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Vasodilator-Stimulated Phosphoprotein: Regulators of Adipokines Resistin and Phenotype Conversion of Epicardial Adipocytes

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Manuscript Preparation E
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Background: Endothelial dysfunction plays a central part in the pathogenesis of coronary atherosclerosis. The adipokine resistin is one of the key players in endothelial cell dysfunction. In addition, the role of epicardial fat in coronary artery endothelial dysfunction is also emphasized.

We investigated whether vasodilator-stimulated phosphoprotein (VASP) is involved in resistin-related endothelial dysfunction and the phenotype conversion of epicardial adipocytes.

Material/Methods: Cell proliferation and migration were evaluated by MTT and Transwell chamber assay, respectively. Next, we took epicardial fat samples from patients with valvular heart disease and non-coronary artery disease. Gene expression was determined by reverse transcription-quantitative polymerase chain reaction and relative abundance of the protein by Western blotting.

Results: Resistin induced endothelial proliferation and migration in a dose-dependent manner. Both resistin-induced cell proliferation and migration were effectively blocked by ablation of VASP. The brown adipose tissue-specific genes for uncoupling protein 1 (UCP-1) and PR-domain-missing16 (PRDM16) decreased, but the white adipose tissue-specific genes for resistin and RIP140 increased in VASP-deficient adipocytes compared with the LV-sictr group. However, disruption of the Ras homolog gene family member A (RhoA) /Rho-associated kinase (ROCK) in VASP-deficient adipocytes with specific inhibitors inverted the adipocyte phenotype existing in VASP-deficient adipocytes. Furthermore, the expressions of proinflammatory cytokines interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1) in VASP-deficient adipocytes were markedly upregulated compared with the LV-sictr group.

Conclusions: These results suggest a physiological role for VASP in coronary atherosclerosis through regulating adipokine resistin and phenotype conversion of epicardial adipose tissue.

MeSH Keywords: **Adipocytes, Brown • Adipocytes, White • Cell Migration • Cell Proliferation • Coronary Artery Disease**

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Background

Coronary atherosclerosis remains the major cause of cardiovascular disease, despite major advances in diagnosis and treatment (mostly preventive) of known risk factors, which indicates the need to further explore the pathogenesis of atherosclerosis [1]. The pivotal role of angiogenesis in atherosclerotic lesions is firmly established [2]. There is no neovascularization in the intima and media of normal arteries, while neovascularization is seen in the intima of human atherosclerotic lesions. The pathological angiogenesis is characterized by persistent proliferation and faster migration of endothelial cells [3], leading to progression of plaque, plaque instability, intraplaque hemorrhage, and subsequently increased risk of athero-thrombotic events.

Epicardial adipose tissue (EAT), given its close anatomical affinity with the coronary, drew our attention. Many studies have demonstrated that EAT exhibits more characteristics of brown adipose tissue (BAT) than white adipose tissue (WAT) in non-coronary atherosclerotic heart disease [4]. BAT mainly produces heat and consumes energy, which is helpful for the maintenance of energy and metabolism balances. However, WAT can secrete a variety of adipokines (such as resistin and leptin) and proinflammatory cytokines (such as IL-6 and IL-8) to facilitate endothelial dysfunction, resulting in progression of coronary atherosclerosis [5]. Resistin, a kind of adipokine or inflammatory cytokine, can accelerate atherosclerotic plaque progression through stimulating endothelial cells. In our previous studies, we demonstrated that during the progression of atherosclerosis, there is a phenotype conversion of EAT from BAT to WAT, which further promotes the focal occurrence and development of atherosclerosis [6]. However, the mechanism of adipokines resistin and phenotype conversion of epicardial adipocytes are unclear.

Vasodilatory-stimulated phosphoprotein (VASP) is an actin-binding protein that plays a crucial role in cell adhesion, proliferation, and migration by regulating F-actin cytoskeleton dynamics [7]. Recently, a body of evidence suggested that resistin can induce human endothelial cell proliferation and migration [8], thereby suggesting a potential for increased angiogenesis [9]. Therefore, we hypothesized that resistin potentiates angiogenesis via VASP signaling molecule in the human coronary endothelial cell line (HCAECs). VASP functions are regulated by phosphorylations, although the specific roles of phosphorylations on functional outcomes are unclear [10]. VASP phosphorylation at serine 239 is not only a marker of cyclic guanosine 3',5'-monophosphate (cGMP)-dependent protein kinase (PKG) activation, but is also a mediator of relevant biological actions exerted by the nitric oxide (NO)/cGMP/PKG pathway [11]. Protein kinase G (PKG) is essential for brown fat cell differentiation and mitochondrial biogenesis [12]. VASP has

recently emerged as an important regulator of differentiation of adipocytes and energy homeostasis [13]. Despite these early studies, the role of VASP in adipocytes is only emerging now, and the mechanism of phenotype conversion of human epicardial adipocytes has not been identified. The aim of our study was to determine the role of VASP in function of resistin in HCAECs and phenotype conversion of epicardial adipose tissue. Our findings may provide evidence for the pathogenesis of coronary atherosclerosis and present new clues for the therapy of coronary atherosclerosis.

Material and Methods

Fat sample

Epicardial fat samples were taken from middle-aged patients (49.32 ± 7.36 y) with valvular heart disease and non-coronary artery disease during heart valve replacement, and most were taken from near the origin of the right coronary artery. Informed consent was obtained from all patients and the study was approved by the Ethics Committee of Nanjing Jinling Hospital.

Cell isolation and adipogenic differentiation

Cell isolation of brown preadipocytes was performed as previously described. In brief, the adipose tissues were digested for 1 h in collagenase buffer [DMEM/F12 containing 0.1% collagenase type I]. After standing for 20 min on ice, cells were centrifuged and washed. The pellet was resuspended in DMEM/F12 supplemented with 10% FBS and 1% P/S, then cells were seeded in 6 wells and grown at 37°C and 5% CO₂. The morphology of primary preadipocytes from human EAT were round and had different sizes. After 7–8 days, cells became oval monolayer-like. When they achieved 80–90% confluence, cells could be amplified by passage culture, but only within the cells of 4 generations.

These brown preadipocyte cells were differentiated by induced liquid of adipose tissue-derived mesenchymal stem cells following the manufacturer's instructions. In brief, when brown preadipocyte cells reached confluence, the medium was refreshed with human adipose-derived stem cell adipogenic differentiation medium A, which mainly consists of glutamine, insulin, IBMX, rosiglitazone, and dexamethasone. After 3 days of induction, the medium was replaced with human adipose-derived stem cell adipogenic differentiation medium B for 1 day, which mainly includes glutamine and insulin. At 3–4 days, cells shrank and became round. After 6–8 days, lipid droplets were present around the nuclei and began to increase and enlarge. Then, the previous steps were repeated until 12–20 days. At 12–20 days, lipid droplets with different sizes were present in 80% of cells. After induction, cells were

Table 1. Short hairpin RNA (shRNA) inserts.

pCDH-U6-VASP-sh1S	GATCCGGGACTCATGGAAGAGATGAACAAGAGTTCATCTCTCCATGAGTCCCTTTTG
pCDH-U6-VASP-sh1A	AATCAAAAAGGGACTCATGGAAGAGATGAACTCTGTTCATCTCTCCATGAGTCCCG
pCDH-U6-VASP-sh2S	GATCCGGACTACAGAGGGTGAAACACAAGAGTGTTCACCTCTGTAGGTCCTTTTG
pCDH-U6-VASP-sh2A	AATCAAAAAGGACTACAGAGGGTGAAACACTCTGTGTTCACCTCTGTAGGTCGG
pCDH-U6-VASP-shNS	GATCCGATGAAATGGATAGAAGTACACAAGAGTGTACTTCTATCCATTTTCATCTTTTG
pCDH-U6-VASP-shNA	AATCAAAAAGATGAAATGGATAGAAGTACTCTGTGTACTTCTATCCATTTTCATCG

treated only with human adipose-derived stem cell adipogenic differentiation medium B for 4–7 days until lipid drops became big enough to be used in subsequent adipocyte-related experiments.

Oil red O staining

When the adipocytes became mature, the medium was removed, and cells were fixed in 10% formalin at room temperature for 1 h. Then, cells were stained with oil red solution for 30 min following manufacturer's instructions. After washing, cells were observed under a light microscope. Oil red O staining showed red granules, which were found as lipid droplets.

Lentivectors construction and lentivirus production

The 2 pairs of shRNA targeting human VASP and a negative control shRNA listed in Table 1 were cloned into pCDH-U6-MCS-EF1-GreenPuro (SBI, Mountain View, CA, USA) to generate the pCDH-U6-VASP-sh1, pCDH-U6-VASP-sh2, and pCDH-U6-VASP-shN lentivectors, respectively. HEK-293T cells were transfected with the constructs along with 3 other plasmids (pGag/Pol, pRev, and pVSVG) into HEK293T cells using TurboFect (Thermo Scientific) according to the manufacturer's protocol. The mature adipocytes or HCAECs were seeded in a 6-well plate. After 24 h, 50% of the medium in each well was removed and polybrene was added to a final concentration of 8 g/ml. The shRNA lentiviruses (MOI=1) were added to the cells. After an overnight incubation at 37°C, the medium was replaced with fresh medium and incubated for another 72 h. Then, cells were used to evaluate the expression of VASP or for further experiments.

Actin cytoskeletal staining

Actin cytoskeletal staining was conducted in the control group (LV-sicntr) and VASP-deficient group (LV-siVASP). Cells were cultured on coverslips in a 6-well plate. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed, and permeabilized with 0.5% Triton X-100 for 5 min. The cells were incubated with Actin Red at room temperature for 20 min. ProLong Gold Antifade reagent and DAPI (Invitrogen, Carlsbad, CA) were used to mount the coverslips to slides.

IL-6 or Y27632 2HCl treatment

The mature adipocytes with different conditions were treated with IL-6 at 100 ng/ml in DMEM/F12 for 24 h at 37°C in an environment with 5% CO₂. Total RNA was extracted, and RT-PCR was employed to detect the mRNA expressions of UCP-1, PRDM16, resistin, and RIP140. Y27632 2HCl, a selective ROCK1 inhibitor, was dissolved in DMSO to a concentration of 30 mM for conservation. Adipocytes were treated with 30 μM for 24 h.

Cell proliferation

The cell proliferation was measured by MTT assay (KeyGEN BioTECH). Serum-starved cells were seeded onto a 96-well plate at 10 000 cells/well. After 24 h, cells were serum-starved for 16 h and treated with various doses of resistin (0 ng/ml, 10 ng/ml, 50 ng/ml, and 100 ng/ml) for an additional 48 h. Then, MTT was added to each well, incubated for 4 h, and dissolved by DMSO at 37°C in a shaking bed. Last, the light density was measured at 490 wavelength using an enzyme meter.

Cell migration

Cell migration was measured using a Transwell chamber assay. Serum-free cell suspension (200 μl, 2×10⁴ cells/well) containing various doses of resistin was added to the Transwell insert (8-μm pore size, Corning, USA), and 10 ng PDGF-β (Sigma, USA) were applied to the bottom chamber as a chemoattractant (600 μl). After 12-h incubation, the chambers were fixed in 2% paraformaldehyde for 5 min and washed in PBS. The non-migrating cells in the upper chamber were wiped off using swabs and washed with PBS. Then, the numbers of cells with various morphologies were counted under a microscope.

RT-PCR

Total RNA was extracted from adipose cells using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol and reverse-transcribed into complementary DNA (cDNA). cDNA was synthesized using the Primescript™ RT reagent kit (Takara Bio, Dalian, China) according to the manufacturer's

Table 2. Primers used in this study.

Target gene	Forward (5'-3')	Reverse (5'-3')
β-actin	CCCGAGCCGTGTTTCCT	GTCCAGTTGGTGACGATGC
UCP-1	TTGGTGTCGGCTCTATC	CCGTGGTCCTTCGTT
PRDM16	AGGTGCCTGTCTTCTATTCCC	GCTTCTTCTCTGCATCCC
RIP140	CTGCCACCTCACCTAA	TAAGTCCAACATCCTT
Resistin	GCAAGACCCTGTGCTCCA	CACTCCAGGCCAATGCTG
VASP	GACCACTCCGAGACCCAACC	GAAGCTTCAATGATTTCCTCT
IL-6	AAGCAGCAAAGAGGCA	TCAAATCTGTTCTGGAGGT
MCP-1	AGAATCACCAGCAGCAAG	GGAATCCTGAACCCACTT
IL-8	AAACCTTTCCACCCC	ACTTCTCCACAACCCCTC
TNF-α	CCACCACGCTCTTCTGC	GCTTGAGGGTTTGCTACAAC

instructions. cDNA served as a template for RT-PCR, and primers used in RT-PCR are shown in Table 2. RT-PCR was performed in 20 μl of the mixture and the amplification conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 58°C for 15 s, and 72°C for 15 s. β-actin served as a loading control.

Western blot analysis

The treated cells were lysed in ice-cold cell lysis buffer containing a protease inhibitor cocktail (Sigma). The protein was separated on a 10% SDS-polyacrylamide gel. The separated proteins were then transferred onto a PVDF membrane (Millipore, MA, USA) and blocked with 5% non-fat dry milk prepared in 1×TBS. The membranes were incubated with the primary antibodies overnight at 4°C. The following antibodies were used: antibodies against UCP-1, PRDM16, and VASP (all from Abcam, Cambridge UK). After 3 washes with TBST, membranes were incubated with the appropriate HRP-conjugated secondary antibodies (Abcam, Cambridge, UK) for 2 h at room temperature, followed by detection with enhanced chemiluminescence (Millipore). The relative band intensity was measured using Image J software.

Statistical analysis

SPSS version 19.0 was used for statistical analysis. Data are expressed as mean ± standard deviation. One-way analysis of variance was used for comparisons among groups and the *t* test was used for comparisons between groups. A value of *P*<0.05 was considered statistically significant.

Results

Gene and protein expression after VASP silence

To test whether VASP is involved in the regulation of HCAECs proliferation and migration or phenotype conversion of epicardial adipocytes, knockdown of VASP by siRNA blocked its expression. Then, RT-PCR analysis and Western blot analysis with VASP-specific antibodies were used to assess expression of VASP. The data revealed that VASP content significantly decreased in LV-siVASP HCAECs compared with the LV-sicntr group (Figure 1).

Loss of VASP inhibited the effect of resistin on cell proliferation

To evaluate the effects of VASP on resistin proliferation, we first verified the effect of resistin on endothelial cell proliferation by MTT assay. The result showed that resistin significantly promoted HCAEC proliferation in a dose-dependent manner compared to the untreated control (Figure 2A). Next, we analyzed cell proliferation of the control group (LV-sicntr) and VASP-deficient group with or without resistin at 100 ng/ml. As shown in Figure 2B, ablation of VASP inhibited the effect of resistin on cell proliferation compared with the LV-sicntr group, showing that resistin induced HCAECs proliferation, at least in part, by interaction with the VASP molecule.

Ablation of VASP disrupts the effect of resistin on cell migration

Previous studies have confirmed that resistin and VASP play an important role in cell migration, but the involvement of VASP in the effect of resistin has not been elucidated. To investigate VASP-dependent regulation of resistin in endothelial cell migration, Transwell chamber assay was used to evaluate migration in various doses of resistin. In line with endothelial cell

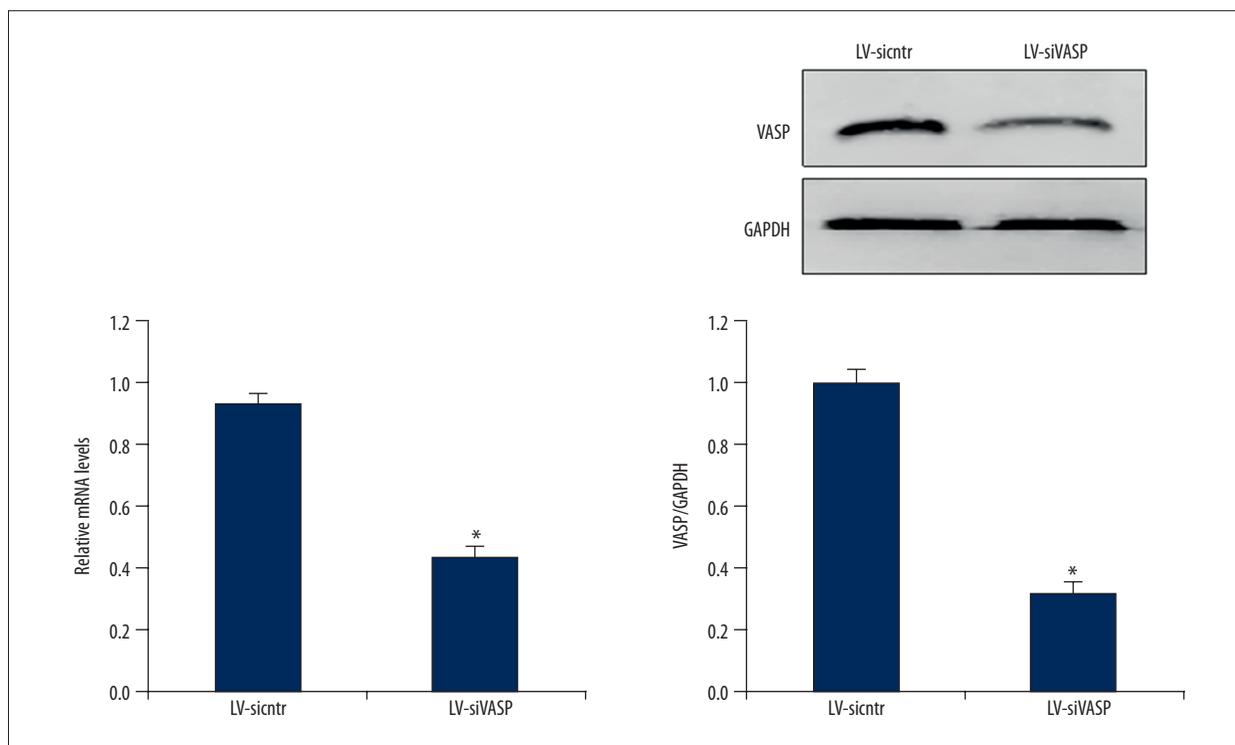


Figure 1. Knockdown of VASP by siRNA. Both gene and protein expressions were obviously lower after knockdown of VASP by siRNA compared with LV-sictr group in HCAECs. * $p < 0.05$.

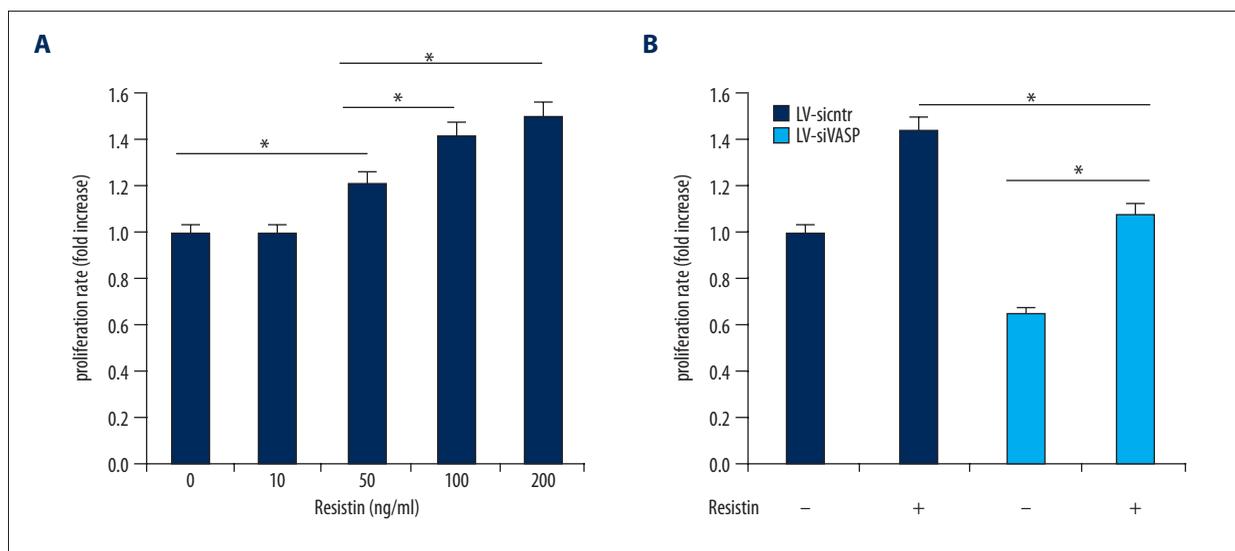


Figure 2. Effect of VASP on proliferation of resistin. (A) After 24h incubation, resistin induced endothelial proliferation in a dose-dependent manner (0–200 ng/ml) with MTT assay. (B) Resistin-induced cell proliferation could be effectively blocked by ablation of VASP with or without 100 ng/ml of resistin. * $p < 0.05$.

proliferation, resistin significantly increased HCAEC migration in a dose-dependent manner compared with the blank control group (Figure 3A). However, knockdown of VASP by siRNA abrogated the effect of resistin at 100ng/ml on endothelial cell migration compared with the LV-sictr group (Figure 3B). Thus, loss of VASP can inhibit endothelial cell migration of resistin.

Knockdown of VASP promotes brown-to-white adipocyte trans-differentiation

Recent studies indicated that inflammation can induce brown-to-white adipocyte phenotype conversion [14]. To prove that there was phenotype conversion of adipocytes in IL-6,

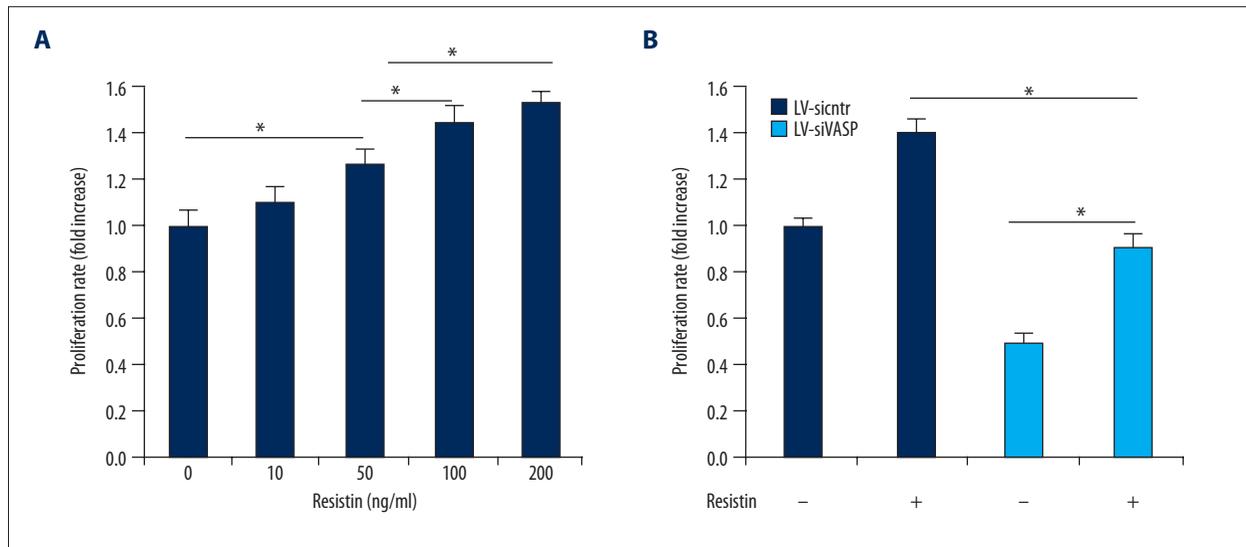


Figure 3. Effect of VASP on migration of resistin. (A) HCAEC migration in different doses of resistin(0–200 ng/ml) was studied by transwell chamber assay. The results showed that resistin promoted migration of HCAECs after 12 h incubation. (B) Ablation of VASP disrupted the function of resistin on cell migration with or without 100 ng/ml of resistin. * $p < 0.05$.

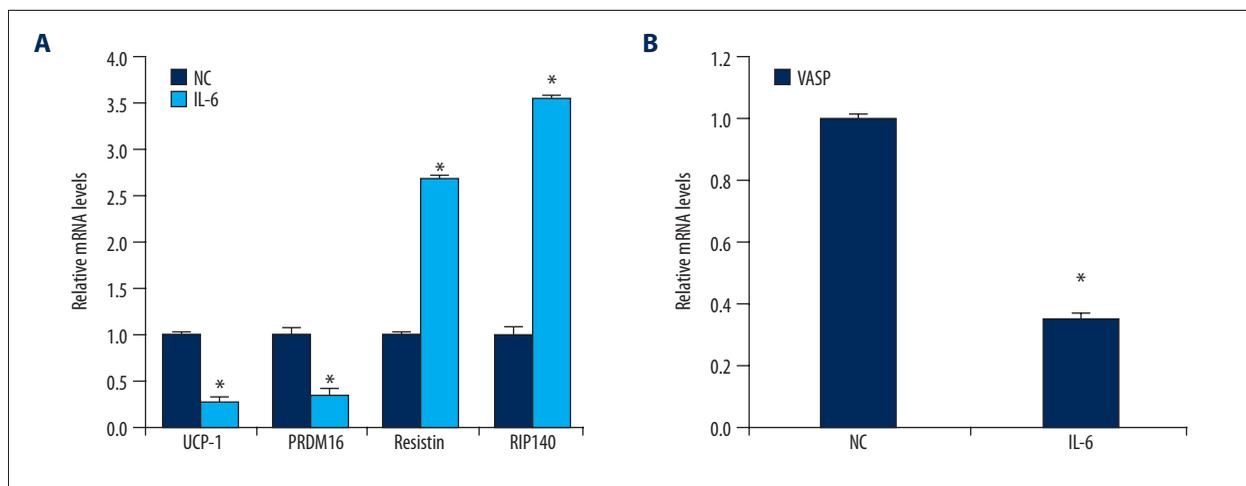


Figure 4. (A) IL-6 induced the differentiation of brown adipocytes into white adipocytes. (B) VASP contents were decreased during brown-to-white adipocyte trans-differentiation phenotype conversion. * $p < 0.05$.

the mature adipocytes were treated with or without IL-6 at 100 ng/ml. We found that the abundance of BAT-specific genes such as UCP-1 and PRDM16 were significantly decreased, but the WAT-specific genes for resistin and RIP140 increased in IL-6 at 100 ng/ml (Figure 4A), which was consistent with previous studies.

Therefore, we measured the contents of VASP during brown-to-white adipocyte trans-differentiation phenotype conversion by RT-PCR. Our results showed that VASP contents were decreased in IL-6 at 100 ng/ml compared with the control groups (Figure 4B). Next, to study the functional consequences of VASP on phenotype conversion of adipocytes, we knocked-down VASP by siRNA blocking its expression in mature brown adipocytes,

and the inhibited effects were almost the same as in HCAEC (data not shown). Because proteins of the enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP) family appear to promote actin filament formation [15], F-actin might exhibit dynamic instability (auto assembly-disassembly) in the absence of VASP. As shown in Figure 5A, fewer actin aggregates and bundled stress fibers were observed in VASP-deficient adipocytes, whereas stress fibers gathered by F-actin appeared long and regular in LV-sicntr adipocytes. Next, we investigated whether VASP was related with phenotype conversion of epicardial adipocytes. Surprisingly, we found obviously decreased expressions of UCP-1 and PRDM16 and increased expressions of resistin and RIP140 in VASP-deficient adipocytes compared with LV-sicntr (Figure 5B). These data show that

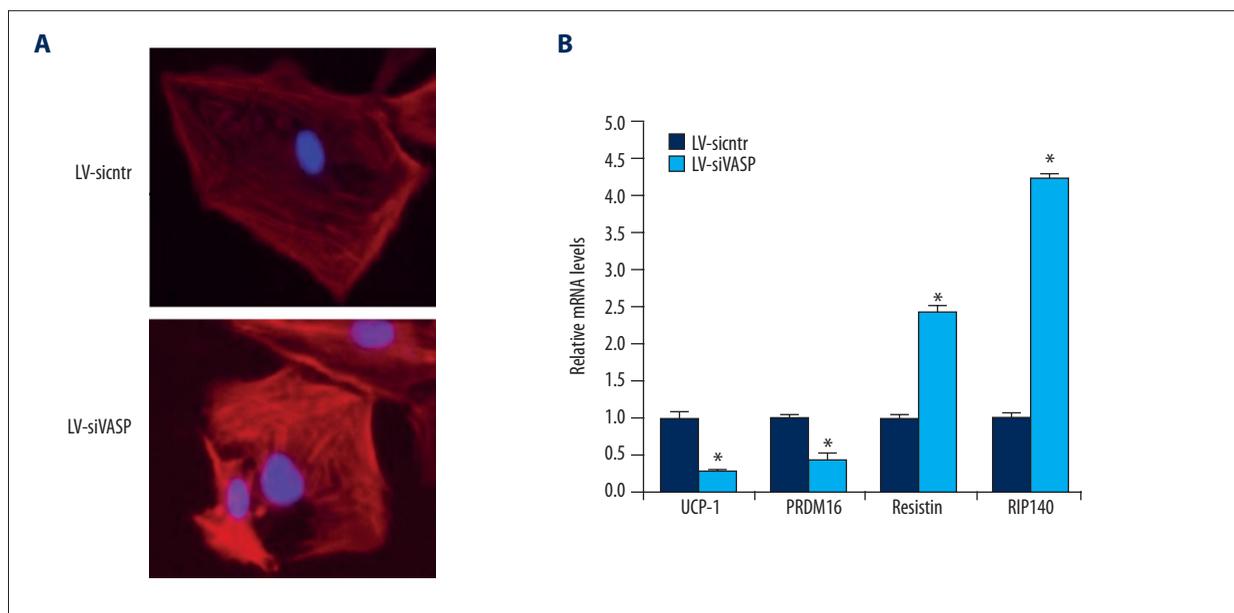


Figure 5. Ablation of VASP promoted brown-to-white phenotype conversion of epicardial adipocytes. **(A)** Actin cytoskeletal staining with or without VASP. **(B)** The abundance of UCP-1 and PRDM16 decreased, but the resistin and RIP140 expressions increased in VASP-deficient adipocytes compared with LV-sicntr group. * $p < 0.05$.

ablation of VASP could not maintain brown phenotype of adipocytes, with a tendency to be easier to convert white adipocytes compared with the LV-sicntr group.

Increased expressions of inflammatory adipocytokines in the absence of VASP

To identify the possible mechanism underlying the upregulation of resistin and RIP140 and downregulation of UCP-1 and PRDM16 in VASP-deficient adipocytes, we examined the expression of several proinflammatory cytokines associated with adipocyte dysfunction. IL-6 and TNF- α transcript expression were higher in VASP-deficient epicardial adipocytes as compared with the LV-sicntr group (Figure 6A, 6B). We also examined IL-8 expression and found high levels of IL-6 in VASP-deficient epicardial adipocytes (Figure 6C). MCP-1 is critically involved in the recruitment of macrophages to adipose tissues [16], and the result showed increased expressions in VASP-deficient epicardial adipocytes compared with the LV-sicntr group (Figure 6D). These results indicate that increased expressions of proinflammatory cytokines in VASP-deficient adipocytes might be responsible for brown-to-white adipocyte trans-differentiation in EAT.

Disruption of RhoA/ROCK activity in VASP-deficient epicardial adipocytes can maintain brown phenotype of adipocytes

Previous studies have shown that RhoA/ROCK inhibits insulin signaling; however, insulin promoted brown adipogenesis and

increases UCP-1 abundance [12]. To investigate the effect of RhoA/ROCK on phenotype conversion in VASP-deficient epicardial adipocytes, we inhibited RhoA/ROCK activity by Y-27632 at a concentration of 30 μ M. As shown in Figure 7, the protein levels of UCP-1 and PRDM16 in Y-27632-treated adipocytes were significantly increased compared to untreated controls in VASP-deficient adipocytes, suggesting that VASP mediated phenotype conversion of EAT associated with RhoA/ROCK activity.

Furthermore, there is increasing evidence that the RhoA signaling pathway plays a critical role in the inflammatory response [17]. In our study, blockade of ROCK activity by Y-27632 inhibited production of proinflammatory cytokines such as IL-6, IL-8, MCP-1, and TNF- α (Figure 6), which is consistent with previous studies. Thus, we demonstrated that disruption of RhoA/ROCK activity upregulates expressions of BAT-specific genes in VASP-deficient epicardial adipocytes involved in reduced inflammatory response.

Discussion

Treatment of coronary atherosclerosis has made little progress in recent years because of its complicated pathogenesis. In our study, we focused on VASP, an actin-binding protein, to provide evidence for the pathogenesis of coronary atherosclerosis and present new clues for the therapy of coronary atherosclerosis.

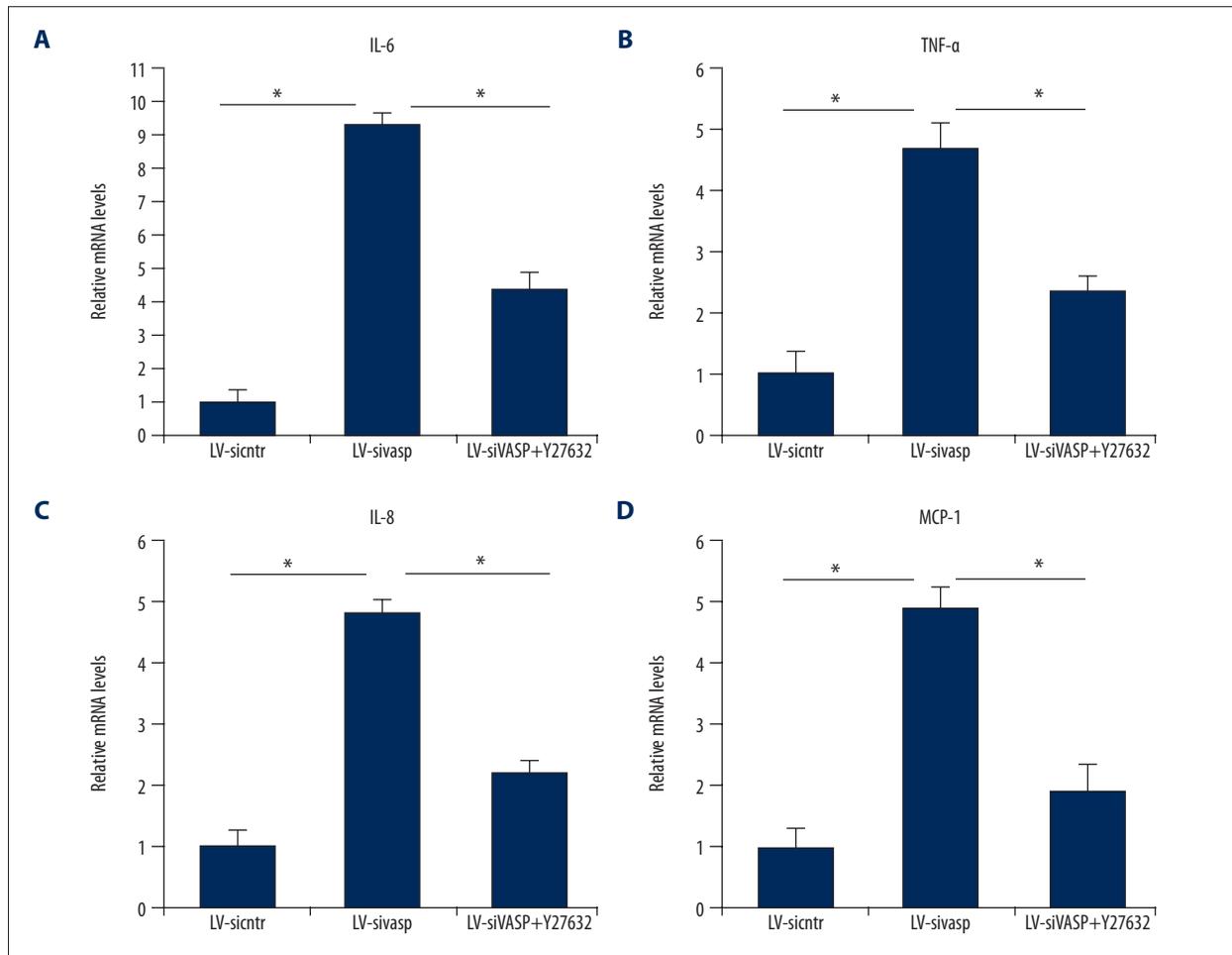


Figure 6. (A–D) Ablation of VASP increased expressions of inflammatory adipocytokines in epicardial adipose tissue. The mRNA expressions of IL-6, IL-8, TNF- α and MCP-1 increased in VASP-deficient adipocytes compared with LV-sicntr group. * $p < 0.05$.

The VASP protein, a member of the Ena/VASP family, consists of 3 functional domains: an N-terminal Ena/VASP homology 1 (EVH1) domain, a central proline-rich domain (PRR), and a C-terminal Ena/VASP homology 2 (EVH2) domain [18]. These functional domains work together to enable VASP to modulate the actin ultrastructure and cellular functions [19] such as shape change, adhesion, migration, and proliferation, while resistin can facilitate angiogenesis by regulating cell migration and proliferation [9]. VASP has been shown to be over-expressed in the neointimal cell layer resulting from surgical endothelial denudation of rat carotid arteries [20]. Therefore, we hypothesized that VASP has crucial role in resistin-induced proliferation and migration in human coronary endothelial cells.

Resistin is an adipokine and was proposed originally to be a link between obesity and type 2 diabetes [21]. Recent experimental studies indicated that resistin aggravates atherosclerosis by inducing vascular inflammation and promoting angiogenesis [22]. It has been reported that lipopolysaccharide, interleukin-1 (IL-1), IL-6, and tumor necrosis factor- α enhance

resistin mRNA expression in human peripheral blood monocytes [23]. Sahmin et al. found that resistin directly binds to adenylyl cyclase-associated protein 1 (CAP1), a functional receptor for human resistin, and upregulate NF- κ B-related transcription of inflammatory cytokines to modulate inflammatory action of monocytes [24]. However, Hong et al. found that resistin induced human endothelial cell proliferation and migration, promoted capillary-like tube formation, and upregulated the expression of vascular endothelial growth factor receptors (VEGFRs) and matrix metalloproteinases (MMPs) [9]. They also investigated potential signaling mechanisms and found that resistin treatment increased phosphorylated extracellular signal regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (p38MAPK, p38) in HCAECs; when specific inhibitors were used to block ERK1/2 and p38, the resistin-induced cell proliferation and migration previously observed were completely blocked [9]. Similarly, treatment of ovary carcinoma cells with resistin induced VEGF production to stimulate endothelial cell tube formation [25]. The effects

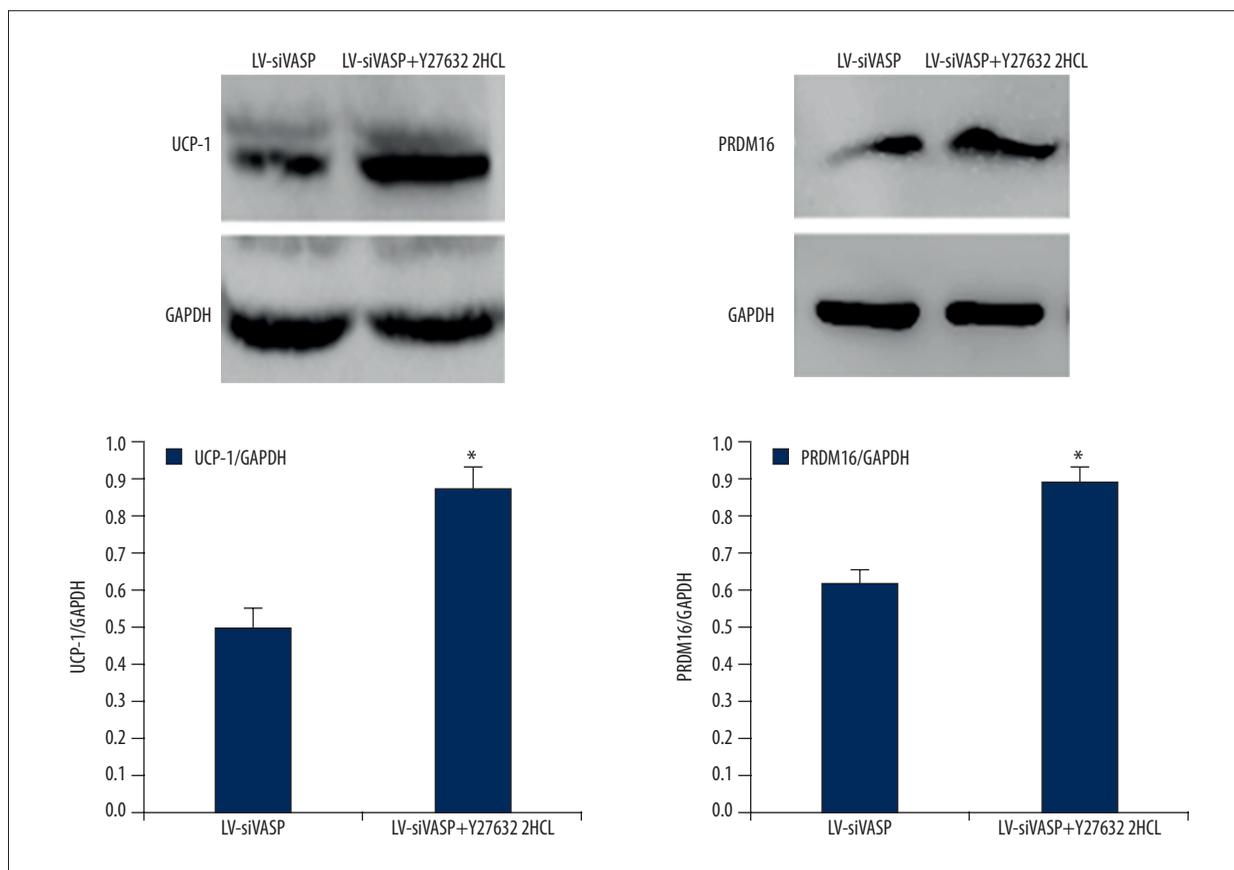


Figure 7. RhoA/ROCK signal was involved in VASP-mediated phenotype conversion. After treatment with RhoA/ROCK specific blocker Y-27632 2HCL in VASP-deficient adipocytes, the expressions of UCP-1 and PRDM16 were significantly increased in protein levels compared with LV-sicntr group. * $p < 0.05$.

of murine resistin and mouse resistin on angiogenesis have been investigated as well [26,27].

To investigate whether VASP is involved in the function of resistin, we observed cell proliferation and migration at various dose of resistin, although previous studies have revealed that resistin induced cell proliferation and migration in endothelial cells and some cancer cells [9,25,28]. In accordance with these results, our data showed that resistin promoted human coronary artery endothelial cells proliferation and migration in a dose-dependent manner. Based on these findings, knockdown of VASP by siRNA in HCAECs was carried out. Then, a fixed dose of resistin (100 ng/ml) was given to the LV-sicntr group and VASP-deficient group. We found that the effect of resistin on endothelial cell proliferation and migration was abolished after ablation of VASP compared with the LV-sicntr group. These data show that resistin induces human endothelial cell proliferation and migration, at least in part, by VASP protein.

Resistin is mainly produced by macrophages and white adipocytes in humans [23,29]. Human epicardial fat was previously referred to as “brown-like fat” and the brown adipose

tissue can change into white adipose tissue with age and obesity [30]. Recently, VASP was proposed to participate in differentiation and VASP knockdown increased inflammation of adipocytes [13,31]. The relationship between VASP and risk factors for atherosclerosis such as hypertension and hyperglycemia have also been investigated [11,32,33]. These findings suggest VASP plays an important role in coronary atherosclerosis. In our study, we took epicardial fat samples from valvular heart disease patients during heart valve replacement to identify whether VASP was associated with phenotype conversion of epicardial adipose tissue. It is well known that the BAT-specific genes include UCP-1, PRDM16, and peroxisome proliferator activated receptor- γ coactivator 1- α (PGC-1 α). The WAT-specific genes include RIP140, resistin, and leptin [6]. In our study, reverse transcription-quantitative polymerase chain reaction (RT-PCR) analysis revealed significantly decreased expression of BAT-specific genes and increased expression of WAT-specific genes in VASP-deficient adipose cell compared with the LV-sicntr group. Interestingly, NO and PKG signaling is required for BAT differentiation and deletion of VASP, which is a major substrate of PKG. Katja et al. found that VASP ablation enhanced BAT function and stimulated the

development of brown-like adipocytes in WAT [13]. In contrast, Handa et al. reported that levels of mRNA encoding proinflammatory cytokines MCP-1, IL-6, and TNF- α were markedly elevated in VASP-/- mice compared with WT controls [31]. More remarkable, recent studies and the present study indicated that inflammation might induce the conversion of EAT from BAT into WAT [6, 14].

Next, we investigated the mechanisms responsible for brown-to-white phenotype conversion in VASP-deficient epicardial adipocytes. A potential reason could be the increased inflammation after ablation of VASP. Epicardial fat is known for its powerful proinflammatory properties associated with adipocyte dysfunction [34]. Secretion of antiinflammatory adiponectin is markedly reduced, whereas that of proinflammatory cytokines IL-6, IL-8, and MCP-1 is markedly increased in perivascular adipocytes surrounding human coronary arteries [35]. NO, produced by nitric oxide synthase (NOS), is involved in obesity-associated adipose tissue inflammation [36]. A growing body of evidence suggests VASP as a downstream mediator of NO signal transduction pathway exerts anti-obesity effects by improving inflammation in various tissues such as the liver, muscle, and adipose tissues [37]. Consistent with these findings, we found that in VASP-deficient epicardial adipocytes, levels of mRNA encoding proinflammatory cytokines MCP-1, IL-6, TNF- α and IL-8 were significantly elevated compared with the LV-sicntr group. However, the result seemed to be contradictory to the fact that blocking VASP could ablate resistin-induced cell proliferation and migration, and resistin served as a pro-inflammation cytokine. Ena/VASP proteins negatively regulates fibroblast migration speed, while within living cells, *Listeria* speeds are reduced by an order of magnitude in the absence of Ena/VASP proteins [38]. Consequently, we suspected that the regulating mechanisms of VASP in different cell type might be different.

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Ena/VASP and Rho GTPases are 2 classes of molecules that regulate the actin cytoskeleton, and a body of evidence suggests that Rho GTPases activity is regulated by Ena/VASP proteins [39,40]. Katja et al. confirmed that in VASP-/- cells, RhoA activity was decreased [13]. To investigate the role of RhoA/ROCK in VASP-deficient adipocytes in phenotype conversion, we disrupted the RhoA/ROCK activities by treating VASP-deficient cells with Y27632. Western blot analysis showed that RhoA/ROCK inhibition in VASP-deficient adipocytes restored reduced crucial markers (UCP-1, PRDM16) of brown fat cells in VASP-deficient adipocytes. After inhibiting RhoA/ROCK activity in VASP-deficient adipocytes for 24 h, increased proinflammatory cytokines such as IL-6, IL-8, TNF- α , and MCP-1 were observed. These findings prove that RhoA/ROCK plays important role in phenotype conversion of EAT in VASP-deficient adipocytes.

Conclusions

Here, we show for the first time that resistin induced HCAECs proliferation and migration, at least in part, by VASP protein. We also showed that VASP regulated phenotype conversion of human epicardial adipocyte associated with inflammation. Therefore, reduced VASP in epicardial adipose tissues might contribute to brown-to-white adipocyte phenotype conversion in the whole body through increased expressions of proinflammatory cytokines like resistin. Furthermore, resistin regulated endothelial cell dysfunction-associated VASP protein. Our study explains the involvement of VASP in the endothelium-driven development of arteriosclerotic diseases and offers new clues for the future therapy of metabolic diseases like obesity-related cardiovascular disease, but the specific mechanism of VASP in other kinds of cells needs further clarification. The modulation of VASP expression could be an opportunity for developing new arteriosclerosis therapies.

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