Ablation of Adenosine Monophosphate-Activated Protein Kinase α1 in Vascular Smooth Muscle Cells Promotes Diet-Induced Atherosclerotic Calcification In Vivo

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Rationale: Atherosclerotic calcification is highly linked with plaque rapture. How calcification is regulated is poorly characterized.

Objective: We sought to determine the contributions of AMP-activated protein kinase (AMPK) in atherosclerotic calcification.

Methods and Results: Aortic calcification was evaluated in aortic roots and brachiocephalic arteries of atherosclerotic prone ApoE−/− mice or in mice with dual deficiencies of ApoE and AMPKα isoforms in whole body (ApoE−/−/AMPKα1−/− and ApoE−/−/AMPKα2−/−) or vascular smooth muscle cell (VSMC)–specific or macrophage-specific knockout of AMPKα1 fed with Western diet for 24 weeks. Genetic deficiency of AMPKα1 but not of AMPKα2 promoted atherosclerotic calcification and the expression of Runx2 (Runx-related transcription factor). Conversely, chronic administration of metformin, which activated AMPK, markedly reduced atherosclerotic calcification and Runx2 expression in ApoE−/− mice but had less effects in ApoE−/−/AMPKα1−/− mice. Furthermore, VSMC-specific but not macrophage-specific ablation of AMPKα1 promoted aortic calcification in vivo. Ablation of AMPKα1 in VSMC prevented Runx2 from proteasome degradation in parallel with aberrant osteoblastic differentiation of VSMC, whereas AMPK activation promoted Runx2 post-translational modification by small ubiquitin-like modifier (SUMO, SUMOylation), which is associated with its instability. Mechanically, we found that AMPKα1 directly phosphorylated protein inhibitor of activated STAT-1 (STAT1) (PIAS1), the SUMO E3-ligase of Runx2, at serine 510, to promote its SUMO E3-ligase activity. Finally, mutation of protein inhibitor of activated STAT-1 at serine 510 suppressed metformin-induced Runx2 SUMOylation and subsequently prevented metformin’s effect on reducing oxidized low-density lipoprotein–triggered Runx2 expression in VSMC.

Conclusions: AMPKα1 phosphorylated protein inhibitor of activated STAT-1 to promote Runx2 SUMOylation and subsequently lead to its instability. AMPKα1 deficiency in VSMC increased Runx2 expression and promoted atherosclerotic calcification in vivo. (Circ Res. 2016;119:422-433. DOI: 10.1161/CIRCRESAHA.116.308301.)

Key Words: atherosclerosis ■ AMP-activated protein kinases ■ muscle, smooth, vascular ■ osteoblasts ■ osteogenesis ■ protein inhibitors of activated STAT ■ Runx2 protein ■ sumoylation

Arterial calcification, the presence of calcium deposits in the vessel wall, is a feature of advanced atherosclerosis and reduces elasticity and compliance of the vessel wall.1 In clinics, it has been observed that arterial calcification, especially atherosclerotic calcification, is linked with plaque instability and predicts cardiovascular outcomes of patients.2,3 Accumulating evidence supports the concept that atherosclerotic calcification is not simply a passive precipitation of crystals, but an active cell-driven process characterized by osteogenic differentiation of vascular cells.4–6 Studies reveal that osteoblastic differentiation of vascular smooth muscle cells (VSMC) plays an essential role in the development of atherosclerotic calcification.6,9 Numerous pathogenic factors, such as oxidative stress,7 high glucose,8 and inflammation,9 are
involved in atherosclerotic calcification. However, the underlying mechanisms that drive the process are poorly characterized.

AMP-activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine kinase that consists of 1 catalytic subunit (α) and 2 regulatory subunits (β and γ).11,12 The α subunit that controls catalytic activity has 2 isoforms, α1 and α2, which are differentially expressed in different tissues.13 For example, the α1 isoform predominates in VSMCs,14 macrophages,15 and adipose tissues,16 whereas cardiomyocytes express much higher amounts of AMPKα2.17 Besides the well-known role in metabolic regulation, recent evidence indicates novel roles for AMPK in the pathogenesis of cardiovascular diseases.18 We and other groups reported that AMPK prevents atherosclerosis.19,20 However, knowledge of the role of AMPK in atherosclerotic calcification is limited.

Several recent studies reported that AMPK activation is negatively associated with chondrogenic and bone development21,22 and osteoblastic differentiation of VSMC in vitro.23 Other studies found that AMPK promotes osteogenesis.24 Hence, the role of AMPK in calcification and osteogenesis is highly controversial, and the contributions of AMPKα subunits to the development of atherosclerotic calcification in vivo remain unknown.

Runt-related transcription factor 2 (Runx2) is a key transcription factor that regulates osteoblastic differentiation, which leads to expression of bone matrix proteins.25 Its expression has been identified in atherosclerotic calcified human vascular tissue specimens and in calcifying aortic smooth muscle cells in mice.26,27 Recent studies further revealed that Runx2 in VSMC is crucial in the development of atherosclerotic calcification in mice.5 It has been reported that AMPK activation negatively regulates Runx2 expression in VSMC in vitro,28 but the mechanism is not clear. Here, we report that AMPK in VSMC plays a key role in regulating the initiation and progression of atherosclerotic calcification in vivo via Runx2 small ubiquitin-related modifer (SUMO)ylation and degradation pathway in vivo.

Methods

ApoE−/-AMPKα1−/- and ApoE−/-AMPKα2−/- mice were generated by crossing AMPKα1−/- or AMPKα2−/- mice with ApoE−/- mice. AMPKα1 VSMC- or macrophage-specific AMPKα1-deficient mice in an ApoE−/- background were generated by crossing AMPKα1 floxed mice (AMPKα1fl) with SM22α or lyzMcα transgenic mice and then crossing into an ApoE−/- background to form ApoE−/-AMPKα1fl/SM22α or ApoE−/-AMPKα1fl/lyzMcα mice.

Atherosclerotic calcification in mice was generated as previously described.1 Briefly, 8-week-old male mice in ApoE−/- background were fed with Western diet for 24 weeks. Calcification in atherosclerotic lesions of aortic roots and brachiocephalic arteries was determined by alizarin red and von Kossa staining. Immunofluorescence staining and Western blot were applied to determine the expression of Runx2.

Quantitative values are expressed as the mean±SD and represent data from at least 3 independent experiments. 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 2 groups and ANOVA followed by Bonferroni multiple comparison test for comparison among ≥3 groups. P values of <0.05 were considered statistically significant. All statistical calculations were performed using SPSS version 17.0 (SPSS Inc, Chicago, IL). Details of materials and experimental procedures are in the Methods in the Online Data Supplement.

Results

Deletion of AMPKα1 Subunit but Not of AMPKα2 Subunit Promotes Diet-Induced Atherosclerotic Calcification In Vivo

ApoE−/-AMPKα1−/- and ApoE−/-AMPKα2−/- mice, and their littermate ApoE−/- were fed with Western diets for 24 weeks. Atherosclerotic lesion calcifications were stained by alizarin red von Kossa staining. Metabolic parameters, including body weight, blood glucose, total plasma cholesterol, and plasma triglycerides, did not differ among ApoE−/- mice, ApoE−/-AMPKα1−/-, and ApoE−/-AMPKα2−/- mice (Table I). As shown in Figure 1A and Online Figure I, calcium nodules were evident in the atherosclerotic lesion areas in the aortic roots and brachiocephalic arteries of ApoE−/- mice after 24 weeks of Western diet treatment, an indication of atherosclerotic calcification in vivo.
ApoE−/− mice. Calcium nodule formation was significantly increased in the aortic roots and brachiocephalic arteries of ApoE−/−/AMPKα1−/− when compared with those in ApoE−/− or ApoE−/−/AMPKα2−/− mice (Figure 1A and 1B; Online Figure I). Moreover, ApoE−/−/AMPKα1−/− but not ApoE−/−/AMPKα2−/− exhibited significantly increased calcium content in aortas in comparison with ApoE−/− mice (Figure 1C). These results suggest that deletion of AMPKα1 promotes atherosclerotic calcification in vivo.

AMPKα1 Deficiency Induces Aortic Runx2 Expression In Vivo
It has been reported that Runx2 in VSMCs is crucial for atherosclerotic calcification. Therefore, we measured this important marker in our Western diet–induced atherosclerotic calcification model. As expected, in parallel with increased atherosclerotic calcification, ApoE−/−/AMPKα1−/− mice showed markedly elevated expression of Runx2 in aortas compared with ApoE−/− mice, as indicated by both immunofluorescence staining (Figure 2A and 2B) and Western blot (Figure 2C). Moreover, ApoE−/−/AMPKα1−/− but not ApoE−/−/AMPKα2−/− exhibited significantly increased calcium content in aortas in comparison with ApoE−/− mice (Figure 1C). These results suggest that deletion of AMPKα1 promotes atherosclerotic calcification in vivo.

Administration of Metformin Suppresses Diet-Induced Atherosclerotic Calcification via AMPKα1 In Vivo
Our data imply that AMPKα1 but not AMPKα2 deletion increases atherosclerotic calcification. Therefore, we further tested whether activation of AMPKα1 may reduce atherosclerotic calcification in vivo. Chronic administration of metformin significantly reduced body weight and triglyceride levels in both ApoE−/− and ApoE−/−/AMPKα1−/− mice, but did not alter other metabolic parameters (Online Table II). More importantly, metformin treatment markedly inhibited calcium nodule formation in atherosclerotic plaque areas of aortic roots and aortic calcium content in ApoE−/− mice (Figure 3A through 3C; Online Figure II). The Runx2 expression was also reduced in ApoE−/− plus metformin-treated group compared with the ApoE−/− group (Figure 3D through 3F; Online Figure II). However, as also shown in Figure 3 and Online Figure II, metformin administration had no effects on the marked elevation in calcium nodule formation, aortic calcium content, and Runx2 expression in ApoE−/−/AMPKα1−/− mice. These results further indicate that the activation of AMPKα1 prevents diet-induced atherosclerotic calcification in vivo.

AMPKα1 Depletion in VSMC but Not in Macrophages Is Essential for Induction of Diet-Induced Atherosclerotic Calcification In Vivo
AMPKα1 is the predominant isoform in VSMC and macrophages. We hypothesized that AMPKα1 in VSMC or
macrophages may play a major role in atherosclerotic calcification. To prove this, we generated ApoE−/−/AMPKα1−/− mice with or without metformin (100 mg/kg per d) were treated with Western diet for 24 wk. A, Representative alizarin red staining of aortic roots of indicated groups. B, ApoE−/−/AMPKα1−/− mice had increased alizarin red staining in atherosclerotic lesion areas of aortic roots compared with ApoE−/− mice. Metformin treatment significantly reduced atherosclerotic calcification in ApoE−/− mice, but had no such effect in ApoE−/−/AMPKα1−/− mice. C, ApoE−/−/AMPKα1−/− mice had increased aortic calcium content compared with ApoE−/− mice. Metformin treatment significantly reduced aortic calcium content in ApoE−/− mice, but had no such effect in ApoE−/−/AMPKα1−/− mice. D, Representative immunofluorescence staining of Runx2 in aortic roots of indicated groups. E, ApoE−/−/AMPKα1−/− had increased Runx2 staining in atherosclerotic lesions compared with ApoE−/− mice. Metformin administration significantly inhibited Runx2 staining in ApoE−/− mice, but the effect was abolished in ApoE−/−/AMPKα1−/− mice. F, Western blot results show ApoE−/−/AMPKα1−/− had increased aortic Runx2 expression compared with ApoE−/− mice. Metformin administration significantly inhibited Runx2 expression in ApoE−/−, but the effect was abolished in ApoE−/−/AMPKα1−/− mice (scale bar: 500 μmol/L for alizarin red staining images and 200 μmol/L for immunofluorescence staining of Runx2. ApoE−/−: n=8; ApoE−/−+metformin: n=7; ApoE−/−/AMPKα1−/−: n=7; ApoE−/−/AMPKα1−/− plus metformin: n=6; *P<0.05; n.s. P>0.05).

Figure 3. Metformin administration inhibits atherosclerotic calcification and Runx2 (Runt-related transcription factor) expression via AMP-activated protein kinase (AMPK) α1 in vivo. ApoE−/− and ApoE−/−/AMPKα1−/− mice with or without metformin (100 mg/kg per d) were treated with Western diet for 24 wk. A, Representative alizarin red staining of aortic roots of indicated groups. B, ApoE−/−/AMPKα1−/− mice had increased alizarin red staining in atherosclerotic lesion areas of aortic roots compared with ApoE−/− mice. Metformin treatment significantly reduced atherosclerotic calcification in ApoE−/− mice, but had no such effect in ApoE−/−/AMPKα1−/− mice. C, ApoE−/−/AMPKα1−/− mice had increased aortic calcium content compared with ApoE−/− mice. Metformin treatment significantly reduced aortic calcium content in ApoE−/− mice, but had no such effect in ApoE−/−/AMPKα1−/− mice. D, Representative immunofluorescence staining of Runx2 in aortic roots of indicated groups. E, ApoE−/−/AMPKα1−/− had increased Runx2 staining in atherosclerotic lesions compared with ApoE−/− mice. Metformin administration significantly inhibited Runx2 staining in ApoE−/− mice, but the effect was abolished in ApoE−/−/AMPKα1−/− mice. F, Western blot results show ApoE−/−/AMPKα1−/− had increased aortic Runx2 expression compared with ApoE−/− mice. Metformin administration significantly inhibited Runx2 expression in ApoE−/−, but the effect was abolished in ApoE−/−/AMPKα1−/− mice (scale bar: 500 μmol/L for alizarin red staining images and 200 μmol/L for immunofluorescence staining of Runx2. ApoE−/−: n=8; ApoE−/−+metformin: n=7; ApoE−/−/AMPKα1−/−: n=7; ApoE−/−/AMPKα1−/− plus metformin: n=6; *P<0.05; n.s. P>0.05).

Taken together, these results indicate that loss of AMPKα1 in VSMC, but not in macrophages, promotes atherosclerotic calcification in vivo.

AMPKα1 Depletion Promotes Runx2 Expression and Induces Osteoblastic Differentiation of VSMC In Vitro

To further confirm the in vivo results, we isolated VSMC from wild-type (WT), AMPKα1−/−, and AMPKα2−/− mice, and cultured them in osteogenic media for 2 weeks. As depicted in Figure 5A, murine AMPKα1−/− VSMC had significantly increased calcium nodule formation in both aortic root and brachiocephalic artery areas, as well as aortic calcium content compared with ApoE−/−/AMPKα1−/− mice (Figure 4A, 4B, and 4D; Online Figure III), which mimicked the phenotype of ApoE−/−/AMPKα1−/− mice (Figure 1). The immunofluorescence staining and Western blot further confirmed markedly enhanced aortic expression of Runx2 in ApoE−/−/AMPKα1−/−CreM22 mice in comparison with ApoE−/− mice (Figure 4A, 4C, and 4E). There was no difference of body weight, blood glucose, total plasma cholesterol, and plasma triglycerides between groups (Online Table III).
increased Runx2 expression compared with VSMC transfected with scramble small interfering RNA (siRNA)-transfected (Online Figure IV).

**AMPKα1 Activation Suppresses Oxidized Low-Density Lipoprotein-Induced Runx2 Expression in VSMC**

Elevated oxidized low-density lipoprotein (ox-LDL) could induce Runx2 in VSMC and is associated with atherosclerotic calcification.\(^{29,30}\) We checked the effect of AMPK activation on Runx2 in human aortic VSMC. As expected, metformin supplementation significantly reduced ox-LDL–induced Runx2 upregulation (Figure 5C). However, although ox-LDL still triggered Runx2 upregulation, metformin did not reverse the effect in AMPKα1-silenced cells (Figure 5C).

**AMPKα1 Deficiency–Induced Osteoblastic Differentiation of VSMC Is Runx2 Mediated**

Runx2 in VSMC is essential for atherosclerotic calcification.\(^5\) We hypothesized that Runx2 may be involved in AMPKα1 depletion–induced osteoblastic differentiation of VSMC. To confirm the role of Runx2 in AMPKα1 deficiency–induced VSMC osteoblastic differentiation, we used lentivirus of Runx2 shRNA to knock down Runx2 in VSMC. As expected, knock down of Runx2 by Runx2 shRNA lentivirus significantly suppressed osteoblastic differentiation induced by AMPKα1 depletion in AMPKα1−/− VSMC as indicated by alizarin red staining (Figure 5D). These data suggest that AMPKα1 deficiency leads to Runx2 induction that is essential for AMPKα1 deficiency–induced osteoblastic differentiation in VSMC.

**AMPKα1 Deficiency Suppresses Runx2 Degradation in VSMC**

We next sought to determine how AMPKα1 depletion upregulates Runx2 in VSMC. AMPKα1 depletion increased Runx2 protein levels (Figure 5B). In contrast, the real-time polymerase chain reaction results indicated that AMPKα1 silencing in human aortic VSMC did not alter Runx2 mRNA levels when compared with scramble siRNA-transfected control.
VSMC (Figure 6A). This suggests that AMPKα1 regulates Runx2 expression in post-translation stage.

The proteasome system is important for intracellular protein degradation, and it has been reported that Runx2 can be degraded by the proteasome. To examine whether AMPKα1 depletion leads to Runx2 upregulation via proteasome degradation, AMPKα1 siRNA-treated human aortic VSMC were coincubated with MG132, a potent 26S proteasome inhibitor. MG132 treatment dramatically increased Runx2 expression in scramble siRNA-transfected cells as early as 4 hours, but had only mild effect in AMPKα1-silenced cells (Figure 6B). These results indicate that the activation of AMPKα1 is involved in promoting Runx2 degradation and AMPKα1 deficiency stabilizes Runx2 and prevents its degradation.

**Activation of AMPK Leads to Runx2 Instability in VSMC**

Because our data demonstrated that AMPKα1 deficiency suppresses Runx2 degradation, we hypothesized that activation of AMPK could lead to Runx2 instability. Indeed, metformin treatment significantly reduced Runx2 half-life (Figure 6C).

Figure 5. AMP-activated protein kinase (AMPK) α1 deficiency promotes osteoblastic differentiation of vascular smooth muscle cell (VSMC) via Runx2 (Runt-related transcription factor). A, Alizarin red staining of calcium nodule formation of murine wild-type (WT), AMPKα1−/−, and AMPKα2−/− VSMC treated with osteogenic media for 14 d. AMPKα1 but not AMPKα2 deficiency significantly induced calcium nodule formation compared with WT VSMC. B, AMPKα1-deficient murine VSMC had significantly increased Runx2 expression compared with WT VSMC. C, ox-LDL (100 μg/mL) significantly induced Runx2 expression, which could be inhibited by metformin (2 mmol/L). AMPKα1 silencing markedly increased Runx2 expression compared with scrambled siRNA-transfected (Scr siRNA) cells. Oxidized low-density lipoprotein (ox-LDL) further induced Runx2 expression, but could not be suppressed by metformin supplementation. D, Representative alizarin red staining of murine WT and AMPKα1−/− VSMC with indicated treatment in osteogenic media for 14 d. AMPKα1 deficiency significantly induced calcium nodule formation compared with WT VSMC. Silencing of Runx2 by lentivirus Runx2 shRNA markedly suppressed the effect (n=3 for each experiment). Lenti-Runx2 shRNA indicates lentivirus Runx2 shRNA; and Lenti-Scr shRNA, lentivirus scramble shRNA. *P<0.05; n.s. P>0.05.

Figure 6. AMP-activated protein kinase (AMPK) α1 enhances Runx2 (Runt-related transcription factor) degradation via proteasome in vascular smooth muscle cell (VSMC). A, AMPKα1 silencing did not alter Runx2 mRNA expression compared with scrambled siRNA-transfected (Scr siRNA). B, MG132 (1 μmol/L) significantly increased Runx2 protein expression in Scr siRNA-transfected VSMC as early as 4 h. The effect of MG132 on Runx2 was mild in AMPKα1-silenced VSMC. C, Metformin treatment markedly reduced Runx2 half-life compared with the control (n=3 for each experiment). CHX indicates cycloheximide. *P<0.05; n.s. P>0.05.
Runx2 SUMOylation Leads to Its Instability and Proteasome Degradation in VSMC

It has been reported that Runx2 can be SUMOylated, and SUMOylation of certain proteins could affect their stability. We checked whether SUMOylation of Runx2 may affect its stability and degradation. We silenced protein inhibitor of activated STAT-1 (PIAS1), the SUMO E3-ligase of Runx2, and ubiquitin conjugating enzyme 9 (UBC9), the unique E2 SUMOylation ligase, to suppress Runx2 SUMOylation. As shown in Figure 7A, silencing of PIAS1 and UBC9 significantly increased Runx2 expression in human aortic VSMC. Moreover, MG132 supplementation significantly increased Runx2 expression in scramble siRNA-transfected cells but had mild effect on PIAS1- or UBC9-silenced cells (Online Figure VB and VC).

We next assayed the effect of Runx2 SUMOylation on its stability. As shown in Online Figure 2D, overexpression of PIAS1 and SUMO1 by electroporation to induce Runx2 SUMOylation in human aortic VSMC led to reduced half-life of WT-Runx2 compared with the control. However, PIAS1 and SUMO1 overexpression did not alter the half-life of K181R-Runx2 mutant, which abolishes Runx2 SUMOylation (Online Figure VD). These results suggest that SUMOylation of Runx2 reduces its stability and leads to proteasome degradation.

AMPK Activation Promotes Runx2 SUMOylation in VSMC

Because both AMPK and SUMOylation lead to Runx2 instability, we further hypothesized that AMPK may promote Runx2 SUMOylation and facilitate its degradation. As shown in Figure 7A, metformin treatment significantly induced Runx2 SUMOylation in human aortic VSMC, which could be abolished by compound C, the AMPK inhibitor.

AMPKα1 Binds With Runx2 SUMO E3-Ligase PIAS1 in VSMC

We explored how AMPKα1 regulates Runx2 SUMOylation. We checked whether there is a link between AMPKα1 and PIAS1, the Runx2 SUMO E3-ligase. As indicated in Figure 7B, binding of AMPKα1/PIAS1 was evident in cell lysate pulled down by either PIAS1 or AMPKα immunoprecipitation.

AMPK Increases Phosphorylation of PIAS1 at Serine 510 in VSMC

Based on our findings that AMPKα1 binds with PIAS1, we proposed a mechanism by which AMPK might directly regulate PIAS1 function. Post-translational modifications of PIAS1, including serine phosphorylation, are known to regulate its SUMO E3-ligase activity. We reasoned that AMPK directly phosphorylate PIAS1 serine(s). To test this hypothesis, we first measured total serine phosphorylation of PIAS1 in VSMCs after metformin treatment. As shown in Figure 7C, exposure of human aortic VSMC to metformin increased the serine phosphorylation of PIAS1. Conversely, inhibition of AMPK by compound C dramatically suppressed the effect (Figure 7C). Neither metformin nor compound C altered the PIAS1 total protein levels (Figure 7C).

Next, we determined the serine residue(s) of PIAS1 to be phosphorylated by AMPKα1. GPS 3.0 was applied first to predict the potential phosphorylation site of PIAS1 by AMPK. To confirm these predicted phosphorylation sites, we generated 3 site-directed mutant constructs of PIAS1 (serine (S) to alanine (A) at S90A, S510A, and S626A). In human aortic VSMC transfected with WT-PIAS1, treatment with metformin significantly increased its serine phosphorylation. This increase was abolished in cells transfected with PIAS1 S510A (Figure 7D).

PIAS1 Serine 510 Phosphorylation Is Required for AMPK Activation–Induced Runx2 SUMOylation

Reports indicate that PIAS1 serine phosphorylation promotes its SUMO E3-ligase activity. We next assayed whether PIAS1 S510 phosphorylation plays a role in AMPK activation-triggered Runx2 SUMOylation. As shown in Figure 8A, metformin treatment to increase AMPKα phosphorylation induced Runx2 SUMOylation in WT-PIAS1–transfected human aortic VSMC. However, overexpression of S510A-mutated PIAS1 in human aortic VSMC, which suppresses the endogenous phosphorylation of PIAS1 at serine 510, markedly abolished metformin-caused Runx2 SUMOylation (Figure 8A). This result suggests that PIAS1 serine 510 phosphorylation is required for AMPK activation–induced Runx2 SUMOylation.

Mutation of PIAS1 Serine 510 to Glutamic Acid Abolishes AMPKα Depletion–Induced Runx2 Upregulation in VSMC

Because AMPKα1 directly phosphorylates PIAS1 at serine 510, which promotes Runx2 SUMOylation, we next tested whether serine 510 phosphorylation of PIAS1 regulates Runx2 expression in VSMC. As indicated in Figure 8B, AMPKα1 silencing increased Runx2 expression in both vector and WT-PIAS1–transfected human aortic VSMC. However, in AMPKα1-silenced human aortic VSMC, simultaneous overexpression of PIAS1 S510E mutant, which mimicked the endogenous phosphorylation of PIAS1 at serine 510, significantly inhibited AMPKα1 silencing–induced Runx2 upregulation compared with WT-PIAS1–transfected cells (Figure 8B).

Mutation of PIAS1 Serine 510 to Alanine Suppresses Reduction Effect of Metformin on ox-LDL–Induced Runx2 Expression in VSMC

Our data indicate that metformin reduces ox-LDL–induced Runx2 expression by activation of AMPKα1, and AMPKα1 phosphorylates PIAS1 to promote Runx2 SUMOylation and degradation. Therefore, we reasoned that blockage of PIAS1 serine 510 phosphorylation may abolished the effect of metformin on reducing ox-LDL–induced Runx2 expression. In the absence of ox-LDL, metformin had only marginal effects on the levels of Runx2 (Figure 8C). In contrast, ox-LDL alone markedly increased the levels of Runx2 in cells overexpressed control vector, WT-PIAS1, or S510A-PIAS1 (Figure 8C). As depicted in Figure 8C, metformin reduced ox-LDL–induced Runx2 expression in both WT-PIAS1– and S510A-PIAS1–overexpressed human aortic VSMC. Overexpression of PIAS1 S510A mutant, which
did not alter the endogenous PIAS1 serine 510 phosphorylation, markedly alleviated metformin’s effect on reducing ox-LDL–induced Runx2 expression (Figure 8C). These data suggest that PIAS1 phosphorylation at serine 510 is required for AMPK activation to inhibit ox-LDL–induced Runx2 expression.

Figure 7. AMP-activated protein kinase (AMPK) α1 phosphorylates protein inhibitor of activated STAT-1 (PIAS1) at serine 510 and promotes Runx2 (Runt-related transcription factor) small ubiquitin-related modifier (SUMO)ylation in vascular smooth muscle cell (VSMC). A, Treatment of metformin (2 mmol/L) for 6 h significantly induced Runx2-SUMO1 expression (∼100-kD band). The effect could be blocked by addition of compound C (10 μmol/L). B, AMPKα1 and PIAS1 in whole-cell lysates were pulled down by the appropriate primary antibody and subjected to Western blot analysis to detect the binding of AMPKα1 and PIAS1. C, Serine phosphorylation of PIAS1 was determined by pull-down PIAS1 antibody and immunoblotting with phosphoserine antibody. Six hours of metformin treatment induced PIAS1 phosphorylation as indicated by pulling down by PIAS1 and immunoblotting with phosphoserine antibody (∼80-kD band). The effect could be abolished by compound C. Neither metformin nor compound C altered PIAS1 expression. D, Myc-tagged WT and site-directed mutants of PIAS1 were transfected into human aortic VSMC then treated with metformin for 6 h. Serine phosphorylation of PIAS1 was determined by pull-down with anti-Myc and Western blot analysis with antiphospho-serine antibody (n=3 for each experiment. *P<0.05).

Figure 8. Protein inhibitor of activated STAT-1 (PIAS1) serine 510 phosphorylation is required for AMP-activated protein kinase (AMPK) α1–activated Runx2 (Runt-related transcription factor) small ubiquitin-related modifier (SUMO)ylation and instability. A, Myc-tagged wild-type (WT) and SS10A-mutated PIAS1 were transfected into human aortic vascular smooth muscle cell (VSCM). Metformin (2 mmol/L) treatment significantly induced Runx2-SUMO1 expression (∼100-kD band) in WT-PIAS1–transfected cells, but had no such effect in SS10A-PIAS1–transfected ones. B, AMPKα1 silencing significantly increased Runx2 expression. The effect could be blocked by transfection with SS10E-mutated PIAS1. C, WT and SS10A-mutated PIAS1 were transfected into human aortic VSMC. Oxidized low-density lipoprotein (ox-LDL) markedly induced Runx2 expression in WT-PIAS1– and SS10A-PIAS1–transfected cells. However, metformin addition could only inhibited ox-LDL–triggered Runx2 expression in WT-PIAS1–transfected cells but not in SS10A-PIAS1–transfected ones. (n=3 for each experiment). Scr siRNA indicates scramble siRNA. *P<0.05; n.s. P>0.05.
PIAS1 Ablation Abolishes the Inhibitory Effect of Metformin on ox-LDL–Induced Osteoblastic Differentiation of VSMC

We further tested whether PIAS1 plays a role in osteoblastic differentiation in VSMC, and whether it could affect metformin’s effect on ox-LDL–induced osteoblastic differentiation. As shown in Online Figure III, ox-LDL significantly promoted calcium nodule formation in human aortic VSMC. This was ablated by coadministration of metformin. Meanwhile, PIAS silencing achieved by lentivirus PIAS1 shRNA transfection markedly induced calcium nodule formation, and ox-LDL further enhanced this effect (Online Figure VI). However, metformin could not inhibit calcium nodule formation in the presence of PIAS1 silencing either in the presence or in the absence of ox-LDL (Online Figure VI).

Discussion

The present study has, for the first time, demonstrated that AMPKα1 activation in VSMC prevents the development of atherosclerotic calcification. Deletion of AMPKα1 but not of AMPKα2 promotes atherosclerotic calcification, and more importantly, the AMPKα1 in VSMC contributes to the effect. Metformin treatment, which activates AMPKα1, significantly inhibited atherosclerotic calcification in vivo. Mechanistically, we found that AMPKα1 binds and phosphorylates PIAS1, the SUMO E3-ligase of Runx2, at serine 510, to promote Runx2 instability via proteasome-mediated degradation.

The first important finding is that AMPKα1 deficiency in VSMC promotes atherosclerotic calcification in vivo. This conclusion is supported by several observations. First, our cellular data showed that AMPKα1 deficiency, rather than AMPKα2 deficiency, stimulates osteoblastic differentiation of VSMC. Next, global deficiency of AMPKα1 but not of AMPKα2 in ApoE−/− mice promotes Western diet–induced atherosclerotic calcification. Furthermore, Runx2 was expressed in VSMC but not in macrophages in plaque areas. It should be noted that we applied CD68 as the macrophage marker. Recent studies indicate that CD68 is not only expressed in macrophages but also in several other cell types. This circumstance might be a limitation of our study. Finally, deficiency of AMPKα1 in VSMC enhances atherosclerotic calcification. These data indicate that AMPKα1 in VSMC plays a protective role in preventing atherosclerotic calcification.

We found that Runx2 plays an important role in AMPKα1 deficiency–caused atherosclerotic calcification. The expression of Runx2 is low in normal arteries, and it has been demonstrated that Runx2 in VSMC is essential for osteoblastic differentiation of VSMC in vitro and atherosclerotic calcification in vivo. Runx2 has been shown to induce ALP activity and the expression of bone matrix protein genes, including osteocalcin, bone sialoprotein, and osteopontin, to promote osteoblastic differentiation of VSMC. Studies also indicate that Runx2 induction in VSMC activates RANKL, which mediates the cross talk between calcifying VSMC and migration and differentiation of macrophages into osteoclast-like cells in the atherosclerotic lesions. Our data show that AMPKα1 depletion dramatically increased VSMC Runx2 expression both in vitro and in vivo. The essential role of Runx2 is further supported by the results that shRNA silencing of Runx2 dramatically prevents osteoblastic differentiation in AMPKα1-depleted VSMC.

One interesting finding is that AMPKα1 activation leads to Runx2 instability in VSMC. Our data demonstrate that AMPKα1 deficiency increased Runx2 protein levels without corresponding increase in Runx2 mRNA levels. This suggests that post-translational regulation of Runx2 is responsible for the process. Indeed, we found that AMPK activation by metformin reduced the half-life of Runx2. The ability of MG132, a proteasome inhibitor, that significantly increased Runx2 in control but not in AMPKα1-silenced cells further indicates that AMPKα1 leads to Runx2 instability and degradation by proteasome.

One plausible hypothesis of AMPKα1-mediated Runx2 instability is that AMPKα1 regulates the post-translation modifications of Runx2 that facilitates its degradation. Because AMPK is a well-known serine/threonine(s) protein kinase, and Runx2 phosphorylation has been shown to be linked with increased instability, it is reasonable to hypothesize that AMPKα1 may phosphorylate Runx2 to confer instability. However, we did not detect binding of Runx2 and AMPKα1 (data not shown). Another hypothesis would be that AMPKα1 promotes Runx2 ubiquitination and degradation. Indeed, it has been reported that several E3 ubiquitin ligases promote Runx2 ubiquitination and degradation in osteoblasts, such as Smurf1, Smurf2, and WWP1. We also checked whether AMPKα1 may regulate Runx2 via these E3 ubiquitin ligase. However, we did not find evidence of AMPKα1 regulating these E3 ubiquitin ligases or these E3 ubiquitin ligases regulating Runx2 in VSMC (data not shown).

It has been reported that Runx2 can be SUMOylated, and certain protein SUMOylation, is associated with degradation. Our results demonstrate that AMPK activation causes Runx2 SUMOylation. Our findings further expand the understanding of Runx2 SUMOylation, indicating that SUMOylation of Runx2 at K181 results in its instability. However, the mechanism by which SUMOylation enhances Runx2 degradation is unclear, but may attribute to the conformational change of the protein, which may facilitate its ubiquitination and attachment to proteasome.

Another important finding is that AMPKα1 binds and phosphorylates PIAS1, the SUMO E3-ligase of Runx2, at serine 510, to promote Runx2 SUMOylation. Phosphorylation of PIAS1 is reported to promote its SUMO E3-ligase activity. We speculated that AMPKα1 may directly phosphorylate PIAS1 and therefore regulate Runx2 SUMOylation. The immunoprecipitation experiments confirmed the binding of AMPKα1 and PIAS1. Phosphorylation of PIAS1 at serine 510 by AMPKα1 was demonstrated by the following evidence: (1) AMPK activation promoted PIAS1 phosphorylation in VSMC; (2) mutation of serine 510 to alanine abolished AMPK-induced PIAS1 serine phosphorylation in VSMC. The involvement of PIAS1 serine 510 phosphorylation in Runx2...
SUMOylation is supported by the data that PIAS1 serine 510 mutation to alanine blocks AMPK activation–induced Runx2 SUMOylation in VSMC.

As all our data indicate the preventive role of AMPKα1 activation in osteoblastic differentiation of VSMC and atherosclerotic calcification, one important question is raised: Could AMPK activation be used as a strategy to inhibit atherosclerotic calcification? Indeed, we found that metformin could activate AMPKα1 to suppress ox-LDL–induced Runx2 upregulation in VSMC, and further prevent ox-LDL–induced osteoblastic differentiation of VSMC via PIAS1 signaling. And more importantly, chronic administration of metformin prevented diet-induced atherosclerotic calcification that is AMPKα1-dependent in vivo. These data not only imply the protective effect of metformin on atherosclerotic calcification but also further support the preventive role of AMPKα1 in atherosclerotic calcification.

Several clinical trials indicate that metformin effectively prevents cardiovascular outcomes of diabetic patients with coronary atherosclerotic heart diseases. According to our study, the beneficial effect of metformin may, at least partially, be associated with reducing atherosclerotic calcification, which subsequently stabilize the plaques. Our study may provide a rationale to pursue AMPKα1 activation as a strategy to prevent atherosclerotic calcification in patients having coronary atherosclerotic heart diseases.

We applied metformin as the strategy to activate AMPK in vivo and in vitro. However, metformin exerts other potentially relevant effects. It has been reported to reduce endoplasmic reticulum stress. Endoplasmic reticulum stress has been shown to be involved in vascular calcification. Also, metformin activates both AMPKα1 and AMPKα2. It might be possible that medications that are selective for AMPKα1 and more potent would have more efficacy in preventing atherosclerotic calcification in vivo.

One report suggests that metformin inhibited calcification in VSMC via the AMPK–eNOS–NO pathway. However, eNOS is majorly expressed in endothelial cell but not in VSMC. Compared with other NOS isoforms, eNOS only produces limited NO. Therefore, although AMPK activation could upregulate eNOS expression in VSMC, the subtle eNOS expression in VSMC could not upregulate much NO production in VSMC and limit this signaling in regulating VSMC osteoblastic differentiation. This suggests that AMPK–eNOS–NO pathway might not been the major signaling involving AMPK–Runx2 regulation.

Studies indicate that Runx2 in VSMC promotes vessel stiffness. Because our study demonstrates that AMPKα1 prevents Runx2 expression in VSMC, it would be possible that AMPKα1 activation may improve vascular compliance, which is closely associated with cardiovascular events, especially in aged populations. We are conducting the study of AMPK and age-related arterial stiffness and will publish the data in the future.

It is interesting to note that the roles of AMPK have opposite roles in osteoblasts and VSMC differentiation. Most studies indicate that AMPK promotes osteogenesis of bone, yet AMPK activation attenuates osteoblastic calcification in VSMC. Exact reasons for these differences are unknown, which might be because of the differences of various enzymes and molecules in different cell types. Recent study reports that AMPK phosphorylates Smurf1, the ubiquitin E3-ligase of Runx2, in osteoblast. This phosphorylation blunts Smurf1’s ubiquitin E3-ligase activity and thus promotes Runx2 expression in osteoblast. However, we found silencing of Smurf1 could not alter Runx2 expression in VSMC (data not shown). This might explain why the roles of AMPK in osteoblastic differentiation are opposite in VSMC and osteoblast. Further investigation is warranted.

In summary, the present studies have demonstrated a novel link between AMPKα1 in VSMC and atherosclerotic calcification and uncovered a novel mechanism underlying the regulation of PIAS1 phosphorylation by AMPKα1. The latter induces Runx2 SUMOylation and instability to prevent VSMC osteoblastic differentiation. These findings have identified AMPKα1 activation as a potential target for preventing atherosclerotic calcification in patients with coronary atherosclerotic heart diseases.

**Sources of Funding**

This study was supported by National Institutes of Health (NIH) grants (HL079584, HL080499, HL089920, HL110488 and AG 047776) from the NIH. This work was, in part, supported by the Georgia Research Alliance. Dr Zou is a Georgia Research Alliance Eminent Scholar in Molecular Medicine.

**Disclosures**

None.

**References**


What Is Known?

- Atherosclerotic calcification is closely linked with plaque instability. Patients with atherosclerotic calcification in coronary arteries have a higher risk of cardiovascular events.
- The serine/threonine protein kinase AMPK participates in the pathogenesis of various cardiovascular diseases.
- It has been reported that metformin and adiponectin inhibit hyperphosphatase-induced VSMC osteoblastic differentiation in vitro.
- The transcription factor Runx2 in VSMC is essential for the development of atherosclerotic calcification in vivo.

What New Information Does This Article Contribute?

- AMPKα1 deficiency in VSMC contributes to diet-induced atherosclerotic calcification in mice.
- Administration of metformin prevents diet-induced atherosclerotic calcification via AMPKα1 in vivo.
- AMPKα1 deficiency promotes osteoblastic differentiation of VSMC via increasing Runx2 protein expression.

Novelty and Significance

Atherosclerotic calcification is an independent risk factor for cardiovascular events. Our study demonstrates a novel link between AMPKα1 in VSMC and atherosclerotic calcification, and reveals that AMPKα1 activation enhances PIAS1 phosphorylation at serine 510, to promote Runx2 SUMoylation resulting in subsequent instability. Deletion of AMPKα1 in VSMC accelerates diet-induced atherosclerotic calcification in vivo. Metformin treatment markedly prevents diet-induced atherosclerotic calcification via AMPKα1 in vivo. Thus, these findings have identified AMPKα1 activation as a potential target for preventing atherosclerotic calcification in patients with coronary atherosclerotic heart diseases.
Ablation of Adenosine Monophosphate-Activated Protein Kinase α1 in Vascular Smooth Muscle Cells Promotes Diet-Induced Atherosclerotic Calcification In Vivo
Zhejun Cai, Ye Ding, Miao Zhang, Qiulun Lu, Shengnan Wu, Huaiping Zhu, Ping Song and Ming-Hui Zou

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Ablation of Adenosine Monophosphate-activated Protein Kinase α1 in Vascular Smooth Muscle Cells Promotes Diet-Induced Atherosclerotic Calcification in vivo

Zhejun Cai, Ye Ding, Miao Zhang, Qiulun Lu, Shengnan Wu, Huaiping Zhu, Ping Song, Ming-Hui Zou
Online Supplemental Methods

Chemicals and antibodies

Metformin, N-Ethylmaleimide (NEM), compound C, cycloheximide (CHX), L-ascorbic acid, and β-glycerophosphate were purchased from Sigma-Aldrich. MG132 was purchased from Cayman Chem (Ann Anbor, MI). Human LDL was purchased from Kalen Biomedical (Montgomery Village, MD). Antibodies against Runx2, p-AMPK, AMPK, AMPKα1, myc-tag, GFP, HA-tag, SUMO1, and PIAS1, were purchased from Cell Signaling Technology (Beverly, MA). Antibody against α-SMA was purchased from Sigma-Aldrich (St. Louis, MO). Antibody against CD68 was purchased from Bio-rad (Hercules, CA). Antibody against PIAS1 and β-actin were purchased from Santa Cruz Biotech (Santa Cruz, CA).

Animals

ApoE−/− (C57BL/6 background) and SM22Cre (C57BL/6 background) mice were purchased from Jackson Laboratories (Bar Harbor, ME). AMPKα1−/− and AMPKα2−/− mice were generated as previously described1, and backcrossed into C57BL/6 background for at least ten generations. AMPKα1 floxed (AMPKα1f/f) mice were kindly provided by Dr. Benoit Viollet. ApoE−/−/AMPKα1−/− and ApoE−/−/AMPKα2−/− mice were generated by crossing AMPKα1−/− or AMPKα2−/− mice with ApoE−/− mice. AMPKα1 VSMC- or macrophage-specific deficient mice in ApoE−/− background, were generated by crossing AMPKα1 floxed mice (AMPKα1f/f) with SM22Cre or IyzMCre transgenic mice, and then crossed into ApoE−/− background to form ApoE−/−/AMPKα1f/f/SM22Cre or ApoE−/−/AMPKα1f/f/IyzMCre mice. The mice will be housed in a controlled environment (20 ± 2 °C, 12-h/12-h light/dark cycle) and have free access to water and diet. The animal protocol was reviewed and approved by Georgia State University Institute Animal Care and Use Committee.

Cell culture

Primary murine VSMC were isolated from mouse aorta and cultured in growth media as described previously2. Human aortic VSMC were purchased from Life Technology (Grand Island, NY) and cultured in M231 medium (Life Technology)
supplemented with growth factors (Life Technology). All experiments were performed with VSMC at passages 3 to 5.

Animal experiments

Male ApoE−/− background mice were fed with western diet for 24 weeks to generate atherosclerotic calcification in vivo. To determine the role of AMPKα1 and AMPKα2 in atherosclerotic calcification, mice were grouped as follow: ApoE−/−: n=8; ApoE−/−/AMPKα1−/-: n=8; and ApoE−/−/AMPKα2−/-: n=7. For the in vivo experiment of metformin, mice were separated into four groups: ApoE−/−: n=8; ApoE−/−+metformin: n=7; ApoE−/−/AMPKα1−/-: n=7; and ApoE−/−/AMPKα1−/-+metformin: n=6. 100mg/kg/d of metformin was added into drinking water. To determine the effect of VSMC-specific knockout of AMPKα1 in atherosclerotic calcification, mice were separated into two groups: ApoE−/−/AMPKα1f/f: n=7; ApoE−/−/AMPKα1f/f/SM22Cre: n=7; ApoE−/−/AMPKα1f/f/lyzMCre: n=6. Mice were sacrificed and blood was collected. Mice were then perfused via the left ventricle with 5 ml PBS prior to tissue collection. Aortic roots, brachiocephalic arteries, and descending aortas were carefully dissected and embedded in optimum cutting temperature compound (OCT; BDH Laboratory Supplies).

Alizarin red staining of aortic roots and brachiocephalic arteries

Sections were cut at 8μm for OCT-embedded samples. Serial cryosections were collected and stained with alizarin red (American Master Tech Scientific Inc., Lodi, CA) and von Kossa (Abcam, Cambridge, MA) according to manufacturer’s instruction and previously reported.

Immunofluorescence staining of aortic roots and brachiocephalic arteries

For the immunofluorescence staining analysis, slides were stained with antibodies specific to Runx2, α-SMA, and CD68. Olympus fluorescence microscope was used for images collecting. Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD) was applied for data quantification.

Determination of aortic calcium content

Calcium content in descending aortas was measured using the calcium detection kit (Abcam) as described previously. The amount of vascular calcium was
normalized by the dry weight of the tissues and expressed as millimolar/gram dry weight.

**Preparation of oxLDL**

OxLDL was achieved by copper oxidation of human LDL\(^4\). Briefly, LDL was incubated with 10mmol/l CuSO\(_4\) for 18h at 37°C and terminated by adding 1mmol/l EDTA. OxLDL was then dialyzed, sterilized by filtering, and diluted to 1mg/ml in PBS. The prepared oxLDL was stored at 4°C and used within 2 weeks.

**Plasmids**

The Myc-PIAS1, GFP-Runx2, HA-SUMO1 and control pCMV6-entry plasmid were purchased from Origene (Rockville, MD). PIAS1 S90A, S510A, S626A, and S510E mutants (Myc tagged) was generated using the QuickChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. The mutation was verified by DNA sequencing.

**Cellular small-interfering RNA and plasmids transfection**

Human aortic VSMC were transfected with control small-interfering RNA (siRNA), PIAS1 siRNA duplex (Santa Cruz Biotechnology), AMPKα1 siRNA duplex, or UBC9 siRNA duplex accordingly with RNAiMax (Life Technology). The plasmids were transfected into cells using P1 Primary Cell 4D-Nucleofector® X Kit L electroporation kit from Lonza (Walkersville, MD), following manufacturer’s instruction.

**Lentivirus transfection**

VSMC were infected with recombinant lentiviruses expressing control shRNA (Santa Cruz Biotechnology), PIAS1 shRNA (Santa Cruz Biotechnology), Runx2 shRNA (Santa Cruz Biotechnology) according to manufacturer’s instruction. Colonies were selected by treatment with 5μg/ml puromycin (Santa Cruz Biotechnology) for 7 days.

**In vitro VSMC calcification**

In vitro VSMC calcification was induced in osteogenic media containing 0.25mmol/L L-ascorbic acid and 10mmol/L β-glycerophosphate for 2 weeks as
described previously. Calcification was determined by alizarin red staining and quantified as described previously.

**Immunoprecipitation and western blot analysis**

For immunoprecipitation, samples were collected and lysed in 20mM Tris-HCl pH 7.5 lysis buffer. Supernatants were immunoprecipitated with 5 µg of anti-myc, anti-AMPK, or anti-PIAS1 overnight accordingly and then incubated with protein A sepharose CL-4B (VWR, Suwanee, GA) for another 3 h at 4 °C. Cell lysates or tissue homogenates were subjected to western blot analysis, as described previously. For detection of SUMOylation, 50mmol/L NEM was added to the lysis buffer to protein extraction. Importantly, the samples cannot be boiled for SUMOylation determination. The intensity (area × density) of the individual bands on Bands were quantified by densitometry using Quantity One Software (Bio-Rad, Hercules, CA).

**RNA extraction and quantitative PCR (qPCR)**

Cell total RNA was extracted using the TRI Reagent® Solution (AM9738; Life technologies) and 1µg of RNA was reverse-transcribed into cDNA using iScript cDNA Synthesis Kits (Bio-Rad). PCR amplification was performed using the SYBR PCR mix (Bio-Rad) with primers of Runx2: 5'-CTTCACAAATCCTCCCCAAG-3'; 5'-GAATGCGCCCTAAATCACTG-3', and GAPDH: 5'-GGAGTCAACGGATTTGGT-3'; 5'-GTGATGGGATTTCCATTGAT-3', which was served as internal calibrator.

**Statistical analysis**

Quantitative values are expressed as the mean ± SD and represent data from at least three independent experiments. 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 ANOVA followed by Bonferroni’s multiple comparison test for comparison among three or more groups. P values of less than 0.05 were considered statistically significant. All statistical calculations were carried out using SPSS version 17.0 (SPSS Inc, Chicago, IL, USA).

**References**


Supplemental Figures

Supplemental Figure 1

Supplemental Figure 1. AMPKα1 ablation increases atherosclerotic calcium in vivo. ApoE\(^+\), ApoE\(^+\)/AMPK\(\alpha1\)\(^+\), and ApoE\(^+\)/AMPK\(\alpha2\)\(^+\) mice were fed with western diet for 24 weeks. (A) Representative images of von Kossa staining of aortic roots and brachiocephalic arteries in indicated groups. ApoE\(^+\)/AMPK\(\alpha1\)\(^+\) but not ApoE\(^+\)/AMPK\(\alpha2\)\(^+\) mice had increased von Kossa staining in atherosclerotic lesion areas of aortic roots (B) and brachiocephalic arteries (C) compared with ApoE\(^+\) mice. (Scale bar: 500μm. ApoE\(^+\): n=8; ApoE\(^+\)/AMPK\(\alpha1\)\(^+\): n=8; ApoE\(^+\)/AMPK\(\alpha2\)\(^+\): n=7. *P<0.05; n.s. P>0.05)
Supplemental Figure 2. Metformin administration inhibits atherosclerotic calcification via AMPKα1 in vivo.

ApoE−/− and ApoE−/−/AMPKα1−/− mice with or without metformin (100mg/kg/d) were treated with western diet for 24 weeks. (A) Representative von Kossa staining of aortic roots and brachiocephalic arteries of indicated groups. (B) ApoE−/−/AMPKα1−/− mice had increased von Kossa staining in atherosclerotic lesion areas of aortic roots compared with ApoE−/− mice. Metformin treatment significantly reduced atherosclerotic calcification in ApoE−/− mice, but had no such effect in ApoE−/−/AMPKα1−/− mice. (C) ApoE−/−/AMPKα1−/− mice had increased von Kossa staining in atherosclerotic lesion areas of brachiocephalic arteries compared with ApoE−/− mice. Metformin treatment significantly reduced atherosclerotic calcification in ApoE−/− mice, but had no such effect in ApoE−/−/AMPKα1−/− mice. (Scale bar: 500μm. ApoE−/−: n=8; ApoE−/−+metformin: n=7; ApoE−/−/AMPKα1−/−: n=7; ApoE−/−/AMPKα1−/− +metformin: n=6. *P<0.05; n.s. P>0.05)
Supplemental Figure 3. VSMC-specific AMPKα1 deficiency promotes atherosclerotic calcification in vivo.

ApoE−/AMPKα1f/f, ApoE−/AMPKα1f/f/SM22Cre, ApoE−/AMPKα1f/f/lyzMCre mice were fed with western diet for 24 weeks. (A) Representative images of von Kossa staining of aortic roots and brachiocephalic arteries. ApoE−/AMPKα1f/f/SM22Cre but not ApoE−/AMPKα1f/f/lyzMCre mice had increased von Kossa staining in atherosclerotic lesion areas of aortic roots (B) and brachiocephalic arteries (C) compared with ApoE−/AMPKα1f/f mice. (Scale bar: 500μM for alizarin red staining images, and 200μM for immunofluorescence staining of Runx2. ApoE−/AMPKα1f/f: n=7; ApoE−/AMPKα1f/f/SM22Cre: n=7; ApoE−/AMPKα1f/f/lyzMCre: n=6. *P<0.05; P>0.05)
Supplemental Figure 4

Supplemental Figure 4. AMPKα1 silencing induces Runx2 expression in human aortic VSMC.

Western blot revealed that AMPKα1 silenced human aortic VSMC had significantly increased Runx2 expression compared with scrambled siRNA transfected cells. (Scr siRNA: scrambled siRNA. n=3. *P<0.05)
Supplemental Figure 5. Runx2 SUMOylation promotes its degradation via proteasome in VSMC.

(A) Silencing of UBC9 or PIAS1 significantly increased Runx2 expression in human aortic VSMC. (B and C) MG132 treatment significantly increased Runx2 expression in scrambled siRNA transfected human aortic VSMC as early as 4 hours. It had only mild effect on UBC9 (B) and PIAS1 (C) silenced cells. (D) Overexpression of PIAS1 and SUMO1 significantly reduced half-life of WT Runx2 in human aortic VSMC. The effect was blocked in K181R-mutated Runx2. (n=3 for each experiment. Scr siRNA: scrambled siRNA. *P<0.05; n.s. P>0.05)
Supplemental Figure 6. PIAS1 silencing suppresses metformin’s effect on inhibiting oxLDL-induced osteoblastic differentiation in VSMC.

Human aortic VSMC were transfected with scrambled shRNA lentivirus or PIAS1 shRNA lentivirus, and then cultured in osteogenic media and treated with oxLDL and/or metformin for 14 days. Calcium nodule formation was determined by alizarin red staining. oxLDL (50μg/mL) significantly increased calcium nodule formation, while metformin (0.5mmol/L) addition abolished the effect. PIAS1 silencing by PIAS1 shRNA lentivirus tranfection markedly increased calcium nodules formation, and oxLDL supplementation further enhanced the effect. However, metformin could not suppress the effect of oxLDL in PIAS1 silenced cells. (n=3 for experiment. Lenti-Scr shRNA: scrambled shRNA lentivirus; Lenti-PIAS1 shRNA: PIAS1 shRNA lentivirus. *P<0.05; n.s. P>0.05)
## Supplemental Tables

### Supplemental Table 1. Metabolic parameters of AMPKα subunit deficient mice in ApoE+/− background treated with western diet for 24 weeks

<table>
<thead>
<tr>
<th></th>
<th>ApoE+/−</th>
<th>ApoE+/−/AMPKα1+/−</th>
<th>ApoE+/−/AMPKα2+/−</th>
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<tr>
<td>Body weight (g)</td>
<td>34.33±0.58</td>
<td>33.98±0.42</td>
<td>34.93±0.71</td>
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<td>Triglyceride (mg/dL)</td>
<td>131.83±12.32</td>
<td>139.31±14.32</td>
<td>144.96±13.77</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>1157.33±74.98</td>
<td>1104.11±72.43</td>
<td>1189.43±91.32</td>
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<tr>
<td>Blood glucose (mg/dL)</td>
<td>144.58±11.20</td>
<td>153.21±18.32</td>
<td>139.33±15.04</td>
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<tr>
<td>Calcium (mg/dL)</td>
<td>9.11±0.20</td>
<td>8.80±0.24</td>
<td>9.20±0.37</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>3.64±0.33</td>
<td>3.82±0.26</td>
<td>4.01±0.36</td>
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</tbody>
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ApoE+/−: n=8; ApoE+/−/AMPKα1+/−: n=8; ApoE+/−/AMPKα2+/−: n=7.
**Supplemental Table 2.** Metabolic parameters of metformin-administrated ApoE^{-/-} or ApoE^{-/-}/AMPKα_{1^{-/-}} with western diet for 24 weeks

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<th>ApoE^{-/-}</th>
<th>ApoE^{-/-}+Metformin</th>
<th>ApoE^{-/-}/AMPKα_{1^{-/-}}</th>
<th>ApoE^{-/-}/AMPKα_{1^{-/-}}+Metformin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>34.56±0.43</td>
<td>31.37±0.55*</td>
<td>34.07±0.59</td>
<td>32.28±0.49†</td>
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<td>Triglyceride (mg/dL)</td>
<td>136.98±14.88</td>
<td>87.32±15.21*</td>
<td>141.12±16.32</td>
<td>96.31±13.21†</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>1204.21±84.32</td>
<td>1120.23±64.22</td>
<td>1123.31±89.21</td>
<td>1178.23±93.21</td>
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<tr>
<td>Blood glucose (mg/dL)</td>
<td>148.09±12.88</td>
<td>138.03±13.43</td>
<td>146.31±15.43</td>
<td>137.31±16.99</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>8.76±0.24</td>
<td>8.93±0.21</td>
<td>9.13±0.33</td>
<td>9.02±0.18</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>3.89±0.23</td>
<td>3.77±0.14</td>
<td>3.95±0.32</td>
<td>3.79±0.25</td>
</tr>
</tbody>
</table>

ApoE^{-/-}: n=8; ApoE^{-/-}+metformin: n=7; ApoE^{-/-}/AMPKα_{1^{-/-}}: n=7; ApoE^{-/-}/AMPKα_{1^{-/-}}+metformin: n=6.

*P<0.05 vs. ApoE^{-/-}; †P<0.05 vs. ApoE^{-/-}/AMPKα_{1^{-/-}}
Supplemental Table 3. Metabolic parameters of ApoE−/−/AMPKα1f/f and ApoE−/−/AMPKα1f/f/SM22Cre mice fed with western diet for 24 weeks.

<table>
<thead>
<tr>
<th></th>
<th>ApoE−/−/AMPKα1f/f</th>
<th>ApoE−/−/AMPKα1f/f/SM22Cre</th>
<th>ApoE−/−/AMPKα1f/f/lyzMCre</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>34.74±0.66</td>
<td>34.25±0.48</td>
<td>33.69±0.87</td>
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<td>Triglyceride (mg/dL)</td>
<td>135.66±14.04</td>
<td>137.79±16.09</td>
<td>142.79±13.14</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>1130.67±58.76</td>
<td>1113.13±59.08</td>
<td>1089.42±71.24</td>
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<tr>
<td>Blood glucose (mg/dL)</td>
<td>148.66±13.09</td>
<td>150.08±15.71</td>
<td>153.59±18.05</td>
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<tr>
<td>Calcium (mg/dL)</td>
<td>8.79±0.26</td>
<td>8.85±0.21</td>
<td>8.66±0.18</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>3.77±0.28</td>
<td>3.92±0.17</td>
<td>3.82±0.26</td>
</tr>
</tbody>
</table>

ApoE−/−/AMPKα1f/f: n=7; ApoE−/−/AMPKα1f/f/SM22Cre: n=7; ApoE−/−/AMPKα1f/f/lyzMCre: n=6.