Phospholipid Transfer Protein Destabilizes Mouse Atherosclerotic Plaque

Ke Zhang, Xiaoling Liu, Yang Yu, Tian Luo, Lin Wang, Chen Ge, Xinxin Liu, Jiantao Song, Xiancheng Jiang, Yun Zhang, Shucun Qin, Mei Zhang

Objective—Phospholipid transfer protein (PLTP) accelerates the development of atherosclerosis in mouse models. We examined the role of PLTP in atherosclerotic plaque stability.

Approach and Results—We prepared apolipoprotein E and PLTP double-knockout (PLTP−/−ApoE−/−) mice. PLTP deficiency significantly decreased lesion size and reduced monocyte/macrophage infiltration, as well as macrophage apoptosis in lesion areas. Moreover, it increased fibrous content in plaques, which suggests that PLTP may affect atherosclerotic plaque stability. Importantly, PLTP overexpression mediated by adenovirus had the reverse effect. It promoted the accumulation of reactive oxygen species in macrophages, which could lead to cell apoptosis and increased the production of inflammatory cytokines and chemokines. PLTP overexpression could promote receptor-interacting protein 3 recruitment of macrophages in cytoplasm, which could induce reactive oxygen species, thus inducing atherogenesis.

Conclusions—PLTP plays an important role in modulating the stability of atherosclerotic plaques. The receptor-interacting protein 3- reactive oxygen species signal pathway could be involved in this PLTP-mediated process.

Key Words: apoptosis ■ phospholipid transfer proteins

In the past 10 years, most human studies, including a recent Framingham Heart study, showed a positive association of plasma phospholipid transfer protein (PLTP) activity and coronary heart disease.1–7 In mouse models, systemic PLTP deficiency reduces atherosclerosis,8 but its overexpression has the opposite effect.9–11 Systemic PLTP deficiency in mice is also associated with a reduced thrombotic response12 and reduced abdominal aortic aneurysm.13 In rabbits, overexpression of PLTP-increased atherosclerotic lesions after a high-fat diet feeding when compared with controls.14 In general, PLTP is a risk factor of atherosclerosis in animal models. However, whether PLTP can affect the stability of atherosclerotic plaque and the mechanism involved are still unknown.

Macrophages play an important role in atherosclerotic plaque destabilization13 and highly express PLTP.16 Apoptotic macrophages, especially in advanced plaques, promote several processes that contribute to plaque instability.17 PLTP could augment apoptosis in THP-1 (human acute monocytic leukemia cell line) cell-derived macrophages18 and PLTP stimulates Janus family kinase 2 phosphorylation,19 involved in apoptosis in several cells, including macrophages.20 Therefore, PLTP-related plaque instability may be mediated by macrophage apoptosis.

In this study, we evaluated the association of PLTP and atherosclerotic plaque stability under PLTP-deficient and PLTP-overexpression conditions. PLTP deficiency could stabilize plaque, and PLTP overexpression had the opposite effect. Furthermore, we explored the underlying mechanisms.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Adenovirus Can Mediate PLTP Expression in Carotid Arteries

To evaluate whether intravenous adenovirus transduction could mediate gene expression in carotid arteries, we injected Ad-GFP or Ad-PLTP into apolipoprotein E (ApoE)−/− or ApoE−/−/PLTP−/− mice and found that green fluorescent protein, thus PLTP, expressed in the tissue (Figure IA...
PLTP Increases Atherosclerosis

To evaluate the effect of PLTP on atherogenesis, we dissected mouse carotid arteries and found plaque area lower in PLTP−/−ApoE−/−+Ad-GFP than in ApoE−/−+Ad-GFP arteries. After Ad-PLTP transduction, plaque area was increased in both mouse groups (Figure 1A and 1D). We then stained carotid arteries with hematoxylin and eosin and found smaller plaque area in PLTP−/−/ApoE−/− mice (P<0.05), which was increased in both mouse groups with Ad-PLTP transduction (P<0.05) when compared with controls (Figure 1B and 1D). Furthermore, we performed en face staining and found less Oil-red-O–stained plaque area for PLTP−/−/ApoE−/− than for ApoE−/− mice (6.5% versus 23.6%; P<0.05), which was increased in both groups with Ad-PLTP transduction when compared with that with Ad-GFP treatment alone (17.9%...
versus 38.9%; *P<0.05; Figure 1C and 1E). Therefore, PLTP accelerated the development of atherosclerotic plaque.

**PLTP Decreases Atherosclerotic Plaque Stability**

We next sought to determine whether PLTP plays a role in atherosclerotic plaque stability. The relative content of collagen, lipids, smooth muscle cells (SMCs), and macrophages in plaques was measured by histological or immunohistochemical staining. Collagen and SMC content were higher, and macrophage content was lower in PLTP−/−ApoE−/−+Ad-GFP than in ApoE−/−+Ad-GFP mice (*P<0.05; Figure 2A and 2B).

Ad-PLTP transduction significantly decreased collagen and SMC content and increased macrophage content in carotid arteries (*P<0.05; Figure 2A and 2B).

Plaque vulnerable index was lower for PLTP−/−ApoE−/− than for ApoE−/− mice (*P<0.05) but was increased in both groups with Ad-PLTP transduction (Figure 2C). Content of monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1, and matrix metalloproteinase-9 (MMP-9) was lower in PLTP−/−ApoE−/− than in ApoE−/− mouse carotid plaques (*P<0.05) but was increased with Ad-PLTP transduction (*P<0.05; Figure 2D and 2E).

**Figure 2.** The effect of phospholipid transfer protein (PLTP) on atherosclerotic plaque stability. A, Cross sections of carotid arteries in ApoE−/− and PLTP−/−ApoE mice stained for collagen (Sirius red), lipids (Oil-red-O), vascular smooth muscle cells (SMCs; α-actin), or macrophages (MOMA-2; magnification ×20). B, Relative content of collagen, lipid, vascular SMCs, and macrophages in plaques. C, Plaque vulnerability index. D, Inflammatory cytokine levels in carotid plaques (magnification ×40). E, Relative content of inflammatory cytokines in plaques. F, Colocalization of apoptotic cells and macrophages. G, Expression of cleaved caspase-3. H, Apoptotic cells in different groups of mice. Data are mean±SD. A−/−+Ad-G (n=18), A−/−+Ad-P (n=18), P−/−A−/−+Ad-G (n=16), P−/−A−/−+Ad-P (n=16). Bar, 100 μm. *P<0.05 vs ApoE−/−+Ad-GFP, #P<0.05 vs PLTP−/−ApoE−/−+Ad-GFP. ICAM-1 indicates intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; and MMP-9, matrix metalloproteinase-9.
To elucidate the involvement of PLTP in atherosclerosis further, we investigated cell apoptosis by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. Colocalization of apoptotic cells and macrophages showed that apoptosis mainly occurred where macrophages aggregated (Figure 2F). Cell apoptosis, as determined by cleaved caspase-3 staining, was lower in PLTP−/−ApoE−/− mice than in ApoE−/− mice but increased with Ad-PLTP rather than with Ad-GFP transduction (Figure 2G). PLTP deficiency significantly decreased but overexpression increased the proportion of apoptotic cells in plaque (P<0.05; Figure 2H). Flow cytometry also showed similar results in peritoneal macrophages (Figure II in the online-only Data Supplement). Thus, PLTP destabilized plaques by increasing inflammatory cytokines activity and promoting macrophage apoptosis.

**Effect of PLTP on Oxidative Stress**

The concentration of reactive oxygen species (ROS) was lower in peritoneal macrophages and bone marrow–derived macrophages from PLTP−/−ApoE−/−+Ad-GFP mice than from ApoE−/−+Ad-GFP mice (P<0.05) and was significantly increased with PLTP overexpression (P<0.05; Figure 3A; Figure IIIA in the online-only Data Supplement). Similar results were observed in vivo (P<0.05; Figure 3B, Figure IV in the online-only Data Supplement). Moreover, the concentration of malonaldehyde, a marker of oxidative stress, was markedly lower in PLTP−/−ApoE−/− than in ApoE−/− macrophages (P<0.05) and was significantly increased with PLTP overexpression (P<0.05; Figure 3C).

**Effect of PLTP Macrophage Inflammatory Responses and Apoptosis**

Protein levels of MCP-1, intercellular adhesion molecule-1, and MMP-9 were lower in PLTP−/−ApoE−/− than in ApoE−/− macrophages (P<0.05), and PLTP overexpression reversed the effect (P<0.05; Figure 4A). As well, MMP-9 activity was lower in PLTP−/−ApoE−/− than in ApoE−/− macrophages and PLTP overexpression significantly increased the MMP-9 activity (Figure VA in the online-only Data Supplement). The level of cleaved caspase-3 was significantly increased with PLTP overexpression (P<0.05; Figure 4C). Poly[ADP-ribose] polymerase 1 was one of the earliest nuclear enzymes to be targeted for degradation by caspas during apoptosis. The expression of poly[ADP-ribose] polymerase 1 was significantly decreased in plaque with Ad-PLTP overexpression (P<0.05; Figure 4B).

Compared with controls, treatment with N-acetyl-L-cysteine (NAC; the ROS scavenger) alone had no significant effect on the activity of inflammatory cytokines and cleaved caspase-3 in peritoneal macrophages and bone marrow–derived macrophages, which was increased with PLTP overexpression plus NAC treatment (P<0.05; Figure 4C; Figure IIIB in the online-only Data Supplement). Treatment with BAY 11 to 7082 (an anti-inflammatory agent) and Z-VAD-Fmk (an irreversible general caspase inhibitor) could reverse the PLTP effect (P<0.05; Figure 4D), which again suggests that PLTP is involved in both inflammation and apoptosis.

**PLTP Regulated Cleaved Caspase-3 Expression by the Receptor-Interacting Protein 3-ROS Pathway**

The receptor-interacting protein 3 (RIP3) has emerged as a critical regulator of apoptosis and ROS production is its downstream event. We found RIP3 level lower in PLTP−/−/ApoE−/− than in ApoE−/− macrophages (P<0.05), and PLTP transduction reversed the effect (Figure 5A; Figure IIA in the online-only Data Supplement). Moreover, PLTP overexpression significantly increased RIP3 levels in the atherosclerotic plaque (Figure VB in the online-only Data Supplement). RIP3 siRNA-mediated knockdown and NAC treatment could attenuate PLTP-activated caspase-3 (P<0.05; Figure 5B and 5C). Similarly, RIP3 siRNA knockdown significantly inhibited the PLTP-increased ROS production (P<0.05; Figure 5D).

**Nuclear Localization of RIP3 Aided PLTP Regulation of Cell Apoptosis**

RIP3 was localized mainly in the nucleus with leptomycin B treatment and was recruited to the cytoplasm with PLTP treatment (Figure 6A). Nucleocytoplasmic shuffling of RIP3 could significantly change the level of cleaved caspase-3 with leptomycin B and PLTP treatment (Figure 6B).

**Plasma Lipid, Inflammatory Cytokine, and Chemokine Levels in PLTP-Deficient and PLTP-Overexpressed Mice**

We measured the lipid levels and body weight in all groups of mice and found that PLTP deficiency reduced cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol.

---

**Figure 3.** The effect of phospholipid transfer protein (PLTP) on reactive oxygen species (ROS) content in peritoneal macrophages and atherosclerotic plaque and the ability of PLTP levels to oxidize low-density lipoprotein (LDL). ROS content in (A) macrophages and (B) atherosclerotic plaque. C, Malonaldehyde (MDA) content in macrophages. Data are representative of 3 independent experiments. Data are means±SD. *P<0.05 vs ApoE−/−+Ad-GFP, #P<0.05 vs PLTP−/−ApoE−/−+Ad-GFP.
levels in the blood; body weight showed similar results (Table I in the online-only Data Supplement). We measured MCP-1, tumor necrosis factor-α, and interleukin (IL)-6 in blood, and we found that PLTP deficiency reduced MCP-1 and IL-6 but not tumor necrosis factor-α levels; PLTP overexpression reversed the effect (Table II in the online-only Data Supplement).

**Discussion**

We found that PLTP overexpression could destabilize atherosclerotic plaque by reducing the local plaque content of collagen and SMCs and increasing intracellular levels of oxidants and macrophage apoptosis (Figure VI in the online-only Data Supplement). Vulnerable plaque is the pathological basis of various cardiovascular clinical events. Apoptosis of macrophages within plaque is one of the most important mechanisms leading to atherosclerotic plaque instability. We aimed to investigate the role of PLTP in the pathogenesis of atherosclerosis instability and the potential mechanism of PLTP-destabilizing atherosclerotic plaque. PLTP affected atherogenicity, which led to pathological changes such as...
increased atherosclerotic lesions; changed content of collagen, SMCs, and macrophages and inflammation activity in plaques; augmented apoptosis-related indexes and apoptotic macrophages; increased RIP3 and ROS levels; and changed RIP3 nuclear localization.

Emerging evidence suggests that elevated plasma PLTP levels increase the number of atherosclerotic lesions, and PLTP deficiency protects against atherosclerosis. Most of our knowledge of PLTP regulating the atherosclerotic process is derived from studies focusing on the formation of atherosclerotic plaque. The involvement of PLTP in the stability of atherosclerotic plaque is poorly understood. Our study demonstrates that PLTP adenovirus transduction could destabilize atherosclerotic plaques by reducing collagen and SMC levels and increasing intracellular levels of the cytokines MCP-1, intercellular adhesion molecule-1, and MMP-9 in mouse plaque. Lipid in plaques is an important factor affecting the stability in atherosclerotic plaques. PLTP transduction could promote lipid deposits in mouse plaque and the accumulation of malonaldehyde in macrophages. These data demonstrate the role of PLTP in atherosclerosis under pathophysiological conditions.

In our study, we observed decreased HDL levels in both PLTP−/−ApoE−/−+Ad-GFP and ApoE−/−+Ad-PLTP mice. Many articles have reported similar results. In PLTP deficiency, the blockade of the transfer of phospholipids and cholesterol from triglyceride-rich lipoproteins into HDL may decrease the formation of mature HDL. In PLTP overexpression, because of the facilitated redistribution of phospholipids and cholesterol among lipoproteins, delipidation of HDL was accelerated.

Apoptosis is a critical process in the physiological organ development and plays an important role in the pathogenesis of many diseases, including atherosclerosis. The receptor-interacting protein 3 (RIP3) is involved in the regulation of cell apoptosis.

Figure 5. Phospholipid transfer protein (PLTP) regulated the cleaved caspase-3 expression by receptor-interacting protein 3 (RIP3)-reactive oxygen species (ROS) pathway. A, RIP3 level in peritoneal macrophages. PLTP-induced cleaved caspase-3 with (B) RIP3 siRNA knockdown and (C) N-acetyl-l-cysteine (NAC) treatment. D, PLTP-induced ROS production with RIP3 siRNA treatment. *P<0.05 vs control, #P<0.05 vs PLTP. Data are means±SD.

Figure 6. Nuclear localization of receptor-interacting protein 3 (RIP3) participated in the regulation of cell apoptosis by phospholipid transfer protein (PLTP). Fluorescence microscopy of (A) RIP3 localization and (B) cleaved caspase-3 content in cells treated with leptomycin B (LMB; 2 ng/mL) or Ad-PLTP (1×10^6 infectious units [ifu]/mL) for 1 hour. Data are means±SD. Bar, 50 μm. *P<0.05 vs control.
of atherosclerosis in terms of stability of plaques, plaque rupture, and thrombosis.\textsuperscript{26,27} We found that PLTP significantly increased the level of inflammatory cytokines and the expression of cleaved caspase-3, involved in apoptosis. NAC treatment of macrophages could reduce the level of inflammatory cytokines and the PLTP-increased expression of cleaved caspase-3. These findings suggest that ROS is involved in regulating PLTP in macrophage apoptosis. ROS are essential mediators of normal cell physiology, but ROS overproduction is associated with inflammation and cell apoptosis. ROS can cause irreversible damage to DNA, proteins, and lipids, thereby altering cell functions.\textsuperscript{28} They can inhibit PI3K activity, which reduces RIP3 (receptor interacting protein 3) level and Akt phosphorylation.\textsuperscript{29} Growing evidence suggests that reducing ROS production can effectively alleviate inflammatory stress.\textsuperscript{30} ROS may be inducers of apoptosis, and antioxidants such as catalase\textsuperscript{31} and NAC could delay cell apoptosis. RIP3 was found crucial for tumor necrosis factor–induced apoptosis in experiments with cells lacking RIP3.\textsuperscript{32,33} As well, RIP3 has a proapoptotic role in several cell lines\textsuperscript{34} by enhancing caspase activity. Our analysis of the signaling components triggered by PLTP revealed an RIP3-ROS pathway. RIP1-RIP3–dependent ROS production is closely associated with cell death.\textsuperscript{35,36} Vitamin E played a role in antioxidant. PLTP is involved in vitamin E transfer to different lipoproteins and tissues.\textsuperscript{12,37} For example, the bioavailability of vitamin E levels could be regulated by PLTP and protected against the formation of atherosclerotic plaques\textsuperscript{38} although most human studies have been negative.\textsuperscript{39,40} Whether vitamin E is involved in PLTP-dependent changes in oxidative is not clear. Apoptosis of macrophages within plaque is an important mechanism leading to atherosclerotic plaque instability. We found that PLTP destabilized atherosclerotic plaque through an RIP3-ROS signal pathway. In summary, our findings demonstrate that PLTP could promote the aggregation of ROS in macrophages and ROS levels could be elevated by increasing the expression of RIP3. These results reveal an RIP3-ROS signal pathway involved in regulating PLTP in macrophage apoptosis. However, the mechanism of how PLTP intervenes in RIP3 expression remains to be determined. RIP3 recruitment to the nucleus could significantly change the PLTP-increased cleaved caspase-3 level. The relationship between the increased expression of RIP3 and PLTP-induced nucleocytoplasmic shuttling of RIP3 remains for further study. PLTP may play an important role in modulating the stability of atherosclerotic plaques. PLTP increases the apoptosis of cells in plaques, especially macrophages. We also confirm an RIP3-ROS signal pathway and RIP3 localization involved in the PLTP regulation of macrophage apoptosis. Our data bring new insights into a role for PLTP in the stability of atherosclerotic plaques.

Acknowledgments

We thank Dr Han (Xiamen University) for kindly providing the anti-RIP3 antibody.

Sources of Funding

The study was supported by the National Basic Research Program of China (973 Program, 2011CB503906), the National Natural Science Foundation of China (No. 30970709, 81270404), and the Taishan Scholars Foundation of Shandong Province (200811).

Disclosures

None.

References

Phospholipid transfer protein may play an important role in modulating the stability of atherosclerotic plaque. Phospholipid transfer protein increases the apoptosis of cells in plaque, especially macrophages. The receptor-interacting protein 3-reactive oxygen species signal pathway and receptor-interacting protein 3 localization are involved in the phospholipid transfer protein regulation of macrophage apoptosis. Our data bring new insights into a role for phospholipid transfer protein in the stability of atherosclerotic plaques.
Phospholipid Transfer Protein Destabilizes Mouse Atherosclerotic Plaque

Ke Zhang, Xiaoling Liu, Yang Yu, Tian Luo, Lin Wang, Chen Ge, Xinxin Liu, Jiantao Song, Xiancheng Jiang, Yun Zhang, Shucun Qin and Mei Zhang

Arterioscler Thromb Vasc Biol. 2014;34:2537-2544; originally published online October 16, 2014;
doi: 10.1161/ATVBAHA.114.303966

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/12/2537

An erratum has been published regarding this article. Please see the attached page for:
/content/35/1/e2.full.pdf

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
In the article by Zhang et al, which appeared in the December 2014 issue of the journal (Arterioscler Thromb Vasc Biol. 2014;34:2537–2544. DOI: 10.1161/ATVBAHA.114.303966), a correction was needed.

The running header short title was incorrect. It has been changed to “PLTP Destabilizes Plaque.”

The authors apologize for the error.

The online version of the article has been corrected and is available at http://atvb.ahajournals.org/content/34/12/2537.
Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Reagents and antibodies
PLTP adenovirus (Ad) was constructed by Life Technologies Corp. (China). Low-density lipoprotein (LDL) was purchased from Sigma (St. Louis, MO). N-acetyl-L-cysteine (NAC) was obtained from Beyotime Technology (China). Antibody against cleaved caspase-3 was from Cell Signaling Technology (Beverly, MA) and antibodies against GAPDH, macrophage chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), poly[ADP-ribose] polymerase 1 (PARP-1), and matrix metalloproteinase-9 (MMP-9) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against RIP3 was kindly provided by Dr. Jiahuai Han (Xiamen University, China). siRNA to knock down RIP3 expression was from GenePharma (Shanghai). Z-VAD-Fmk was from Sigma (St. Louis, MO). Leptomycin B (LMB) was from Santa Cruz Biotechnology. RAW264.7 cells were provided by Dr. Yun Zhang (Shandong University Qilu Hospital, Jinan, China).

Animal model
We crossed our PLTP-/- mice\(^1\) with ApoE-/- mice (Peking University, Beijing). All mice were in the C57BL/6J background. Male mice were used in all experiments.

PLTP heterozygous mice (PLTP+/-) were provided by Dr. Xiancheng Jiang (SUNY Downstate Medical Center, NY, USA). ApoE/- mice were obtained from Peking University (Beijing). All mice were in the C57BL/6J background. ApoE/- mice were used as control mice. We crossbred PLTP+/- and ApoE/- mice and obtained PLTP-/-ApoE/- mice.

Animals were housed in colony cages in a temperature-controlled environment (20-25°C). All mice were allowed a 1-week acclimatization period before entry into any experimental protocol. The atherosclerotic model was previously described.\(^2\) In brief, atherosclerotic lesions were induced by placing a perivascular constrictive silica collar on the right common carotid artery of mice. Mice 8 weeks old were divided into 4 groups for treatment: ApoE/- mice transfected with empty adenovirus (Ad) (ApoE/- + Ad-GFP) (n =18) or PLTP adenovirus (ApoE/- + Ad-PLTP) (n=18), and PLTP-/-ApoE/- mice transfected with empty adenovirus (PLTP-/-ApoE/- + Ad-GFP) (n=16) or PLTP adenovirus (PLTP-/-ApoE/- + Ad-PLTP) (n=16). All groups were fed a high-fat diet (0.25% cholesterol and 15% cocoa butter) for 10 weeks. Ad-GFP or Ad-PLTP (100 μl, \(1 \times 10^9\) infectious units [ifu]/ml) was transduced by tail-vein injection at 8 weeks after carotid-artery surgery. Two weeks after transduction, all mice were sacrificed. All animal protocols were in accordance with the Guide for Care and Use of Laboratory Animals published by the European Commission (Directive 2010/63/EU, revised 2010) and were approved by the Animal Care Committee of Shandong University.
Atherosclerotic plaque composition determined by immunohistochemistry

Carotid samples were dissected and embedded in OCT compound, then cut into pieces 6 µm thick at 50-µm intervals. Sections were stained with hematoxylin and eosin (H&E), Oil-red O or Sirius red, then incubated with the rabbit anti-mouse α-smooth muscle cell (α-SMC) (Sigma) for smooth muscle cells (SMCs) or rat anti-mouse MOMA-2 (Sigma) for macrophages. The collagen content of plaques was viewed under polarized light microscopy. The mean area of collagen, SMCs, extracellular lipid deposits and macrophages was recorded as percentage positive area divided by plaque area in at least 20 high-power fields (20×). The vulnerability index was calculated as (macrophage staining % + lipid staining %)/(SMC % + collagen %). Plaque area and ratio of cap to core were determined by use of ImagePro Plus 6.0 (Media Cybernetics, USA) attached to a color CCD video camera.

In situ detection of apoptotic cells

Apoptotic cells and apoptotic macrophages in carotid cryosections were determined by use of an in situ apoptosis detection kit (Roche, USA).

Peritoneal macrophage culture

ApoE-/− or PLTP-/−ApoE-/− mice were injected intraperitoneally with 1 ml of 3% thioglycollate (Taigemei Biotechnology, Beijing). After 2 days, peritoneal cells were harvested by lavage from the mouse peritoneal cavity with use of phosphate buffered saline. Macrophages were collected by spinning cells at 1000 rpm for 5 min, then resuspending them in RPMI 1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum and plating at 2×10⁶ cells/well in six-well plates in supplemented RPMI 1640 medium.

Adenovirus and siRNA transfection

siRNA (100 nM) for suppression of RIP3 was used to transfect macrophages in 6-well plates for 24 h by the Lipofectamine 2000 method (Invitrogen, Carlsbad, CA). The siRNA sequence targeting RIP3 was 5’-CACAGGGUUGGUAAUACAUATT-3’. The mock siRNA sequence was 5’-UUCUCCGAACGUGUCACGUTT-3’. Transfected cells were treated with or without PLTP adenovirus for 24 h.

Experimental macrophage groups

To observe the effect of levels of PLTP on reactive oxygen species (ROS), low-density lipoprotein (LDL) oxidation, inflammatory cytokines, and the expression of RIP3 and cleaved caspase-3, peritoneal macrophages from mouse groups were divided into 4 groups for treatment: ApoE-/− mouse macrophages transfected with empty Ad (ApoE-/− + Ad-GFP) or PLTP Ad (ApoE-/− + Ad-PLTP), and PLTP-/−ApoE-/− mouse macrophages transfected with empty Ad (PLTP-/−ApoE-/− + Ad-GFP) or PLTP Ad (PLTP-/−ApoE-/− + Ad-PLTP).

To determine whether the RIP3-ROS signal pathway is involved in the effect of PLTP on cleaved caspase-3, RAW264.7 cells were divided into 4 groups for
treatment: control (Ad-GFP), Ad-PLTP, RIP3 siRNA (NAC), and Ad-PLTP + RIP3 siRNA (NAC). In culture medium, the final concentration of Ad-GFP, Ad-PLTP, RIP3 siRNA, and NAC was 1×10^6 ifu/ml, 1×10^6 ifu/ml, 100 pmol/L, and 10 mmol/L, respectively.

**Oxidant assay**
Cellular ROS levels in macrophages were determined by use of the ROS Assay Kit (Beyotime, Haimen, China). Macrophages were placed in 6-well dishes, incubated with 100 μg/ml LDL and 2.5 μmol/L CuSO₄ for 6 h at 37 °C, then oxidation was determined by the Lipid Peroxidation Malonaldehyde (MDA) Assay Kit (Beyotime, China) measuring the amount of thiobarbituric acid-reactive substances.

**Western blot analysis**
After treatment, macrophages were lysed in buffer containing protease inhibitors, and total protein was extracted. Protein concentration was measured by the Bio-Rad DC Protein Assay Kit (Pierce, Rockford, IL). Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were blocked in 5% skim milk for 60 min at room temperature. Blots were incubated with antibodies for GAPDH (1:1000), MCP-1 (1:200), ICAM-1 (1:200), MMP-9 (1:100), cleaved caspase-3 (1:100) or RIP3 (1:1000), then horseradish peroxidase-conjugated secondary antibodies. After an extensive washing with tris buffered saline-tween, membranes were detected by use of enhanced chemiluminescence plus reagents (Amersham Biosciences, UK).

**Immunofluorescence staining and microscopy**
Cells were grown on glass coverslips and treated with LMB (2 ng/ml) or Ad-PLTP (1×10^6 ifu/ml), then washed with phosphate buffered saline, fixed with 4% paraformaldehyde for 10 min, and permeabilized with 0.2% Triton X-100 for 5 min. Coverslips were blocked with 1% bovine serum albumin, incubated with primary antibody, washed with phosphate buffered saline, then incubated with Texas red-labeled secondary antibody, then with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 0.1 μg/mL) for 15 min to label nuclei. Slides were examined under a Leica DMLS epifluorescence microscope equipped with a Leica DC 100 digital camera, and data were analyzed by use of ImagePro Plus v4.5 (Media Cybernetics).

**Measurement of PLTP activity**
An assay kit (BioVision) was used for measurement of PLTP activity.

**Statistical analysis**
Data analysis involved use of SPSS v16.0 (SPSS Inc., Chicago, IL) and results were from at least 3 independent experiments. Comparisons were analyzed by Student t test or one-way ANOVA. All results are expressed as mean ± SEM and a value of P<0.05 was considered statistically significant.
References

1. Qin S, Kawano K, Bruce C, Lin M, Bisgaier C, Tall AR, Jiang X. Phospholipid transfer protein gene knock-out mice have low high density lipoprotein levels, due to hypercatabolism, and accumulate apoa-iv-rich lamellar lipoproteins. *Journal of lipid research.* 2000;41:269-276


**Supplemental Figure I.** PLTP adenovirus transfection efficiency and PLTP activity. A, GFP fluorescence to monitor transfection efficiency in the carotid plaque. B, PLTP activity in ApoE-/- and PLTP-/-ApoE-/- mice. Bar=50 µm. *P<0.05 compared with Ad-GFP transfection.
Supplemental Figure II. Impact of PLTP on macrophage apoptosis in different groups in peritoneal macrophages. Data are mean ± SD. *P<0.05 vs ApoE-/- + Ad-GFP, ^P<0.05 vs PLTP-/-ApoE-/- + Ad-GFP.
Supplemental Figure III. Impact of PLTP on the expression of ROS, MCP-1, MMP-9, cleaved caspase 3 and RIP 3 among different group in cultured bone marrow derived macrophages. A, ROS content in bone marrow derived macrophages. B, Effect of NAC treatment on level of inflammatory cytokines and cleaved caspase-3 level induced by PLTP. C, RIP3 and cleaved caspase 3 level in bone marrow derived macrophages. Data are representative of 3 independent experiments. Data are mean ± SD. *P<0.05 vs ApoE-/- + Ad-GFP, "P<0.05 vs PLTP-/-ApoE-/- + Ad-GFP.
Supplemental Figure IV. Impact of PLTP on the SOD activity. A/- + Ad-G (n =18), A/- + Ad-P (n =18), P/-A/- + Ad-G (n=16), P/-A/- + Ad-P (n=16). Data are mean ± SD. *P<0.05 vs ApoE/- + Ad-GFP, #P<0.05 vs PLTP/-ApoE/- + Ad-GFP.
Supplemental Figure V. A, Impact of PLTP on the activity of MMP-9. B, the expression of RIP3 in plaque. Data are mean ± SD. *P<0.05 vs ApoE−/− + Ad-GFP, #P<0.05 vs PLTP−/−ApoE−/− + Ad-GFP.
Supplemental Fig. VI

**Supplemental Fig. VI.** PLTP changes the stability of atherosclerotic plaque by increasing intracellular oxidant content and macrophage apoptosis.
**Supplemental Table I.** The changes of plasma lipid and body weight in different mice. (X ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>TC</th>
<th>TG</th>
<th>HDL-C</th>
<th>BW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mmol/L)</td>
<td>(mmol/L)</td>
<td>(mmol/L)</td>
<td>(g)</td>
</tr>
<tr>
<td>ApoE/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Ad-GFP(n=18)</td>
<td>19.11±1.65</td>
<td>3.77±1.92</td>
<td>1.68±0.15</td>
<td>29.03±2.01</td>
</tr>
<tr>
<td>ApoE/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Ad-PLTP(n=18)</td>
<td>21.63±1.98*</td>
<td>4.61±1.67*</td>
<td>0.76±0.12*</td>
<td>29.68±2.13</td>
</tr>
<tr>
<td>PLTP/-ApoE/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Ad-GFP(n=16)</td>
<td>17.63±1.83*</td>
<td>2.36±1.15*</td>
<td>0.87±0.08*</td>
<td>25.02±1.77*</td>
</tr>
<tr>
<td>PLTP/-ApoE/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Ad-PLTP(n=16)</td>
<td>19.61±1.37#</td>
<td>3.62±1.08#</td>
<td>1.19±0.13&quot;</td>
<td>25.72±1.61*</td>
</tr>
</tbody>
</table>

* P<0.05 vs. ApoE/- + Ad-GFP; #, P<0.05 vs. PLTP/-ApoE/- + Ad-GFP.
**Supplemental Table II.** The changes of plasma MCP-1, TNF-α and IL-6 in different mice. (\(\bar{x} \pm \text{SEM}\))

<table>
<thead>
<tr>
<th></th>
<th>MCP-1 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE/-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Ad- GFP (n=18)</td>
<td>436.76±17.98</td>
<td>674.27±20.17</td>
<td>203.17±18.87</td>
</tr>
<tr>
<td>ApoE/-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Ad- PLTP (n=18)</td>
<td>601.31±18.17*</td>
<td>637.39±21.38</td>
<td>212.13±20.15</td>
</tr>
<tr>
<td>PLTP/-ApoE/-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Ad- GFP (n=16)</td>
<td>227.78±12.79*</td>
<td>623.93±26.12</td>
<td>129.86±16.06*</td>
</tr>
<tr>
<td>PLTP/-ApoE/-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Ad- PLTP (n=16)</td>
<td>461.53±15.64*</td>
<td>603.18±18.86</td>
<td>226.54±19.31*</td>
</tr>
</tbody>
</table>

*, P<0.05 vs. ApoE/- + Ad-GFP; #, P<0.05 vs. PLTP/-ApoE/- + Ad-GFP.