

## Original Article

# Increased ADRP expression in human atherosclerotic lesions correlates with plaque instability

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**Abstract:** Adipose differentiation-related protein (ADRP) is intrinsically associated with the surface of lipid droplets implicated in the development of atherosclerosis. We analyzed expression of ADRP in human popliteal artery plaques. Atherosclerotic plaque tissue from the popliteal artery was obtained from 18 patients undergoing lower extremity amputation for arteriosclerosis obliterans, and with either stable (n = 6) or unstable (n = 12) atherosclerotic plaques. Plaques were histologically classified as either unstable ( $\geq 40\%$  lipid core plaque area) or stable ( $< 40\%$  lipid core plaque area). Control tissues consisted of sections of mesenteric arteries obtained from 10 patients without a history of atherosclerosis, but undergoing a subtotal gastrectomy. Plaque tissues were analyzed for expression of ADRP and protein kinase C (PKC) protein by immunohistochemical methods, and ADRP mRNA expression was measured using the reverse transcription polymerase chain reaction (RT-PCR). Immunohistochemical analyses showed that ADRP expression was higher in samples of unstable plaque when compared with expression in stable plaque (gray intensities  $103.56 \pm 1.187$  vs  $106.95 \pm 1.389$ , respectively,  $P < 0.05$ ) and that ADRP expression was associated with increased PKC expression (gray intensities  $102.32 \pm 1.730$  vs  $104.70 \pm 0.959$ , respectively,  $P < 0.01$ ). Consistent with ADRP protein expression, expression of ADRP mRNA was also higher in unstable plaque compared to expression in stable plaque (relative expression  $1.17 \pm 0.15$  vs  $0.81 \pm 0.03$ , respectively,  $P < 0.05$ ). In conclusion: this study demonstrated that increased expression of ADRP in human atherosclerosis was associated with plaque instability.

**Keywords:** Adipose differentiation-related protein, protein kinase C, atherosclerosis, plaque instability

## Introduction

Atherosclerotic plaque instability, resulting in plaque rupture and thromboembolism, is a major cause of acute coronary syndrome (ACS) and sudden coronary death. Development of atherosclerotic plaque is a multistep process that includes phases of plaque formation, maturation, and resultant clinical complications [1]. Plaque rupture usually occurs in areas of a lesion that are enriched in foam cells [2]. Differences in the cellular compositions of stable and ruptured plaques are well established. Despite advances in the understanding the mechanism of atherosclerosis development, the series of events which lead to atherosclerotic plaque progression and resultant clinical complications remain to be elucidated. An ability to assess the likelihood that a plaque will

rupture would be very useful for planning therapeutic interventions and preventing atherothrombotic events.

Atherosclerotic plaque progression from pathologic intimal thickening to fibroatheroma formation is thought to be related to macrophage infiltration [3, 4]. Adipose differentiation-related protein (ADRP or adipophilin) has been reported to be associated with macrophage-derived foam cells in atherosclerotic lesions [5]. ADRP is ubiquitously expressed in many cells and tissues as a major component of lipid droplets [6]. ADRP has also been reported to regulate fatty acid mobilization and formation or stabilization of lipid droplets in adipocytes and other cells [7], play a role in transfer of lipids between cells [8], and induce formation of lipid droplets [9]. It is therefore possible that ADRP might play a

role in destabilization of atherosclerotic plaque and the development of atherosclerosis.

Protein kinase C (PKC) was initially identified as a nucleotide-independent, Ca<sup>2+</sup>-dependent serine kinase [10], and comprises a family of at least 12 isoforms of serine and threonine kinases. PKC isoforms are potentially involved in the process of atherosclerosis [11]. PKC activation increases expression of transforming growth factor- $\beta$ , which is one of the most important growth factors regulating extracellular matrix production. PKC expression in the smooth muscle cells of aortas from atherosclerotic rabbits was significantly higher than expression in a normal control group [12, 13]. Increased intima-media thickness in rabbit atherosclerotic lesions may be associated with an imbalance between proliferation and apoptosis mediated by PKC, and also with lipid-accumulation mediated by ADRP [14].

Together, these findings suggest ADRP and PKC as potential factors which regulate stability of atherosclerotic plaque. Here, we investigated the expression of ADRP and PKC in specimens of human popliteal artery obtained from patients undergoing lower extremity amputation for arteriosclerosis obliterans, to explore the relationship between ADRP and PKC expression and plaque instability.

### Materials and methods

#### *Human tissue specimens*

Samples of atherosclerotic plaque from the popliteal artery were obtained from 18 patients (14 males and 4 females; aged 48-77 years) undergoing lower extremity amputation for arteriosclerosis obliterans. All tissue samples were washed in PBS and divided longitudinally into 2 pieces. One piece was immediately frozen and stored at -80°C until RNA extraction, and the other piece was fixed in 4% paraformaldehyde until hematoxylin-eosin (H&E) staining and immunohistochemical analysis. Control tissues were obtained from the mesenteric arteries of 10 individuals (6 males and 4 females; aged 31-42 years) without a history of atherosclerosis, but a undergoing subtotal gastrectomy. Each sample of popliteal or mesenteric artery was 4-7 cm in length. Written informed consent was obtained from all patients, and the study was approved by the Ethical

Committee of Jilin University and conducted in agreement with institutional guidelines. Samples of atherosclerotic plaque were classified into categories of either stable or unstable plaque based on an arbitrary criterion of whether the plaque contained a core lipid area consisting of > 40% of the plaque area as described by de Bore [15].

#### *Reagents*

Anti-ADRP polyclonal antibody and monoclonal antibodies against human PKC were from Beijing Bios Biological Co., Beijing, China. Reagent kits used for total RNA extraction were purchased from BioDev Technology Biology Co., Beijing, China. TaqDNA polymerase, MMV reverse transcriptase, and DNA markers were purchased from Dalian Takara Biology Co., Dalian, China. The primer sequences of ADRP were synthesized by Shanghai Sangon Biology Technology Co., Shanghai, China.

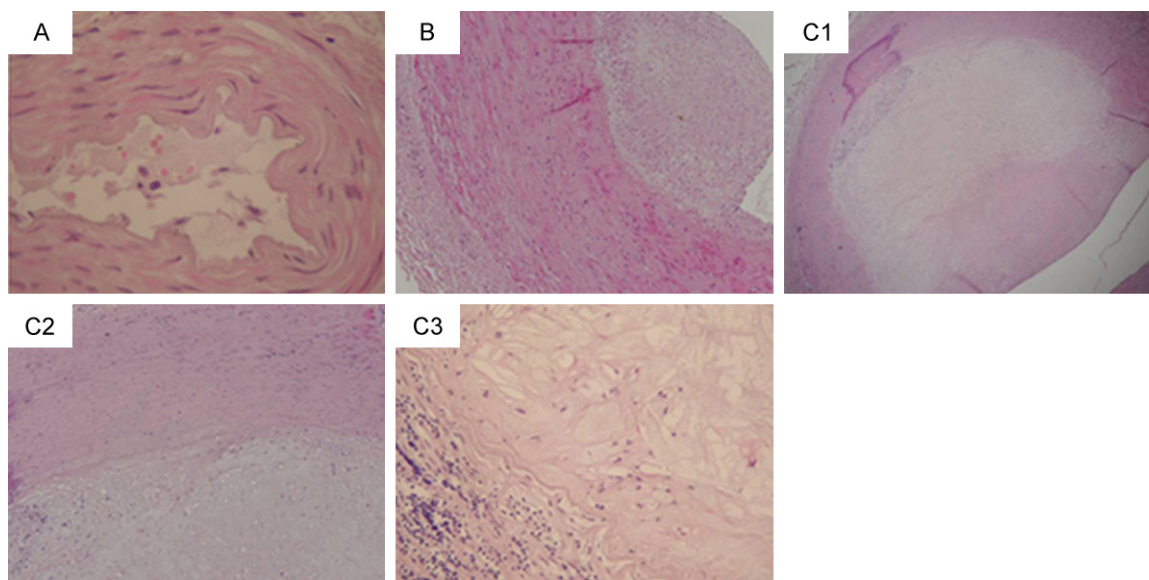
#### *Histological examination*

Fixed specimens were demineralized with 12.5% disodium ethylenediamine tetraacetic acid (EDTA) at room temperature for 1 week, and then cut into 3  $\mu$ m sections for subsequent hematoxylin-eosin (H&E) staining. The stained tissues were then assessed for morphological changes using a light microscope at  $\times$  100 magnification. All analyses were performed by an investigator blinded to the identity of the tissues.

#### *Immunohistochemical analyses of PKC and ADRP protein*

Immunohistochemical staining was used to determine expression of PKC and ADRP proteins. Briefly, arterial sections (3  $\mu$ m thick) were fixed in 5% formaldehyde for 7 days and dehydrated in a descending series of ethanol concentrations. Following dehydration, the sections were adhered to poly-L-lysine glass slides, washed with phosphate-buffered saline (PBS 0.01 M, pH 7.4), and then treated with hydrogen peroxide to inactivate endogenous peroxides. The samples were then blocked with 1% bovine serum albumin in PBS for one hour at room temperature and incubated with anti-ADRP (1:100 dilution) or anti-PKC (1:100 dilution) antibody for 12 h at 44°C. After washing 3 times in PBS, the sections were incubated with

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**Figure 1.** Histological analysis of atherosclerotic tissues and control tissues. A. H&E stained pathological sections of artery in the normal control group showing integrative structure of vascular wall, smooth intima, arranged endothelial cells, smooth muscle cells arranged without hyperplasia in the media, and an outer layer of loose connective tissue (magnification  $\times 100$ ). B. Stable plaque tissue showing eccentric intimal thickening and no obvious lipid core; plaques consisting of foam cells with pink lipid deposition; internal elastic lamina is clearly visible. Smooth muscle cell (SMC) proliferation, disorder, and inflammatory cells infiltration are observed in the media (magnification  $\times 100$ ). C1. Unstable plaque tissue showing thickening intima, area of plaque lipid core  $> 40\%$  (magnification  $\times 40$ ). C2. Unstable plaque showing numerous uniform pink lipid deposits and cavity after lipid dissolved, slight deposition of calcium (purple); foam cells and destructive internal elastic lamina destruction are observed in the intima; SMC proliferation, disorder, foam cell infiltration and lipid deposition are observed in the media (magnification  $\times 100$ ). C3. Unstable plaque showing inflammatory cell infiltration and cholesterol crystals in the core of plaques (magnification  $\times 200$ ).

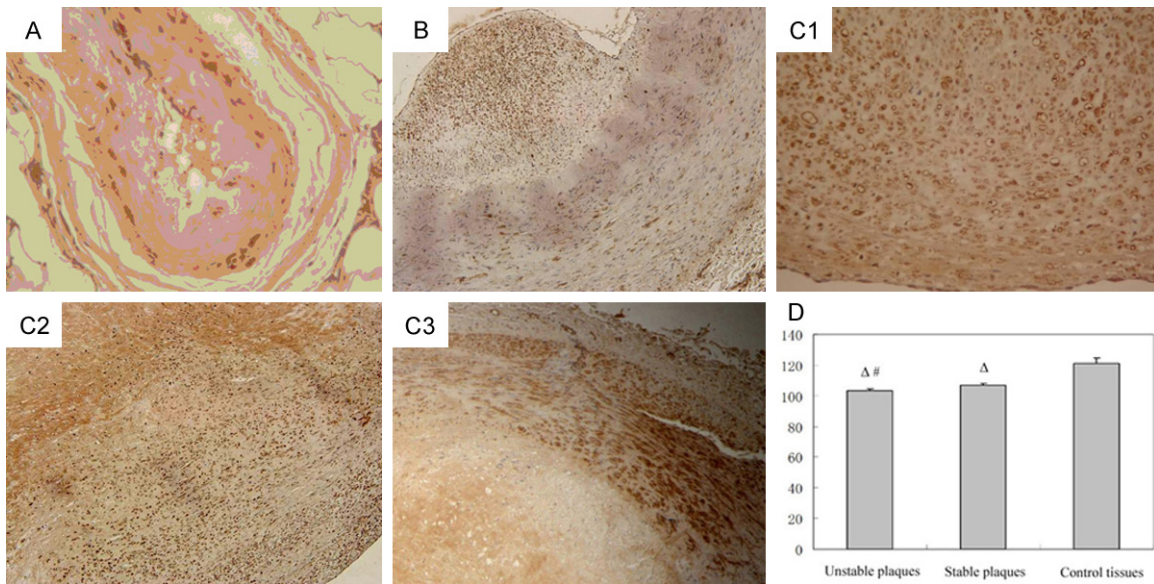
biotinylated secondary antibody for 10 min at  $37^{\circ}\text{C}$ . The sections were then incubated for another 10 min ( $37^{\circ}\text{C}$ ) with horseradish peroxidase labeled streptavidin solution, followed by incubation for 1-2 min with diaminobenzidine (DAB). The cover slips were counterstained with hematoxylin, and the stained sections were examined under light microscopy at magnifications of  $\times 40$ - $\times 100$ . The staining densities for ADRP and PKC immuno-positive nuclei and cytoplasm were determined in 4-5 randomly selected fields by using gray scale analysis (Images Advanced 3.2 systems, Motic Co., China).

### RT-PCR analyses of ADRP

Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis was used to measure expression of ADRP mRNA. Briefly, total RNA was isolated from plaque tissues with Trizol reagent according to the manufacturer's instructions. Total RNA was quantified using the ratio of OD readings at 260 and

280 nm, and sample integrity was checked by 1.8% agarose gel electrophoresis. The primer sequences for ADRP were ADRP-specific primers forward: 5'-CTGCTCACGAGCTGCATCATC-3'; reverse: 5'-TGTGAGATGGCAGAGAACGGT-3'; resulting in a 383 bp fragments. As a housekeeping gene, we used rat GAPDH primers forward: 5'-G GGTGATGCTGGTGCTGAGTATGT-3'; reverse: 5'-AAGA ATGGGTGTTGCTGTTGAAGTC-3', resulting in a 617 bp fragments. The temperature cycling conditions used for amplification were as follows: for ADRP, an initial step of  $45^{\circ}\text{C}$  for 30 min, denaturation at  $95^{\circ}\text{C}$  for 5 min, 30 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $52^{\circ}\text{C}$  for 30 s, elongation at  $72^{\circ}\text{C}$  for 30 s, and a final extension at  $72^{\circ}\text{C}$  for 10 min. For GAPDH, an initial step of  $45^{\circ}\text{C}$  for 30 min, denaturation at  $95^{\circ}\text{C}$  for 5 min, 28 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $57^{\circ}\text{C}$  for 30 s, elongation at  $72^{\circ}\text{C}$  for 30 s, and a final extension at  $72^{\circ}\text{C}$  for 10 min. RT-PCR was performed using the Gene Amplify PCR System. The amplification products were electrophoresed on a 1.8% agarose gel, visualized

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**Figure 2.** Immunohistochemical analyses of ADRP. A. The normal control group showing no positive ADRP staining in the intima and uniform yellowish brown ADRP staining in cytoplasm of SMC in the media (magnification  $\times 100$ ). B. Stable plaque tissue showing foam cell infiltration in the intima and dark brown granular ADRP staining (magnification  $\times 100$ ). C1. Unstable plaque tissue showing large amount of ADRP staining in the intima or media, and a small amount of staining in the lipid core (magnification  $\times 40$ ). C2. Unstable plaque showing obvious intimal thickening, large accumulation of foam cells, and deep brown granular ADRP staining in the foam cells (magnification  $\times 100$ ). C3. Unstable plaque showing large amount of dark brown granular ADRP staining in the foam cells of intima and SMC of media (magnification  $\times 200$ ). D. Results of gray scale analysis. Values are expressed as mean  $\pm$  SD ( $n = 6$ ).  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$  vs. control group;  $\#P < 0.05$ ,  $\#\#\#P < 0.01$  vs. stable plaque group. Positive ADRP gray staining intensities were significantly less in unstable plaques compared to stable plaques tissues ( $P < 0.05$ ). The results indicate that ADRP protein expression is up-regulated in unstable plaques to stable plaques.

by ethidium bromide (0.5 g/mL) staining, and photographed. Gel images were scanned using an image analysis system (Tanon-4200, Science and Technology Ltd., Shanghai, China); the intensities of specific PCR bands were determined using the relative quantitation method expressing results in relation to GAPDH bands from the same sample.

### Data analyses

Data were expressed as mean  $\pm$  SD. One-way analysis of variance followed by Bonferroni's multiple comparison tests were conducted using SPSS10.0 software.  $P$  values  $< 0.05$  were considered to be statistically significant.

## Results

### Histological analysis of tissues

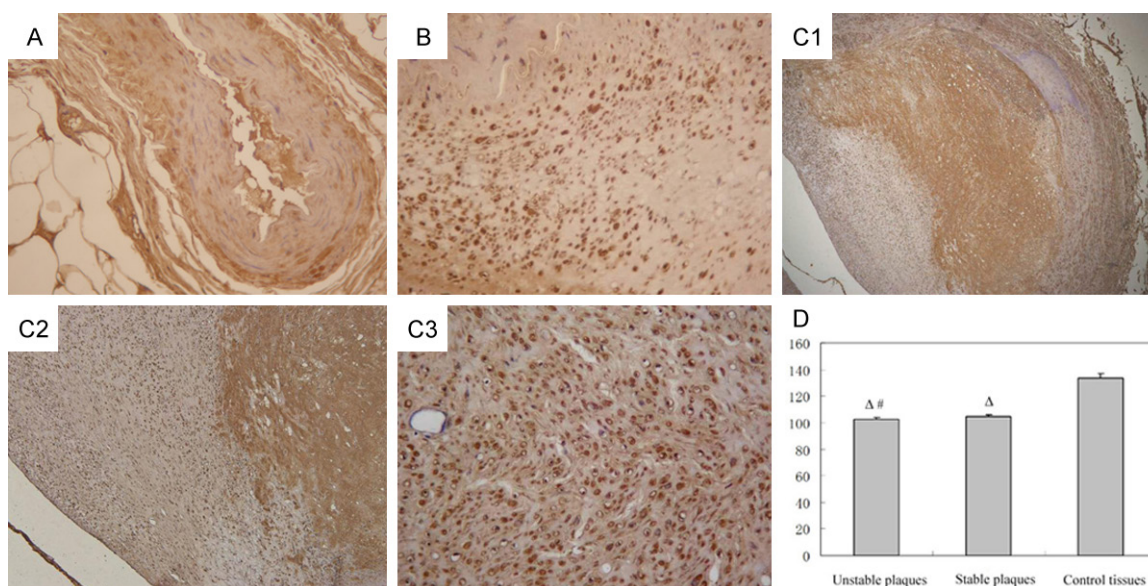
Based on whether the lipid core of a plaque was  $> 40\%$  of the total plaque area, 6 pieces of atherosclerotic tissue were classified as stable plaque and 12 pieces were classified as unstable plaque. **Figure 1A** showed the normal control tissues. Endothelial cell is put in order,

endomembrane is smoothing. No medial thickening was found, and there was no lipid core in samples of normal control mesenteric artery. As shown in **Figure 1B**, the lipid core was not obvious in samples of stable plaque. Most samples of stable plaque contained proliferating endothelial cells, and the internal elastic lamina could be observed. In samples of unstable plaque (**Figure 1C**), intimal thickening was observed and the lipid core area was  $> 40\%$  of the total plaque area. Additionally, there were proliferative endothelial cells with foam cells and large amounts of lipid deposits in the intima. Inflammatory cell infiltration, the presence of cholesterol crystals, and internal elastic lamina destruction were also observed in unstable plaques, along with hyperplasia of smooth muscle cells and large lipid deposits.

### ADRP protein expression

Positive blue staining for ADRP protein expression was found in the nucleus of cells and both blue and brown staining was found in the cytoplasm. As shown in **Figure 2A**, there was no positive staining for ADRP expression in intimal

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**Figure 3.** Immunohistochemical analysis of PKC. A. The normal control group showing no positive PKC staining in the intima and only slight yellowish brown positive PKC staining in cytoplasm of SMC in the media (magnification  $\times 100$ ). B. Stable plaque tissue showing large amounts of dark brown granular PKC staining in the intima and only slight granular positive PKC staining in the media (magnification  $\times 100$ ). C1. Unstable plaque tissue showing large amounts of dark brown PKC staining in the intima and destruction of media (magnification  $\times 40$ ). C2. Unstable plaque tissue showing deep brown granular or patch PKC staining in foam cells and necrotic lipid core (magnification  $\times 100$ ). C3. Unstable plaque tissue showing positive PKC staining in macrophages, proliferative SMC, and lipid deposition (magnification  $\times 200$ ). D. Results of gray scale analysis. Values are expressed as mean  $\pm$  SD ( $n = 6$ ).  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$  vs. control group;  $\#P < 0.05$ ,  $\#\#P < 0.01$  vs. stable plaque group. Positive PKC gray intensities were significantly less in unstable plaque tissue compared with stable plaque tissue ( $P < 0.05$ ). Results indicate that PKC protein expression is up-regulated in unstable plaque compared to stable plaque.

cells; however, some slight ADRP expression was shown in medial smooth muscle cells in normal control tissues. There were full of brown particle or fragmented brown staining in stable plaques tissues (**Figure 2B**) and unstable (**Figure 2C**).

As shown in **Figure 2D**, gray scale analyses showed that the intensity of positive gray staining for ADRP was significantly less in both unstable ( $103.56 \pm 1.187$ ) and stable ( $106.95 \pm 1.389$ ) plaque tissues compared with gray staining in normal control tissues ( $121.08 \pm 3.624$ ;  $P < 0.01$ ). Also, the intensity of gray staining positive for ADRP was significantly less in unstable plaque tissue compared with staining in stable plaque tissue ( $P < 0.05$ ). These results indicated that ADRP protein expression was significantly up-regulated in plaque tissue compared to expression in normal tissue, especially in unstable plaque tissue.

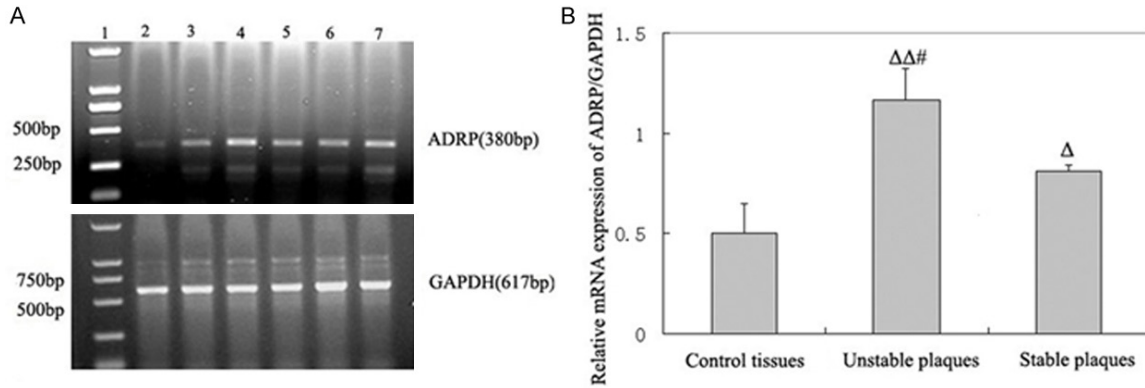
### PKC protein expression

Positive staining for PKC protein expression was found in the nucleus cells of popliteal

artery, along with blue and brown staining in the cytoplasm. As shown in **Figure 3A**, there was no positive staining for PKC protein expression in intimal cells; however, slight positive staining was found in the medial smooth muscle cells of normal control mesenteric artery. Large amounts of positive staining for PKC protein expression were found in the intima, and slight positive staining was found in the medial smooth muscle cells of stable plaques of popliteal artery (**Figure 3B**). There were also particles or fragmented positive staining in the intima, media, and lipid cores of unstable plaques (**Figure 3C**).

As shown in **Figure 3D**, gray scale analyses showed that positive PKC gray intensities were significantly less in both unstable ( $102.32 \pm 1.730$ ) and stable ( $104.70 \pm 0.959$ ) plaques compared to normal control tissues ( $133.88 \pm 3.411$ ;  $P < 0.01$ ). Positive PKC gray intensities were significantly less in unstable plaque compared to stable plaque ( $P < 0.05$ ). These results indicate that expression of PKC protein is significantly up-regulated in plaques tissues, compared to expression in normal control tissue.

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**Figure 4.** A. ADRP mRNA expression in atherosclerotic tissue. ADRP mRNA expression was determined by real time RT-PCR. RT-PCR was performed using total RNA from artery tissues. Lane 1, DNA markers; lanes 2 and 3, normal control group; lanes 4 and 5, unstable plaques; lanes 6 and 7, stable plaques. B. ADRP mRNA expression is expressed relative to the corresponding densities of GAPDH bands. Values are expressed as mean  $\pm$  SD ( $n = 6$ ).  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$  vs. control group;  $*P < 0.05$ ,  $##P < 0.01$  vs. stable plaque group. Results indicate that ADRP mRNA expression is up-regulated in unstable plaque compared to stable plaque.

### ADRP mRNA expression

Products of the amplification RNA were subjected to electrophoresis on 1.8% agarose gels and visualized with ethidium bromide. As shown in **Figure 4A**, RT-PCR analysis revealed that the product size for ADRP was 383 bp. Expression of ADRP mRNA was significantly enhanced in both unstable and stable plaques compared with expression in normal control tissues. As shown in **Figure 4B**, the band intensities were quantified using an image analysis system (Tanon-4200, Science and Technology Ltd., Shanghai, China). The results for determinations of ADRP mRNA expression are expressed relative to the corresponding densities of GAPDH bands. Expression of ADRP mRNA in unstable plaque was significantly increased compared to expression in normal control tissues ( $1.17 \pm 0.15$  vs  $0.5 \pm 0.15$ , respectively,  $P < 0.01$ ) and stable plaque ( $1.17 \pm 0.15$  vs  $0.81 \pm 0.03$ , respectively,  $P < 0.05$ ). Also, ADRP mRNA expression in stable plaque was increased compared to expression in normal control tissue ( $P < 0.05$ ). These results indicate that ADRP mRNA expression is significantly increased in both stable and unstable plaque tissue, compared to expression in normal control tissue.

### Discussion

In this study, the extent of ADRP expression in atherosclerotic plaque appear to be related to the degree of plaque stability. Also, ADRP mRNA

and PKC protein expression are increased in unstable plaque. PKC protein expression was up-regulated in plaque from patients with atherosclerosis compared to expression in artery samples obtained from control subjects, and was more prominent in unstable plaque. Our results contribute to understanding the relevance of the PKC-ADRP signaling pathway in regulation of atherosclerotic plaque stability.

Atherosclerosis is a chronic inflammatory disorder characterized by deposition of lipids in the arterial intima. Plaque rupture with luminal thrombosis is the most common cause of ACS and sudden coronary death. Despite advances in understanding the pathogenesis of atherosclerosis, the factors that determine atheromatous plaque instability have remained unclear. Intra-plaque hemorrhage and rupture of immature neointima might be critical in promoting instability of atherosclerotic plaque [16, 17]. Pathology studies have identified several characteristics of plaques that are associated with plaque rupture and subsequent thrombosis. One such characteristic was that the plaque core was occupied by lipid droplets composed of cholesterol esters and triglycerides. Additionally, the unstable plaques were often quite large and had a necrotic core that comprised a significant portion of the plaque.

ADRP is a 53 kDa protein and was encoded by a cDNA originally cloned by differential hybridization of murine adipocytes. ADRP has served as a sensitive marker of lipid accumulation [6,

18], and increased cytosolic ADRP might be indicative of an increase in lipid droplets [19]. ADRP was highly expressed in a subset of lipid-rich foam cells in human atherosclerotic lesions [5], and promoted accumulation of lipids in foam cells and increased accumulation of lipids atherosclerotic plaque. An increase of lipids in the core region of plaques reduced plaque stability. In the current study, expression of ADRP mRNA and protein were up-regulated in both stable and unstable plaque compared with normal tissue. Furthermore, ADRP mRNA and protein expression were more prominent in unstable plaques than in stable plaques. Immunohistochemical analyses showed that positive staining for ADRP expression was mainly found in foam cells. Our findings support the hypothesis that increasing ADRP expression occurs in parallel with increases in plaque instability. The current study identified ADRP as a potential de-stabilizing factor for atherosclerotic plaques.

PKC exists as a family of serine/threonine kinases which regulate a host of cellular responses [20], and members of the PKC family are major effectors of lipid second messengers [21]. Our results showed that expression of PKC protein was significantly increased in atherosclerotic plaque, and that expression of PKC in unstable plaque was significantly higher than that in stable plaque. We therefore suggest that increased expression of PKC is associated with plaque instability. Plaque instability promoted by PKC might be involved in regulation of foam cell formation, smooth muscle cell proliferation, and endothelial dysfunction. Our findings suggest important roles for both ADRP and PKC in regulation of plaque stability and support the need for further research into the mechanism of atherosclerotic plaque instability. In addition, peroxisome proliferator-activated receptor (PPAR) $\gamma$  might serve as a bridge between PKC and ADRP [22], and the PKC-PPAR  $\gamma$ -ADRP signal pathway might play an important role in regulating the stability of atherosclerotic plaque.

It should be noted that our current preliminary study does have several limitations. First, the vessels examined were from the popliteal artery; however, the most clinically significant vascular disease occurs in smaller vessels of the cardiovascular system, and the characteristics of atherosclerotic lesions in the popliteal

artery may more closely correlate with those found in the aorta. Second, the distinction between stable and unstable plaques was based on an arbitrary criterion that the plaque core comprised at least 40% of the plaque area. However, the size of a plaque core can show considerable variation; and therefore, a selection bias may have affected our results. Third, we did not investigate the effects of PPAR- $\gamma$  on atherosclerotic plaque. PPAR- $\gamma$  may play a role in the atherosclerotic process by affecting adipocyte differentiation and lipid storage [23-25], and future studies will explore the impact of PPAR- $\gamma$  on the stability of atherosclerotic plaque.

In conclusion, this study demonstrated that increased expression of ADRP in human atherosclerosis was associated with plaque instability. Given the role of ADRP in regulation of plaque stability, our findings are important for the diagnosis and monitoring of atherosclerotic vascular disease.

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### Disclosure of conflict of interest

None.

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