP activity. The evaluation of MMP activities in response to AngII was performed as described previously. Five micrograms of protein in culture medium or tissue homogenates was electrophoresed in SDS-PAGE gels containing 1 mg/ml gelatin. Gels were washed in 2.5% Triton X-100 for 30 min and incubated overnight in zymography developing buffer at 37 °C. Subsequently, the gel was stained with Coomassie brilliant blue.

Cell culture. At 70-80% confluency, HASMC grown in M231 medium were treated with different agents, as indicated. Primary mouse VSMC collected from aortas of WT and AMPKα2−/− mice were cultured as described previously. Purity of VSMC was confirmed through positive staining for SMA-α. In all experiments, cells were between passages 3 and 10.

PCR for mRNA expression. Total RNA was isolated using Qiagen reagent and reverse transcribed to cDNA with specific antisense primers using the ThermoScript RT-PCR system protocol (Invitrogen). The primers used for RT-PCR are listed in Supplementary Table 3.

DNA constructs and luciferase reporter assays. The DNA fragments from the human KLF4 promoter were amplified by PCR and cloned into pGL-3 Basic (Promega) to generate the KLF4-luciferase constructs, with different following primers: pGL-KLF4-2600 (p2600) forward primer 5'-agtgcgagctccggcgaggagtggaaaaatcag-3', pGL-KLF4-1583 (p1583) forward primer 5'-agtgcgagctcggcggaggttcagtgcagctgag-3', and reverse primer 5'-actaagcttggcgacggccggtact-3'. Mutant constructs of the predicted binding site for NF-κB in the KLF4 promoter were generated by site-directed mutagenesis (Promega) using the following primers: 5'-gtcacttcccacttttagggggctg-3' and 5'-ccccccctaacagtggaaagtgcagctgag-3'. All the plasmids were verified by sequencing. The luciferase reporter plasmids along with the AMPKα2 siRNA were transfected into HASMC using electroporation.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) analyses of WT and AMPKα2−/− VSMC were performed using the Magna ChIP A/G Chromatin Immunoprecipitation Kit, following the manufacture’s recommendations. Briefly, cells were cross-linked using formaldehyde and then DNA was sheared by sonication followed by incubation with 1 μg of normal rabbit IgG, or anti-NF-κB p65 antibody. Immunoprecipitation was performed with the magnetic beads. For PCR, 2 μl of the 50-μl total immunoprecipitated DNA was analyzed with the following oligonucleotides: 5'-agactgcctttccccccc-3' and 5'-ggagatacttttcaccagggagtct-3'.

Statistics. Quantitative results are expressed as mean ± SEM. Chi-Square test was applied to comparisons of intraplaque hemorrhage, buried fibrous cap and discontinuity of the fibrous cap incidence. After confirming that all variables were normally distributed by the Kolmogorov-Smirnov test followed by Q-Q plots analysis, statistical differences were determined by Student’s t-test for comparison between two groups and two-way ANOVA analysis followed by Bonferroni’s multiple comparison test for comparison among multiple groups. P values of less than 0.05 were considered statistically significant.


Supplemental Figure I. AMPKα2 deficiency increases atherosclerotic plaque size significantly in the BA. Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>AMPK<sub>α2</sub><sup>−/−</sup> mice were fed with western diet for 10 weeks, and BA were collected. (A) Oil red O staining of representative BA of Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>AMPK<sub>α2</sub><sup>−/−</sup> mice. Scale bar=100 µm (B) Quantification of atherosclerotic plaque size in the BA in Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>AMPK<sub>α2</sub><sup>−/−</sup> mice. (C) Quantification of plaque area percentage of internal elastic lamina area in Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>AMPK<sub>α2</sub><sup>−/−</sup> mice. (D) Quantification of lumen area in Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup>AMPK<sub>α2</sub><sup>−/−</sup> mice. n=20-21 in each group. Values represent the means ± SEM. *, P<0.05 vs. Apoe<sup>−/−</sup> mice.
Supplemental Figure II. AMPKα2 deficiency upregulates MMP2 expression in atherosclerotic plaque in the BA. Representative images of IF staining of MMP2 and quantification of total plaque MMP2 expression at BA in Apoe−/− and Apoe−/−AMPKα2−/− mice. Scale bar=50 µm. n=10 in each group. Values represent the means ± SEM. *, P<0.05 vs. Apoe−/− mice.
Supplemental Figure III. VSMC is the major cell origin of increased KLF4. Representative images of IF staining of KLF4 (red), SMA-α (green) and CD68 (blue) at BA in Apoe\(^{-}\) and Apoe\(^{-}\) AMPKa2\(^{-}\) mice. Scale bar=200 µm.
Supplemental Figure IV

Supplemental Figure IV. Western blot analysis of AMPKα2 expression in the aorta from Apoe\(^{-/-}\)AMPKα2\(^{m/+}\) and Apoe\(^{-/-}\)AMPKα2\(^{m/-}\) mice (n=2).
Supplemental Figure V

Supplemental Figure V. Oil red O staining of representative BA from Apoe<sup>−/−</sup>AMPKa<sup>2<sup>sm+/+</sup></sup> and Apoe<sup>−/−</sup>AMPKa<sup>2<sup>sm−/−</sup></sup> mice treated with or without pravastatin. Scale bar=100 µm.
Supplemental Figure VI. Macrophage-specific AMPKα2 deficiency has no effect on atherosclerotic plaque formation and plaque instability. Apoe<sup>−/−</sup>AMPKα2<sup>f/f</sup> and Apoe<sup>−/−</sup>AMPKα2<sup>f/f</sup>LyzM<sup>cre</sup> mice were fed with western diet for 10 weeks. (A) Representative images of H&E staining and Masson trichrome staining of BA. (B-D) Quantification of atherosclerotic plaque size (B), necrotic core size (C) and collagen content (D) in the BA of Apoe<sup>−/−</sup>AMPKα2<sup>f/f</sup> and Apoe<sup>−/−</sup>AMPKα2<sup>f/f</sup>LyzM<sup>cre</sup> mice. Scale bar=200 µm, n=8 in each group.
Supplemental Figure VII. Activation of AMPK by statin is ROS dependent. (A) Pravastatin enhances superoxide anions generation. Intracellular superoxide anions was detected by the DHE fluorescence as described in methods. (n=6. *, P<0.05 vs. control; #, P<0.05 vs. pravastatin). (B) Dose-dependent effects of pravastatin on AMPK-Thr172 phosphorylation. (C) Time course of pravastatin induced AMPK phosphorylation at Thr172. (D) Phosphorylation of AMPK (Thr172) induced by 30 min treatment of pravastatin was attenuated by 100 μM Tempol. (E) Phosphorylation of AMPK (Thr172) induced by 30 min treatment of pravastatin was inhibited by 10 μM mito-Tempol.
Supplemental Figure VIII

**A** Representative images of IF staining of KLF4 (red), SMA-α (green) and CD68 (blue) at BA in Apoe−/−AMPKα2sm+/+ and Apoe−/−AMPKα2sm−/− mice with or without pravastatin treatment. Scale bar=200 µm. **B** Quantification of plaque macrophage content in BA based on CD68 IF staining (blue) of Apoe−/−AMPKα2sm+/+ and Apoe−/−AMPKα2sm−/− mice with or without pravastatin treatment. n=10 in each group. Values represent the mean ± SEM. *, P<0.05 vs. Apoe−/−AMPKα2sm+/+ mice without pravastatin treatment. #, P<0.05 vs. Apoe−/−AMPKα2sm−/− mice without pravastatin treatment. **C** WT and AMPKα2−/− VSMCs isolated from mice aorta were treated with 80 µg cholesterol for 72 h. After 72 h cholesterol loading, mRNA level of the macrophage marker Igals3 in WT VSMCs was detected by Real time PCR. n=6. *, P<0.05 vs. WT-0 µg. #, P<0.05 vs. AMPKα2−/−-0 µg.
Supplemental Figure IX

Supplemental Figure 9. AMPKα2 deficiency upregulates extracellular matrix and MMP2 in cultured VSMCs. (A) WT and AMPKα2−/− VSMCs were isolated from mice aorta. MMP2, Collagen I and Collagen IV were detected by western blot. MMP2 activity was detected by Zymography. (B) HASMCs were transfected with con siRNA or AMPKα2 siRNA for 48 h. MMP2 and Collagen I were detected by western blot.
Supplemental Figure X.

Western blot analysis of protein expression of canonical NF-κB pathway markers in WT and AMPKa2−/− mouse VSMC (n=5). *, P<0.05 vs. WT.
**Supplemental Table I.** Serum cholesterol and triglyceride level of VSMC-specific AMPKα2 knockout mice treated with or without pravastatin

<table>
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<th>Total Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
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<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;AMPKα2&lt;sub&gt;sm+/+&lt;/sub&gt; +Control</td>
<td>895.45±25.3</td>
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<tr>
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<td>920.1±30.4</td>
<td>174.6±46.0</td>
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<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;AMPKα2&lt;sub&gt;sm−/−&lt;/sub&gt; +Pravastatin</td>
<td>917.4±26.4</td>
<td>175.6±33.8</td>
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n=10 in each group. Data are expressed by mean ± SEM
**Supplemental Table II.** Body weight of VSMC-specific AMPKα2 knockout mice treated with or without pravastatin

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<th>Apoe&lt;sup&gt;+&lt;/sup&gt;AMPKα2&lt;sup&gt;sm+/+&lt;/sup&gt;</th>
<th>Apoe&lt;sup&gt;+&lt;/sup&gt;AMPKα2&lt;sup&gt;sm-/-&lt;/sup&gt;</th>
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<tr>
<td>Control</td>
<td>31.05±1.68</td>
<td>31.85±0.75</td>
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<tr>
<td>Pravastatin</td>
<td>31.28±0.66</td>
<td>31.6±2.75</td>
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n=10 in each group. Data are expressed by mean ± SEM
**Supplemental Table III.** Primer sequences used in real-time quantitative PCR.

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<tr>
<th>Sequence no.</th>
<th>Gene name</th>
<th>Primer sequence</th>
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<td>1.</td>
<td>Mouse GAPDH</td>
<td>F-5’-AACCTTTGGCATTTGTGGAAGG-3’&lt;br&gt;R-5’-ACACATTGGGGGTAGGAACA-3’</td>
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<tr>
<td>2.</td>
<td>Mouse SMA-α</td>
<td>F-5’-CTGACAGAGGACACCTGAA-3’&lt;br&gt;R-5’-GAAATAGCCAAGCTCAGT-3’</td>
</tr>
<tr>
<td>3.</td>
<td>Mouse Calponin</td>
<td>F-5’-GCACATTTAACCAGGTC-3’&lt;br&gt;R-5’-TGACCTTTCTCACAGAACC-3’</td>
</tr>
<tr>
<td>4.</td>
<td>Mouse SM-MHC</td>
<td>F-5’-TGGACACCATGTCAAGGAA-3’&lt;br&gt;R-5’-ATGGCACAAGCTCAGTCTC-3’</td>
</tr>
<tr>
<td>5.</td>
<td>Mouse Vimentin</td>
<td>F-5’-AGGAAATGGCTCGTCACCTCCGTGAATA-3’&lt;br&gt;R-5’-GGAGTGTCCGGTTTAAAGAATAGAGCT-3’</td>
</tr>
<tr>
<td>6.</td>
<td>Mouse osteopontin</td>
<td>F-5’-CTTTCTACCTCAATCGTCCCTAC-3’&lt;br&gt;R-5’-GCTCTCTTTGTGGAATGCTCAAGT-3’</td>
</tr>
<tr>
<td>7.</td>
<td>Mouse KLF4</td>
<td>F-5’-GAAATTCGCCCGCTCCGATGA-3’&lt;br&gt;R-5’-CTGTGTGTTGTGCCGTAGTGCC-3’</td>
</tr>
<tr>
<td>8.</td>
<td>Human SMA-α</td>
<td>F-5’-CCTGACTGAGGCGCGTATT-3’&lt;br&gt;R-5’-GATGAAGGATGGCTGAAACA-3’</td>
</tr>
<tr>
<td>9.</td>
<td>Human Calponin</td>
<td>F-5’-GCCCAAGATAGTACCACCA-3’&lt;br&gt;R-5’-TGATGAATGGCTGCTGACC-3’</td>
</tr>
<tr>
<td>10.</td>
<td>Human SM-MHC</td>
<td>F-5’-TGGAGGCCGAAAGACAGAGAAC-3’&lt;br&gt;R-5’-TCCGGCGACGGTGATGAAAGA-3’</td>
</tr>
<tr>
<td>11.</td>
<td>Human Vimentin</td>
<td>F-5’-TCCAGCAGCTTCCCTGATTG-3’&lt;br&gt;R-5’-CCCTCACCTGGAAGTGGAT-3’</td>
</tr>
<tr>
<td>12.</td>
<td>Human osteopontin</td>
<td>F-5’-AGGAGAAGGGCAGAGCACA-3’&lt;br&gt;R-5’-CTGATTAGGCGCAGGATGTG-3’</td>
</tr>
<tr>
<td>13.</td>
<td>Human KLF4</td>
<td>F-5’-GGTGCAGCTTGCAGCAGTAA-3’&lt;br&gt;R-5’-AAATCTAGGTCCAGGAGGTCT-3’</td>
</tr>
<tr>
<td>14.</td>
<td>Human GAPDH</td>
<td>F-5’-GGAGTCAACGGATTGGT-3’&lt;br&gt;R-5’-GTGATGGGATTGTGCTGTTGAT-3’</td>
</tr>
<tr>
<td>15.</td>
<td>Mouse Lgals3</td>
<td>F-5’-AGGAGAAGGGAATGATGGCC-3’&lt;br&gt;R-5’-GGTTTGGCACTCTCAAAGGG-3’</td>
</tr>
</tbody>
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