Macrophage Liver Kinase B1 Inhibits Foam Cell Formation and Atherosclerosis

Zhaoyu Liu, Huaiping Zhu, Xiaoyan Dai, Cheng Wang, Ye Ding, Ping Song, Ming-Hui Zou

Rationale: LKB1 (liver kinase B1) is a serine/threonine kinase and tumor suppressor, which regulates the homeostasis of hematopoietic cells and immune responses. Macrophages transform into foam cells upon taking-in lipids. No role for LKB1 in foam cell formation has previously been reported.

Objective: We sought to establish the role of LKB1 in atherosclerotic foam cell formation.

Methods and Results: LKB1 expression was examined in human carotid atherosclerotic plaques and in western diet-fed atherosclerosis-prone Ldlr−/− and ApoE−/− mice. LKB1 expression was markedly reduced in human plaques when compared with nonatherosclerotic vessels. Consistently, time-dependent reduction of LKB1 levels occurred in atherosclerotic lesions in western diet-fed Ldlr−/− and ApoE−/− mice. Exposure of macrophages to oxidized low-density lipoprotein downregulated LKB1 in vitro. Furthermore, LKB1 deficiency in macrophages significantly increased the expression of SRA (scavenger receptor A), modified low-density lipoprotein uptake and foam cell formation, all of which were abolished by blocking SRA. Further, we found LKB1 phosphorylates SRA resulting in its lysosome degradation. To further investigate the role of macrophage LKB1 in vivo, ApoE−/−LKB1fl/flLysMcre and ApoE−/−LKB1fl/fl mice were fed with western diet for 16 weeks. Compared with ApoE−/−LKB1fl/fl wild-type control, ApoE−/−LKB1fl/flLysMcre mice developed more atherosclerotic lesions in whole aorta and aortic root area, with markedly increased SRA expression in aortic root lesions.

Conclusions: We conclude that macrophage LKB1 reduction caused by oxidized low-density lipoprotein promotes foam cell formation and the progression of atherosclerosis. (Circ Res. 2017;121:1047-1057. DOI: 10.1161/CIRCRESAHA.117.311546.)

Key Words: aorta ■ atherosclerosis ■ foam cells ■ homeostasis ■ macrophages

Atherosclerosis is the primary cause of most cardiovascular diseases, such as coronary artery disease, myocardial infarctions, and strokes. The formation and development of atherosclerotic lesions is a chronic process characterized by the deposition of excessive cholesterol in the arterial intima. As the major component in atherosclerotic lesions, macrophages play a critical role in the development of atherosclerosis. During atherogenesis, circulating monocytes transmigrate into the subintima and differentiate into macrophages that can uptake large amounts of modified lipoproteins through the scavenger receptor pathway. Uncontrolled accumulation of lipoproteins transforms the macrophages into lipid-rich foam cells, which is a hallmark of atherosclerosis and results in lesion expansion. Although the formation of foam cells in the arterial wall has long been recognized as a critical step in the development of atherosclerosis, the molecular mechanisms underlying this process are not fully understood.

LKB1 (liver kinase B1) is a conserved serine/threonine kinase that was first identified as a tumor suppressor responsible for Peutz-Jeghers syndrome. However, expression of LKB1 is ubiquitous in many cell types and tissues, suggesting that LKB1 may be a multifunctional protein. Indeed, LKB1 plays a critical role in embryonic development as global Lkb1 knockout (KO) is embryonically lethal in mice. Various tissue- and cell-specific KO mice models have revealed multiple protective roles of LKB1 in cardiovascular diseases and metabolic disorders. Recently, we have found a novel role of LKB1 in macrophages suppressing lipopolysaccharide-induced inflammation. However, the contribution of LKB1 in atherosclerosis, a chronic inflammatory disease, remains unknown.
Novelty and Significance

What Is Known?
- Atherosclerosis is the primary cause of many cardiovascular diseases.
- The serine/threonine protein kinase LKB1 (liver kinase B1) is a well-known tumor suppressor, which has recently been implicated in cardiovascular diseases.
- SRA (scavenger receptor A) is one of the major scavenger receptors responsible for binding and uptake of oxidized and acetylated forms of low-density lipoproteins by macrophages leading to the formation of foam cells.

What New Information Does This Article Contribute?
- LKB1 expression is reduced in macrophages with atherosclerotic progression.
- LKB1 deficiency in myeloid cells promotes foam cell formation and development of atherosclerosis.
- LKB1 phosphorylates SRA and promotes its degradation through the lysosome.

Foam cell formation is crucial for atherosclerosis and fuels the development of vulnerable plaques. We found that the levels of LKB1 are reduced during the development of atherosclerosis and that LKB1 phosphorylates SRA and promotes its degradation by a lysosome-dependent mechanism. These findings reveal an important role of LKB1 in foam cell formation and provide a novel link between LKB1 and atherosclerosis. LKB1 could be a potential therapeutic target for preventing atherosclerotic disease.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>AcLDL</td>
<td>acetylated low-density lipoprotein</td>
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<tr>
<td>BafA</td>
<td>bafilomycin A1</td>
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<tr>
<td>BMDM</td>
<td>bone marrow–derived macrophage</td>
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<tr>
<td>CQ</td>
<td>chloroquine</td>
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<tr>
<td>KO</td>
<td>knockout</td>
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<td>LKB1</td>
<td>liver kinase B1</td>
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<td>OxLDL</td>
<td>oxidized low-density lipoprotein</td>
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<tr>
<td>SRA</td>
<td>scavenger receptor A</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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<td>WT</td>
<td>wild type</td>
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of SRA in the vessel wall is highly induced during cholesterol feeding and the formation of atherosclerotic lesion.20 However, the underlying mechanism is not completely understood. It has been shown that SRA can be regulated by a variety of stimuli such as modified lipoproteins, macrophage colony-stimulating factor, and lipopolysaccharides at the mRNA level.21–23 Recently, it has been reported that the phosphorylation level of SRA is negatively associated with its abundance,24 suggesting a role for phosphorylation in regulating SRA protein level.

Methods

Macrophase-specific LKB1-deficient mice in an ApoE−/− background were generated by crossing LKB1-floxed mice (LKB1fl/fl) with LysMcre transgenic mice and then crossing with ApoE−/− mice to form ApoE−/−LKB1fl/flLysMcre mice. Eight-week-old male ApoE−/−LKB1fl/flLysMcre mice were then retro-orbitally injected with bone marrow cells (5×10⁶) to eliminate endogenous bone marrow stem cells and bone marrow–derived cells. Mice were then treated with the western diet (D12079B, Research Diets) for 16 weeks.

For bone marrow transplantation experiments, 8-week-old male Ldlr−/− mice were subjected to 11-Gy lethal total body irradiation (2 Gy per fraction over 10–15 min) to eliminate endogenous bone marrow stem cells and bone marrow–derived cells. Mice were then retro-orbitally injected with bone marrow cells (5×10⁶) isolated from wild-type (WT) or macrophage-specific LKB1 KO mice. Mice were allowed to recover for 6 weeks after bone marrow transplantation and then fed a western diet for 18 weeks. Atherosclerotic lesions of thoracic aorta, aortic arch, and aortic root were determined by Sudan IV staining and Oil Red O staining.

Results

Expression of LKB1 Is Decreased in Human Atherosclerotic Carotid Artery

To determine the role of LKB1 in the initiation and progression of atherosclerosis, we first measured the expression of LKB1 in human healthy vessels (internal mammary artery) and carotid atherosclerotic plaques. As shown in Figure 1A, LKB1 level was dramatically decreased in atherosclerotic plaques, suggesting an important role of LKB1 in the development of atherosclerotic plaque.

Time-Sensitive Reduction of LKB1 in Western Diet–Fed Atherosclerosis-Prone Mice

To validate this finding, we further examined the expression of LKB1 in atherosclerosis-prone mice that had been fed a western diet for different durations. As shown in Figure 1B, Ldlr−/− mice fed a western diet for 8 weeks showed a 30% reduction in LKB1 protein levels when compared with mice fed a normal chow diet. In mice fed a western diet for 16 weeks, the levels of LKB1 were reduced 70%. The mRNA level of LKB1 was also significantly decreased after 8 weeks of western diet feeding and was further decreased after 16 weeks of western diet feeding (Figure 1C).

In another atherosclerosis-prone mouse model ApoE−/− mice, LKB1 protein level and mRNA level were also significantly decreased after 5 weeks of western diet feeding and were further decreased after 10 weeks of western diet feeding (Figure 1D and 1E). LKB1 level was further analyzed in atherosclerotic lesions by immunofluorescence staining. As shown in Figure 1F, western diet feeding resulted in massive macrophage accumulation in the atherosclerotic lesion area, whereas LKB1 was dramatically decreased in mice fed a western diet for 16 weeks, especially in the lesion area. All these results suggest a potential role for LKB1 in the development of atherosclerosis.

Downregulation of LKB1 Occurs Markedly in Macrophages Compared With Vascular Smooth Muscle Cells

Macrophages play a prominent role in atherosclerotic plaque formation.3 To determine whether LKB1 is decreased in...
macrophages within atherosclerotic plaques, costaining of LKB1 and macrophage marker CD68 was analyzed. As shown in Figure 1F, there was much less costaining of LKB1 and CD68 in mice fed a western diet for 16 weeks than that in mice fed a western diet for 8 weeks, suggesting a potential role of macrophage LKB1 in atherosclerosis development.

Vascular smooth muscle cells (VSMCs) are also involved in atherosclerotic foam cell formation. We thus further examined LKB1 expression in VSMCs during the development of atherosclerosis. To this end, LKB1 was costained with SMA (smooth muscle actin), a smooth muscle cell marker. As shown in Online Figure I, costaining of LKB1 and SMA changed little after 8 weeks of western diet feeding.

**Macrophage LKB1 Is Downregulated by OxLDL**

To further evaluate the effects of atherosclerotic stimuli on LKB1 expression in macrophages and smooth muscle cells, Raw264.7 macrophages, primary peritoneal macrophages, or VSMCs were incubated with OxLDL. As shown in Figure 2A, LKB1 protein level was downregulated to 55% after 12 hours of OxLDL treatment in Raw264.7 macrophages, and it was further decreased to 40% after 24 hours of OxLDL treatment. Quantitative real-time polymerase chain reaction analysis showed that mRNA levels of LKB1 were also downregulated in response to OxLDL in Raw264.7 macrophages in a time-dependent manner (Figure 2B). Consistently, in primary macrophages, both protein and mRNA levels of LKB1 were
downregulated by OxLDL treatment in a time-dependent manner (Figure 2C and 2D). In contrast, 12 or 24 hours of OxLDL treatment had no effects on VSMC LKB1 levels (Online Figure IIA and IIB). Prolonged exposure of VSMCs to OxLDL (48 or 72 hours) also had no effects on LKB1 levels (Online Figure IIC and IID). Taken together, these data suggested that OxLDL downregulates LKB1 specifically in macrophages but not in VSMCs.

Figure 2. LKB1 (liver kinase B1) is downregulated by oxidized low-density lipoprotein (OxLDL) in macrophages in vitro. A, Western blot analysis of LKB1 protein expression in Raw264.7 cells treated with OxLDL (50 µg/mL) for the indicated time. B, Quantitative real-time polymerase chain reaction (RT-PCR) analysis of LKB1 mRNA levels in Raw264.7 cells. C, Western blot analysis of LKB1 protein expression in peritoneal macrophages treated with OxLDL for the indicated time. D, Quantitative RT-PCR analysis of LKB1 mRNA levels in peritoneal macrophages (n=6, *P<0.05, **P<0.01, ***P<0.001).

Figure 3. Macrophage LKB1 (liver kinase B1) deficiency promotes foam cell formation. A, Upper, Representative images of Oil Red O staining of Raw264.7 cells silenced with control or LKB1 siRNA and incubated with/without oxidized low-density lipoprotein (OxLDL; 50 µg/mL) for 48 h. Lower, Quantification of Oil Red O staining (n=6). B, Upper, Representative images of Oil Red O staining of bone marrow–derived macrophages (BMDMs) from wild-type (WT) or LKB1 knockout (KO) mice incubated with/without OxLDL (50 µg/mL) for 48 h. Lower, Quantification of Oil Red O staining (n=5). C, Upper, Representative images of filipin staining of Raw264.7 cells silenced with control or LKB1 siRNA and incubated with OxLDL (50 µg/mL) for 48 h. Lower, Quantification of filipin staining (n=6). D, Upper, Representative images of filipin staining of BMDMs from WT or LKB1 KO mice incubated with OxLDL (50 µg/mL) for 48 h. Lower, Quantification of filipin staining (n=5, *P<0.05).
OxLDL is known to induce inducible nitric oxide synthase (iNOS) expression in macrophages, and we had previously found that NO-mediated s-nitrosylation could promote LKB1 degradation. Thus, we first tested whether OxLDL differentially induces iNOS expression in macrophages versus VSMCs. As shown in Online Figure IIIA through IIIC, iNOS expression was robustly induced by OxLDL in Raw264.7 macrophages, whereas iNOS was almost nondetectable in VSMCs. In addition, OxLDL dramatically increased s-nitrosylation of LKB1 in macrophages but not in VSMCs (Online Figure IIID), suggesting that iNOS induction in macrophages promoted LKB1 degradation.

**Macrophage LKB1 Deficiency Promotes Foam Cell Formation**

Macrophage-derived foam cell formation is a crucial step in the development of atherosclerosis. Tissue macrophages become foam cells when exposed to chemically modified lipoproteins such as OxLDL and AcLDL. To determine whether macrophage LKB1 deficiency affects foam cell formation, both macrophage cell line Raw264.7 cells and primary bone marrow–derived macrophages (BMDMs) isolated from mice were treated with OxLDL. After the treatments, both Oil Red O staining and filipin staining were performed in OxLDL-treated macrophages.

As shown in Figure 3A through 3C, Raw264.7 cells silenced with LKB1 siRNA showed a significant increase in both Oil Red O staining and filipin staining when compared with those silenced with control siRNA (for siRNA efficiency, Online Figure IV). Similarly, primary BMDMs from LKB1 KO mice showed higher levels of both Oil Red O staining and filipin staining than their counterparts from WT mice (Figure 3B through 3D). These results indicated that LKB1 deficiency dramatically increases the capacity of macrophages to form foam cells.

**Macrophage LKB1 Deficiency Increases the Uptake of Modified Lipoproteins**

Macrophage foam cells are generated by uncontrolled uptake of modified lipoproteins and impaired cholesterol efflux. Therefore, we next investigated whether the increased uptake of modified LDL and impaired cholesterol efflux could account for the increased foam cell formation in LKB1-deficient macrophages. To this end, uptake of
fluorescently labeled AcLDL (Alexa 488-AcLDL) was performed in both Raw264.7 macrophages and BMDMs. As shown in Figure 4A, the uptake of Alexa 488-AcLDL, as indicated by remarkable increase in fluorescence intensity, was significantly higher in LKB1-silenced Raw264.7 cells than in cells transfected with control siRNA. Similarly, primary macrophages isolated from LKB1 KO mice exhibited much more Alexa 488-AcLDL uptake than cells isolated from WT mice (Figure 4B).

To further confirm these results, we examined the uptake of AcLDL using flow cytometry analysis. As shown in Figure 4C and 4D, the mean fluorescence intensity of AcLDL was significantly higher in both LKB1-deficient Raw264.7 cells and primary macrophages than in LKB1-positive counterparts, suggesting that LKB1 deficiency promotes AcLDL uptake.

Macrophage LKB1 Deficiency Does Not Affect Its Cholesterol Efflux

Next, we determined whether LKB1 affects cholesterol efflux by performing the cholesterol efflux assay. As shown in Figure 4E and 4F, there is no difference in cholesterol efflux between control and LKB1-deficient macrophages. Together these results suggest that LKB1 deficiency promotes foam cell formation through increased uptake of modified lipoproteins.

LKB1 Deficiency Promotes Lipoprotein Uptake and Foam Cell Formation Through Upregulating SRA

Macrophage scavenger receptors, particularly CD36 and SRA, are the principal receptors responsible for the uptake of modified lipoproteins and have been implicated as factors contributing to early foam cell formation and progression toward more complex vulnerable plaques. As shown in Figure 5A, LKB1 deficiency in Raw264.7 cells significantly increased SRA level while having no effect on CD36 level. Importantly, primary BMDMs showed a similar pattern (Figure 5B). We further investigated the abundance of surface SRA and CD36 by using flow cytometry. As shown in Figure 5C and 5D, surface expression of SRA but not CD36 was significantly increased in LKB1-deficient macrophages when compared with WT.

We next determined whether LKB1 deficiency promotes modified lipoprotein uptake and foam cell formation through SRA. AcLDL uptake and foam cell formation were investigated in BMDMs isolated from WT and LKB1 KO mice and preincubated with and without blocking antibody against SRA. As shown in Figure 5E, LKB1-deficient BMDMs had...
significantly increased uptake of AcLDL, which was abrogated specifically by pre-treatment using SRA blocking antibody. Further, LKB1 deficiency in BMDMs significantly increased foam cell formation as indicated by Oil Red O staining (Figure 5F). Pre-treatment with SRA blocking antibody abrogated foam cell formation in LKB1-deficient macrophages. Taken together, these data suggest that LKB1 deficiency promotes modified lipoprotein uptake and foam cell formation through upregulating SRA.

**LKB1 Promotes Lysosome Degradation of SRA**

Next, we sought to investigate how LKB1 regulates SRA expression in macrophages. LKB1 deficiency increased SRA protein levels (Figure 5A through 5D). In contrast, quantitative real-time polymerase chain reaction results showed that LKB1 deficiency in both Raw264.7 macrophages and primary BMDMs did not alter mRNA levels of SRA when compared with control (Figure 6A and 6B), suggesting that LKB1 regulates SRA post-transcription.

Proteasome and lysosome are 2 major systems for intracellular protein degradation. To determine how SRA is degraded, Raw264.7 macrophages were treated with proteasome or lysosomal inhibitors. As shown in Figure 6C, treatment of MG132, a potent 26S proteasome inhibitor, did not change SRA level in macrophages. However, inhibition of lysosomal function by bafilomycin A1 (BafA) or chloroquine (CQ) significantly increased SRA levels (Figure 6D and 6E). These data indicated that SRA is degraded through a lysosome-dependent pathway.

To further examine whether LKB1 downregulates SRA through the lysosome-dependent pathway, macrophages were overexpressed with LacZ or LKB1 plasmid and then treated with/without lysosomal inhibitors BafA or CQ. As shown in Figure 6F and 6G, overexpression of LKB1 significantly decreased SRA expression, whereas treatment with lysosomal inhibitors BafA or CQ abrogated this decrease. Overall, these results suggest that LKB1 promotes lysosome degradation of SRA.

**Phosphorylation of SRA by LKB1 Promotes Its Degradation**

Phosphorylation of scavenger receptors can modulate their stability. Specifically, phosphorylation changes in the cytoplasmic domains of these receptors can affect their degradation. As LKB1 is a conserved serine/threonine kinase, we hypothesized that LKB1 may phosphorylate SRA and thus regulate its degradation. To test this hypothesis, SRA from total protein extracts of Raw264.7 macrophages silenced with control or LKB1 siRNAs was immunoprecipitated and detected for phosphorylation and total expression of SRA. As shown in Figure 7A, the amount of phosphorylated SRA was much lower in LKB1-silenced macrophages even though more total SRA protein was present, suggesting a decreased phosphorylation of SRA. This result was confirmed in primary BMDMs isolated from WT and LKB1 KO mice (Figure 7B). To further investigate whether LKB1 is the upstream kinase of SRA, an in vitro kinase assay was performed. As shown in Figure 7C, LKB1 significantly increased the phosphorylation of SRA, suggesting that LKB1 functions as the upstream kinase and can directly phosphorylate SRA.

**Figure 6. LKB1 (liver kinase B1) promotes SRA (scavenger receptor class A) degradation through lysosome-dependent pathway.**

A, B, Quantitative real-time polymerase chain reaction (RT-PCR) analysis of mRNA levels of SRA in Raw264.7 cells silenced with control or LKB1 siRNAs (A) or bone marrow–derived macrophages (B; n=6). C–E, Western blot analysis of SRA protein levels in Raw264.7 cells treated with DMSO or 1 µmol/L MG132 (C), or with DMSO or 10 nmol/L Bafilomycin A (BafA; D) or with/without 5 µmol/L chloroquine (CQ; E) for 48 hours. F, Raw264.7 cells were overexpressed with LacZ or LKB1 plasmid and then treated with DMSO or BafA for 48 h. G, Raw264.7 cells were overexpressed with LacZ or LKB1 plasmid and then treated with/without CQ for 48 h. The blot is a representative of 3 individual experiments. (P<0.05.) KO indicates knockout; and WT, wild type.
To further examine whether phosphorylation of SRA by LKB1 regulates its protein stability, SRA protein sequences were analyzed by phosphorylation prediction software GPS 2.1. As shown in Figure 7D, human and mouse SRA sequences showed a conserved LKB1 phosphorylation site in the cytoplasmic domain (Ser-48 and Ser-53 in human and mouse SRA sequences, respectively). We found that SRA expressed by a phosphorylation mouse mutant (serine 53 was replaced with alanine, S53A) was more stable than WT SRA. Conversely, SRA expressed by a phosphomimic mutant (serine was mutated into aspartic acid, S53D) was less stable than the WT SRA (Figure 7E), suggesting that the phosphorylation status of SRA at serine 53 affects its protein stability. Next, we examined whether LKB1 regulates SRA degradation through phosphorylation of serine 53. As shown in Figure 7F, overexpression of LKB1 downregulated WT SRA but had no effect on S53A mutant SRA. All these results suggested that phosphorylation of SRA by LKB1 promotes its degradation.

Macrophage LKB1 Deficiency Promotes the Development of Atherosclerosis In Vivo

Finally, to determine the role of macrophage LKB1 in atherosclerosis in vivo, Ldlr−/− mice were transplanted with bone marrow from WT or macrophage-specific LKB1 KO mice and fed a western diet for 18 weeks. As shown in Online Figure VA through VC, there is no difference in body weight, serum triglyceride, and serum cholesterol levels between Ldlr−/− mice receiving WT and KO bone marrows. However, Ldlr−/− mice receiving LKB1 KO bone marrows developed more lesions in the aortic arch area than those receiving WT bone marrows (Online Figure VD). Sudan IV staining of aorta showed that mice receiving LKB1 KO bone marrows had a 111% increase in en face thoracic aorta lesion when compared with WT control (Online Figure VE). Analysis of Oil Red O staining of the aortic root also showed a significant increase (52%) in lesion formation in mice receiving LKB1 KO bone marrows (Online Figure VF), suggesting a protective role of macrophage LKB1 in the development of atherosclerosis. SRA levels were further detected in the aortic root lesion, and results showed that Ldlr−/− mice receiving LKB1 KO bone marrow had markedly increased SRA expression in aortic root lesion (Online Figure VG).

To confirm our hypothesis, ApoE−/−LKB1fl/flLysMcre mice, which were macrophage-specific KO’s of LKB1 in an ApoE−/− background, were generated. These mice, together with ApoE−/−LKB1fl/fl mice, were fed with western diet for 16 weeks. As shown in Figure 8A through 8C, no difference in body weight, serum triglyceride, and serum cholesterol levels was observed in the 2 groups of mice, whereas ApoE−/−LKB1fl/flLysMcre mice developed much more lesions in whole aorta and aortic root area compared with ApoE−/−LKB1fl/fl mice (Figure 8D through 8F). Similarly, SRA levels were dramatically increased in the aortic root lesion in ApoE−/−LKB1fl/flLysMcre mice (Figure 8G). Taken together, all these data...
demonstrated that macrophage LKB1 deficiency promotes atherosclerosis.

In addition, macrophage content was increased within plaques from ApoE<sup>−/−</sup>LKB1<sup>fl/fl</sup>LysM<sup>cre</sup> mice compared with ApoE<sup>−/−</sup>LKB1<sup>fl/fl</sup> control mice (Online Figure VIA), whereas smooth muscle cell content and plaque collagen content were decreased in ApoE<sup>−/−</sup>LKB1<sup>fl/fl</sup>LysM<sup>cre</sup> mice relative to ApoE<sup>−/−</sup>LKB1<sup>fl/fl</sup> control mice (Online Figure VIB and VIC), suggesting that LKB1 deficiency in macrophages may promote plaque instability.

**Discussion**

The present study has revealed the essential role of conserved serine/threonine kinase LKB1 in suppressing the development of atherosclerosis. LKB1 is downregulated primarily in macrophages during the development of atherosclerosis, and macrophage LKB1 deficiency decreases phosphorylation and lysosomal degradation of SRA and promotes foam cell formation and the development of atherosclerosis.

Foam cell formation is a hallmark of atherosclerotic lesions through all the stages of this disease. The formation and retention of the lipid-rich macrophages in the lesion area exacerbates the disease and fuels the development of vulnerable plaques. Therefore, strategies targeting the formation of foam cells can slow lesion progression and may serve as a potential adjunctive treatment for standard lipid-lowering therapies. However, the mechanisms controlling foam cell formation during atherosclerotic progression remain incompletely understood. Here, we report that the well-known tumor suppressor, LKB1, functions as a negative regulator in this process. Importantly, we found that LKB1 is downregulated in response to proatherosclerotic stimuli in vitro and during the progression of atherosclerosis. Because of the essential role of LKB1 in protecting against hyperlipidemia-induced atherosclerotic plaque formation, this downregulation of LKB1 may play an important causative role in the progression of atherosclerosis.

The accumulation of lipids is essential for the differentiation of macrophages and the formation of foam cells. SRA...
is one of the most important scavenger receptors expressed on the cell surface of macrophages and has been strongly implicated in pathological lipid deposition. It has been reported that SRA is highly expressed in the vessel wall during the induction of atherosclerotic lesion formation. However, the underlying mechanism is poorly understood. Our data demonstrate that LKB1 deficiency increases SRA protein levels without corresponding increase in SRA mRNA levels, which suggests that a post-translational regulation is involved. In fact, it has been previously reported that mimicking phosphorylation of human SRA at serine 48 strongly reduces surface SRA expression. Ricci et al also found that phosphorylation level of SRA is negatively associated with its protein abundance. Here, we found that LKB1 functions as the upstream kinase of SRA and can directly phosphorylate SRA and promote its degradation. This regulation of SRA by LKB1 helps to explain the elevated SRA expression in atherosclerotic lesions and provides a novel mechanism for foam cell regulation.

Although both macrophages and smooth muscle cells have been demonstrated to play important roles in foam cell formation and atherosclerotic progression, we found that macrophages are the primary target of proatherosclerotic stimuli for LKB1 loss. OxLDL exposure causes a time-dependent downregulation of LKB1 in macrophages, but not in VSMCs. We found that OxLDL can robustly induce iNOS expression in macrophages, which is consistent with a previous report; however, we cannot detect iNOS expression in VSMCs after OxLDL exposure (Online Figure II). We have previously found that NO-mediated s-nitrosylation promotes LKB1 degradation. It is thus possible that OxLDL-induced iNOS expression in macrophages may promote LKB1 degradation through s-nitrosylation, whereas VSMCs, which lack iNOS expression, undergo no such effect. However, it is also possible that increased exposure of macrophages to OxLDL triggers the transcriptional downregulation of LKB1. The underlying mechanisms warrant further investigation.

In summary, the present study has demonstrated a novel role for macrophage LKB1 in preventing the development of atherosclerosis and uncovered a novel mechanism underlying the regulation of SRA. LKB1 phosphorylates SRA and promotes its lysosomal degradation, which results in less modified lipoprotein uptake and foam cell formation. These findings identify the modulation of macrophage LKB1 as a novel approach for reducing atherosclerosis.

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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL

MATERIAL AND METHODS

Reagents
Antibodies for western blotting directed against LKB1, SRA, GAPDH, and actin were from Santa Cruz Biotechnology (Dallas, TX, USA). Antibody against CD36 was from Cell Signaling Technology (Danvers, MA, USA). Antibody for flow cytometry against SRA and CD36 was from Biolegend (San Diego, CA, USA). Antibody against CD68 and blocking antibody against SRA was from AbD Serotec (Kidlington, UK). Human oxidized LDL was from Kalen Biomedical (Montgomery Village, MD, USA). Alexa 488-conjugated acetylated LDL was from Life technologies (Carlsbad, CA, USA).

Cell culture
Raw264.7 macrophages were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine in a humidified atmosphere of 5% CO2 at 37°C. Bone marrow-derived macrophages (BMDMs) were obtained from bone marrow cells isolated from femurs and tibias and cultured in DMEM medium containing 10% FBS and 20 ng/mL macrophage colony stimulating factor (MCSF) for 5-7 days. Peritoneal macrophages were collected from peritoneal exudates 4 days after injecting mice with 1.5 ml of 4% BBL thioglycollate brewer (BD), and then cultured in RPM1640 supplemented with 10% FBS.

Animals
Macrophage-specific LKB1 KO mice were generated as we had described previously [1]. Ldlr<sup>-/-</sup> and ApoE<sup>-/-</sup> mice were obtained from Jackson laboratory (Bar Harbor, ME). Macrophage-specific LKB1 deficient mice in an ApoE<sup>-/-</sup> background were generated by crossing LKB1 floxed mice (LKB1<sup>fl/fl</sup>) with LysM<sup>cre</sup> transgenic mice, and then crossing with ApoE<sup>-/-</sup> mice to form ApoE<sup>-/-</sup>LKB1<sup>fl/fl</sup>LysM<sup>cre</sup> mice. All mice were housed in a controlled environment (20±2°C, 12-hour/12-hour light/dark cycle), where they were maintained on a standard chow diet with free access to water. The animal protocol was reviewed and approved by the Animal Care and Use Committee at the University of Oklahoma Health Sciences Center.

Foam cell formation assay
Macrophages were incubated with 50 μg/ml OxLDL (Kalen Biomedical, Cat No. 770252) for 48 h. Cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Cells were then incubated with isopropanol for 5 min, stained with 0.5% Oil red O in isopropanol for 30 min, washed with 85% isopropanol. After washing with water, macrophages were photographed under a microscope at ×400 magnifications.

AcLDL uptake assay
Macrophages were incubated with Alexa 488-conjugated AcLDL (Life Technologies, Cat No. L23380, 1 µg/mL) at 37°C for 4 hours. Fluorescence intensity was examined by a fluorescence microscopy (Leica, Germany). Images were acquired at identical exposure time. Alexa 488-AcLDL uptake was also analyzed by flow cytometry with at least 10,000 events acquired for each sample.

Cholesterol efflux assay
Macrophages were labeled with 0.5 μCi/ml 1,2-[3H]cholesterol for 24 h and then washed 3 times with phosphate-buffered saline and incubated for 2 h at 37°C with serum-free medium. The medium was replaced with serum-free medium with or without HDL (20 µg/ml) and incubated at 37°C for 6 h. The media were then collected and analyzed by liquid scintillation counting. Cells were dissolved in 0.2 N NaOH for determination of protein and radioactivity. The rate of cholesterol efflux is expressed as % cholesterol efflux, calculated as \[ \frac{\text{dpm}_{\text{medium}}}{\text{dpm}_{\text{medium+lysate}}} \times 100\% \].

Quantitative RT-PCR
RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-rad). Individual quantitative RT-PCR was performed using gene-specific primers as shown in Online Table I.

SiRNA transfection
Raw 264.7 cells were seeded into plate and cultured to 30% confluence. Cells were then transfected with gene specific siRNA or control siRNA using Lipofectamine™ RNAi MAX transfection reagent according
to the manufacturer’s instructions. The medium was replaced with fresh medium 6 hours after transfection. Cells were cultured for 48h before treatment with the indicated agents.

**Plasmid Construction and Transfection**

SRA Mouse cDNA Clone (also called Mr1, NM_031195) was purchased from Origene Company (Rockville, MD). Phosphorylation mutant S53A and phosphomimic mutant S53D were generated using a QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Primers used for point mutation are listed in Online Table II. All mutations were verified by DNA sequencing. Amaxa Nucleofection system from Lonza (Köln, Germany) was used for transfection of Raw 264.7 cells.

**In vitro kinase assay**

*In vitro* kinase assay was carried out as previously described [2]. Briefly, SRA pulled down from Raw264.7 cells was incubated in the absence or presence of recombinant LKB1/STRADα/MO25α protein (a kind gift from Dr. Dietbert Neumann of Maastricht, the University in Netherlands) in the kinase buffer (50 mM Tris, pH 7.5/10 mM MgCl₂/1 mM DTT/100 µm ATP) for 20 min at 30°C. Reactions were stopped by addition of SDS sample buffer, and samples were then subjected to SDS-PAGE and western blot analysis.

**Biotin switch assay**

S-nitrosylated proteins were detected by the S-nitrosylated protein detection assay kit (Cayman Chemical Company). In brief, free thiols were first blocked by incubation with thiol-specific methylthiolation reagent. After the blocking of free thiols, nitrosothiols were selectively decomposed and labeled with maleimide-biotin. Biotinylated proteins were then detected by avidin-coupled reagent.

**Immunofluorescence staining**

For tissue staining, sliced aortic root tissue were rinsed with ddH₂O and then fixed in ice-cold aceton for 10 minutes. After fixation, tissue slides were washed with PBS and blocked with 1%BSA for 1 hour at room temperature. For staining, we incubated primary antibody (anti-LKB1, LS Bio, 1:100; anti-CD68, Bio-rad, 1:200; anti-SRA, AbD Serotec, 1:100) with slides at 4°C overnight. Samples were washed 3 times with PBS for 15 minutes and incubated with corresponding fluorescence secondary antibodies (Life technologies, 1:400) for 1 hour at room temperature. Samples which are only incubated with secondary antibody were used as negative control. After 3 times wash with PBS, samples were mounted with ProLong™ Gold antifade reagent with DAPI for fluorescence photography.

**FACS analysis of cell surface expression of SRA and CD36**

24 hours after siRNA silencing, Raw274.7 cells were scraped from 6 well-plates and washed with cold PBS and cell suspensions were incubated with fluorescence anti-SRA antibody and anti-CD36 for 30 minutes on ice, washed with cold PBS for three times and followed by fluorescent secondary antibody staining for another 30 minutes on ice. Unstained cells were served as a negative control. Cells are washed three times with cold PBS. Flow cytometry was performed on a FACS Calibur (BD Biosciences). Data were analyzed with BD Cell Quest Pro software.

**Bone marrow transplantation**

8 week-old male Ldlr<sup>−/−</sup> mice were subjected to 11-Gy lethal total-body irradiation (two doses of 5.5 Gy within an interval of 4 h) to eliminate endogenous bone marrow stem cells and bone marrow-derived cells. Mice were then retroorbitally injected with bone marrow cells (5×10<sup>6</sup>) from wild type or macrophage specific LKB1 KO mice. Mice were allowed to recover for 6 weeks after bone marrow transplantation and then fed with western diet.

**Quantification of atherosclerotic lesions**

After being fed with western diet for 18 weeks, mice were fasted for 14 h, and then anesthetized and euthanized. The heart and aortic tissues were removed from the ascending aorta to the ileal bifurcation and fixed in 4% paraformaldehyde. After fixation, the adventitia was thoroughly cleaned under a dissecting microscope. To analyze the lesion area in the aortic root, the heart was dissected from the aorta, embedded in OCT compound, and sectioned (8-µm thickness). Six serial cryosections were collected from each mouse and stained with Oil Red O for neutral lipids, and then counterstained with hematoxylin to visualize nuclei. Images of plaques were captured under the Olympus microscope, and quantitative analysis was performed with Image J software (National Institute of Health) by averaging the lesion areas in the six sections. To
analyze the lesion area in the aortic arch, the intimal surface was exposed by a longitudinal cut from the ascending arch to an area that was 5 mm distal to the left subclavian artery. The aorta was rinsed for 5 min in 75% ethanol, stained with 0.5% Sudan IV in 35% ethanol and 50% acetone for 15 min, destained in 75% ethanol for 5 min, and then rinsed with phosphate-buffered saline.

**Quantification of lipid levels**

Total cholesterol and triglycerides in mice serum were measured by Infinity™ Cholesterol Liquid Stable Reagent and Infinity™ Triglycerides Liquid Stable Reagent (Thermo Fisher Scientific) respectively. HDL cholesterol, LDL cholesterol, total cholesterol and triglycerides and in human serum were measured by HDL-C kit, LDL-C kit, CHO kit, and TG kit purchased from Biosino Bio-technology Company (China).

**Statistical analysis**

Data are presented as the means ± SEM. Statistical analyses were performed using Prism5 software (GraphPad). The statistical significance of differences between two groups was analyzed by Student's t-tests. For comparing more than two means, one-way analysis of variance (ANOVA) with the Newman-Keuls post-hoc analysis was employed. Values of P<0.05 were considered statistically significant.

**REFERENCE**

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<th>Gene name</th>
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Online Table II. Primers for site-directed mutagenesis of SRA

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Online Table III. Clinical characteristics of the patients

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BW: body weight; BG: blood glucose; SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL cholesterol: high density lipoprotein cholesterol; LDL cholesterol: low density lipoprotein cholesterol; TG: triglyceride; TC: total cholesterol
Online Figure I. Co-staining of LKB1 and smooth muscle actin (SMA) in aortic root lesion from Ldlr−/− mice fed a western diet for the indicated time (Scale bar, 50 μm, n=7).
Online Figure II. Effect of OxLDL on LKB1 expression in vascular smooth muscle cells (VSMCs). A and C, Western blot analysis of LKB1 protein levels in VSMCs treated with OxLDL (50 µg/mL) for the indicated time. B and D, RT-PCR analysis of LKB1 mRNA levels in VSMCs treated with OxLDL (50 µg/mL) for the indicated time. (n=5).
Online Figure III. Effect of OxLDL on iNOS prosuction and LKB1 s-nitrosylation in macrophages and vascular smooth muscle cells. A, RT-PCR analysis of iNOS mRNA levels in Raw264.7 cells and VSMCs treated with OxLDL (50 µg/mL) for 24 hours. B, Western blot analysis of iNOS protein levels in Raw264.7 cells and VSMCs treated with OxLDL (50 µg/mL) for the indicated time. C, Griess assay of nitrite concentrations in the culture supernatants of Raw264.7 cells and VSMCs treated with OxLDL (50 µg/mL) for 24 hours. D. Biotin switch assay of s-nitrosylated LKB1 in Raw264.7 cells and VSMCs treated with OxLDL (50 µg/mL) for 24 hours. (n=4, *, p<0.05).
Online Figure IV. Efficiency of LKB1 siRNA in Raw264.7 cells. A, RT-PCR analysis of LKB1 mRNA levels. B, Western blot analysis of LKB1 protein levels. (n=3, **p<0.01)
Online Figure V. Macrophage LKB1 deficiency promotes the development of atherosclerosis. Irradiated Ldlr⁻/⁻ mice were transplanted with bone marrows from WT or macrophage specific LKB1 KO mice and fed a western diet for 18 weeks. A, Body weight of mice were measured. B-C, Serum levels of triglycerides (B) and cholesterol (C) were measured (n=9-10). D, Representative images of aortic arch regions with white plaques (yellow arrows). E, En face Sudan IV staining of aortic arch and thoracic aorta area. Lesion area was quantified as percentage of total surface area of aortic arch and thoracic aorta (n=5 per genotype). F, Oil Red O stained cross sections at the aortic root. Aortic root lesion area was quantified as percentage of lumen area (n=14 per genotype). G Immunostaining of SRA in aortic root lesion (Scale bar, 100 μm, n=5 per genotype) (*, P<0.05, **, P<0.01).
Online Figure VI. Atherosclerotic plaque composition in ApoE^{-/-}LKB1^{fl/fl} and ApoE^{-/-}LKB1^{fl/fl} LysM^{Cre} mice fed with western diet for 16 weeks. A, Representative images and quantification of macrophage content in aortic root lesions based on CD68 staining. B, Representative images and quantification of smooth muscle cell content in aortic root lesions based on smooth muscle actin(SMA) staining. C, Representative images and quantification of collagen content in aortic root lesions based on masson trichrome staining. (n=6, *, p<0.05)