Biochemical and Biophysical Research Communications 423 (2012) 844–849

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel adipocytokine, vaspin inhibits platelet-derived growth factor-BB-induced migration of vascular smooth muscle cells

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ARTICLE INFO

Article history:
Received 5 June 2012
Available online 17 June 2012

Keywords:
Adipocytokine
Vascular smooth muscle
Migration
Reactive oxygen species
Signal transduction

ABSTRACT

Vaspin is a novel adipocytokine originally identified in visceral white adipose tissues of Otsuka Long-Evans Tokushima fatty rats, an animal model of type 2 diabetes. We have previously shown that vaspin has anti-inflammatory effects in vascular smooth muscle cells (SMCs). SMCs migration is an important process for development atherosclerosis. However, effects of vaspin on SMCs migration remain to be clarified. Rat mesenteric arterial SMCs were treated with platelet-derived growth factor (PDGF)-BB (10 ng/ml, 90 min) in the absence or presence of vaspin (0.01–10 ng/ml, pretreatment for 2 h). SMCs migration was evaluated by a Boyden chamber assay. Western blotting was performed to analyze cellular signals. Reactive oxygen species (ROS) generation was fluorometrically measured using 2′,7′-dichlorofluorescein diacetate. Vaspin significantly inhibited PDGF-BB-induced SMCs migration. Vaspin significantly inhibited PDGF-BB-induced phosphorylation of p38 and heat shock protein (HSP) 27 as well as ROS generation. SMCs migration was blocked by an inhibitor of p38 or an anti-oxidant drug, *N*-acetyl-L-cysteine (NAC). NAC significantly inhibited the PDGF-BB-induced phosphorylation of p38 and HSP27. In addition, vaspin inhibited PDGF-BB-induced actin cytoskeletal reorganization (lamellipodia formation) as revealed by a rhodamine phalloidin staining. The present study for the first time revealed that vaspin inhibits PDGF-BB-induced SMCs migration through inhibiting p38/HSP27 signals via preventing the ROS generation.

1. Introduction

Obesity leads to increased risks of many metabolic diseases, including type 2 diabetes mellitus, dyslipidemia, atherosclerosis and hypertension [1]. It is associated with inflammatory conditions with both the production and the secretions of inflammatory cytokines, termed adipocytokines from activated adipose tissue [2,3].

Vaspin is a serine protease inhibitor family protein that is originally identified in white visceral adipose tissue of obese type 2 diabetic Otsuka Long-Evans Tokushima Fatty rats at the age when obesity and plasma insulin concentrations reach a peak. It improves glucose tolerance and enhances insulin sensitivity in mice [4]. Furthermore, serum vaspin concentrations elevated in obese subjects when compared with lean subjects. Therefore, vaspin represents a novel biomarker for obesity and impaired insulin sensitivity in humans [5].

Vascular smooth muscle cells (SMCs) migration plays a crucial role in the development of atherosclerosis [6]. Platelet-derived growth factor (PDGF) is one of the most important stimulators for migration of SMCs from media into the neointimal lesions [7]. Vascular inflammatory response is another key pathogenic feature of atherosclerosis [8,9]. Recently, we have reported that vaspin plays anti-inflammatory roles in vascular SMCs [10]. Nevertheless, the effects of vaspin on migration of vascular SMCs remain to be clarified. Therefore, we investigated the effects of vaspin on PDGF-induced vascular SMCs migration and explored possible signaling mechanisms in the current study.

2. Materials and methods

2.1. Materials

Reagent sources were as follows: recombinant vaspin and PDGF-BB (PeproTech, Inc., Rocky Hill, NJ, USA); *N*-acetyl-L-cysteine (NAC) (Sigma–Aldrich, St.Louis, MO, USA); SB203580 (Jena Bioscience Gmbh, Germany). Antibody sources were as follows: phospho-p38 (Promega, Madison, WI, USA); total p38 (Santa Cruz Biotech, Santa Cruz, CA, USA); phospho-heat shock protein (HSP) 27 (Enzo Life Science, Plymouth Meeting, PA, USA); α-smooth muscle actin (DAKO, Glostrup, Denmark).

Abbreviations: SMCs, smooth muscle cells; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; HSP, heat shock protein; NAC, *N*-acetyl-L-cysteine; H_2DCFDA, 2′,7′-dichlorofluorescein diacetate.

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http://dx.doi.org/10.1016/j.bbrc.2012.06.052

2.2. Cell culture

Vascular SMCs were isolated from rat mesenteric artery by an explant method and cultured in DMEM supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) [10]. SMCs at 90% confluence were growth arrested by incubating in DMEM containing 0.5% FBS for 24 h before stimulation. Animal care and treatment were conducted in conformity with the institutional guidelines of The Kitasato University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Western blotting

Western blotting was performed as described previously [10]. Protein lysates were obtained by homogenizing SMCs with Triton-based lysis buffer. Protein concentration was determined using the bicinchoninic acid method. Equal amounts of proteins (10–18 μg) were separated by SDS–PAGE 8-12% and transferred to a nitrocellulose membrane. After being blocked with 3% bovine serum albumin (for phosphorylation antibody) or 0.5% skim milk (for total antibody), membranes were incubated with primary antibody (1:500 dilution) at 4 °C overnight, and the membrane-bound proteins were visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 h) and the EZ-ECL system (Biological Industries, Kibbutz beit, Haemek, Isarel). Equal loading of protein was confirmed by measuring total protein or α-smooth muscle actin expression. The results were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

2.4. Measurement of reactive oxygen species (ROS) production

Intracellular ROS production was assessed by using 2′,7′-dichlorofluorescein diacetate (H_2DCFDA, Invitrogen) [10]. After stimulated with PDGF-BB (10 ng/ml, 90 min) in the absence or presence of vaspin (0.01–10 ng/ml, pretreatment for 2 h), SMCs were incubated with H_2DCFDA (10 μM) for 30 min at 37 °C. Fluorescence images were obtained using fluorescence microscope (BX-51, Olympus, Tokyo, Japan) equipped with cooled CCD camera. The fluorescence intensity was measured using Image J software, averaged, and normalized to the control value.

2.5. Boyden chamber assay

Boyden chamber assay was performed in Transwell chambers (Costar, Cambridge, MA, USA) as described previously [11]. The polycarbonate membranes with an 8 μm pores were coated with 2% gelatin for 30 min at 37 °C. SMCs were harvested using trypsin–EDTA and suspended in DMEM containing 0.5% FBS. A total of 600 μl of 0.5% FBS/DMEM was added in the lower chamber. The upper chamber was added with 5×10^4 cells in 100 μl media/well. After vaspin (0.01–10 ng/ml), SB203580 (30 μM) or NAC (10 mM) was pretreated for 2 h, PDGF-BB (10 ng/ml, 90 min) was added to the lower chamber. The membranes were fixed with methanol for 2 min at room temperature and then stained with Giemsa (Nacalai Tesque, Kyoto, Japan). After the membranes were washed several times with water, non-migrated cells were wiped with cotton-swab. The number of migrated cells through the membranes was randomly counted in × 100 fields under light microscope (CKX31, Olympus) and averaged.

2.6. Phalloidin staining

To investigate the reorganization of actin cytoskeleton, SMCs seeded on coverslips were stained with rhodamine phalloidin (Cytoskeleton, Inc., Denver, CO, USA). After SMCs were pretreated with vaspin (10 ng/ml), NAC (10 mM) or SB203580 (30 μM) for 2 h, they were stimulated with PDGF-BB (10 ng/ml, 90 min). After SMCs were fixed with 4% paraformaldehyde at 4 °C for 10 min, they were permeabilized with 0.2% Triton X-100 (Sigma–Aldrich) in TBS for 1 min at room temperature and blocked with 1% bovine

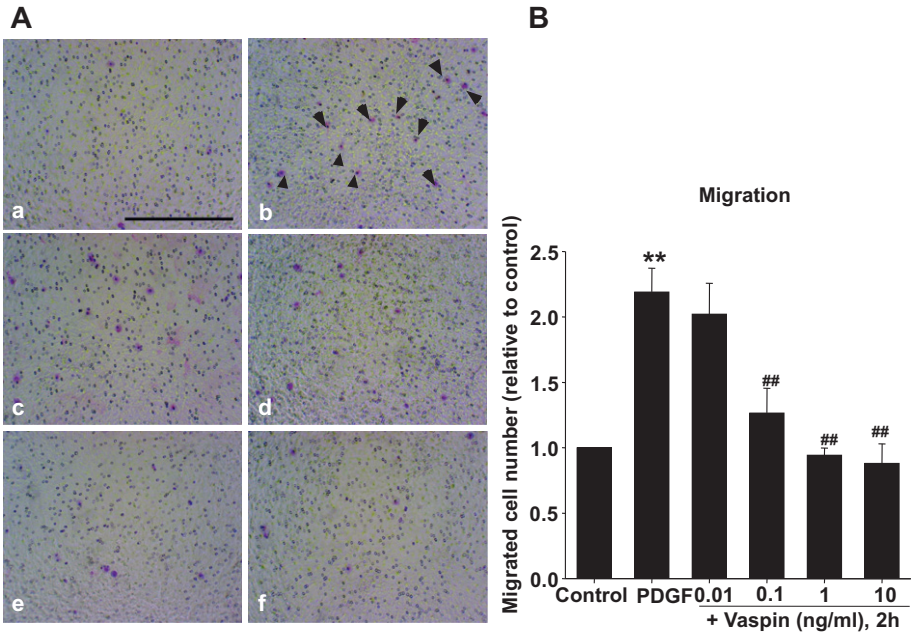

Fig. 1. Effects of vaspin on platelet-derived growth factor (PDGF)-BB-induced smooth muscle cells (SMCs) migration. Migration of SMCs was determined by a Boyden chamber assay. (A) Representative photomicrographs of SMCs pretreated with vaspin (b: 0; c: 0.01; d: 0.1; e: 1; f: 10 ng/ml, 2 h) before PDGF-BB (10 ng/ml, 90 min) stimulation were shown. (a) shows the control non-stimulated cell. After the membrane was fixed with methanol, it was stained with Giemsa. Arrow heads show migrated cells through the membrane. Scale bar: 50 μm. (B) The number of migrated cell is shown as fold increase relative to control ($n = 4$). $^{**}P < 0.01$ vs. Control; $^{\#\#}P < 0.01$ vs. PDGF-BB.

serum albumin. After SMCs were incubated with 1:5000 dilution of rhodamine phalloidin for 30 min at room temperature in dark, fluorescence images were obtained using fluorescence microscope.

2.7. Statistical analysis

Data were shown as mean ± SEM. Statistical evaluations were performed using one-way analysis of variance followed by Tukey's test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of vaspin on PDGF-BB-induced SMCs migration

To clarify the effects of vaspin on SMCs migration, we assessed migration of SMCs using a Boyden chamber assay. As shown in Fig. 1A a-b, SMCs migrated through the polycarbonate membrane after treatment with PDGF-BB (10 ng/ml, 90 min). Vaspin pretreatment (0.01–10 ng/ml, 2 h) before PDGF-BB stimulation significantly attenuated SMCs migration (Fig. 1A and B, PDGF-BB: 2.19 ± 0.18-fold relative to control, $n = 4$ vs. PDGF-BB + vaspin (0.1 ng/ml): 1.27 ± 0.19-fold relative to control, $n = 4$, $P < 0.01$; PDGF-BB + vaspin (1 ng/ml): 0.94 ± 0.05-fold, $n = 4$, $P < 0.01$; PDGF-BB + vaspin (10 ng/ml): 0.88 ± 0.15-fold, $n = 4$, $P < 0.01$).

3.2. Effects of vaspin on PDGF-BB-induced activation of p38 or HSP 27

Activation of p38 plays an important role in migratory response of vascular SMCs [12]. To determine the mechanisms of vaspin to inhibit PDGF-BB-induced SMCs migration, we examined the effects of vaspin on PDGF-BB-induced activation of p38. Vaspin pretreatment (0.01–10 ng/ml, 2 h) significantly inhibited the PDGF-BB (10 ng/ml, 90 min)-induced p38 phosphorylation (Fig. 2A, PDGF-BB: 1.34 ± 0.05-fold relative to control, $n = 7$ vs. PDGF-BB + vaspin (0.1 ng/ml): 0.98 ± 0.08-fold relative to control, $n = 7$, $P < 0.01$; PDGF-BB + vaspin (1 ng/ml): 0.98 ± 0.1-fold, $n = 7$, $P < 0.01$; PDGF-BB + vaspin (10 ng/ml): 0.81 ± 0.07-fold, $n = 7$, $P < 0.01$). To confirm whether PDGF-BB-induced SMCs migration is mediated via activation of p38, we pretreated SMCs with a p38 inhibitor, SB203580. SB203580 (30 μM, 2 h) significantly inhibited the PDGF-BB (10 ng/ml, 90 min)-induced SMCs migration (Fig. 2B, PDGF-BB: 3.05 ± 0.42-fold relative to control, $n = 3$ vs. PDGF-BB + SB203580: 0.94 ± 0.13-fold relative to control, $n = 3$, $P < 0.01$].

We next examined whether vaspin could inhibit PDGF-BB-induced activation of HSP27 that is down-stream p38 [13]. Vaspin pretreatment (0.01–10 ng/ml, 2 h) significantly attenuated the PDGF-BB (10 ng/ml, 90 min)-induced phosphorylation of HSP27 (Fig. 2C, PDGF-BB: 1.36 ± 0.05-fold relative to control, $n = 6$ vs. PDGF-BB + vaspin (0.1 ng/ml): 0.98 ± 0.06-fold relative to control, $n = 6$, $P < 0.05$; PDGF-BB + vaspin (1 ng/ml): 0.98 ± 0.12-fold, $n = 6$,

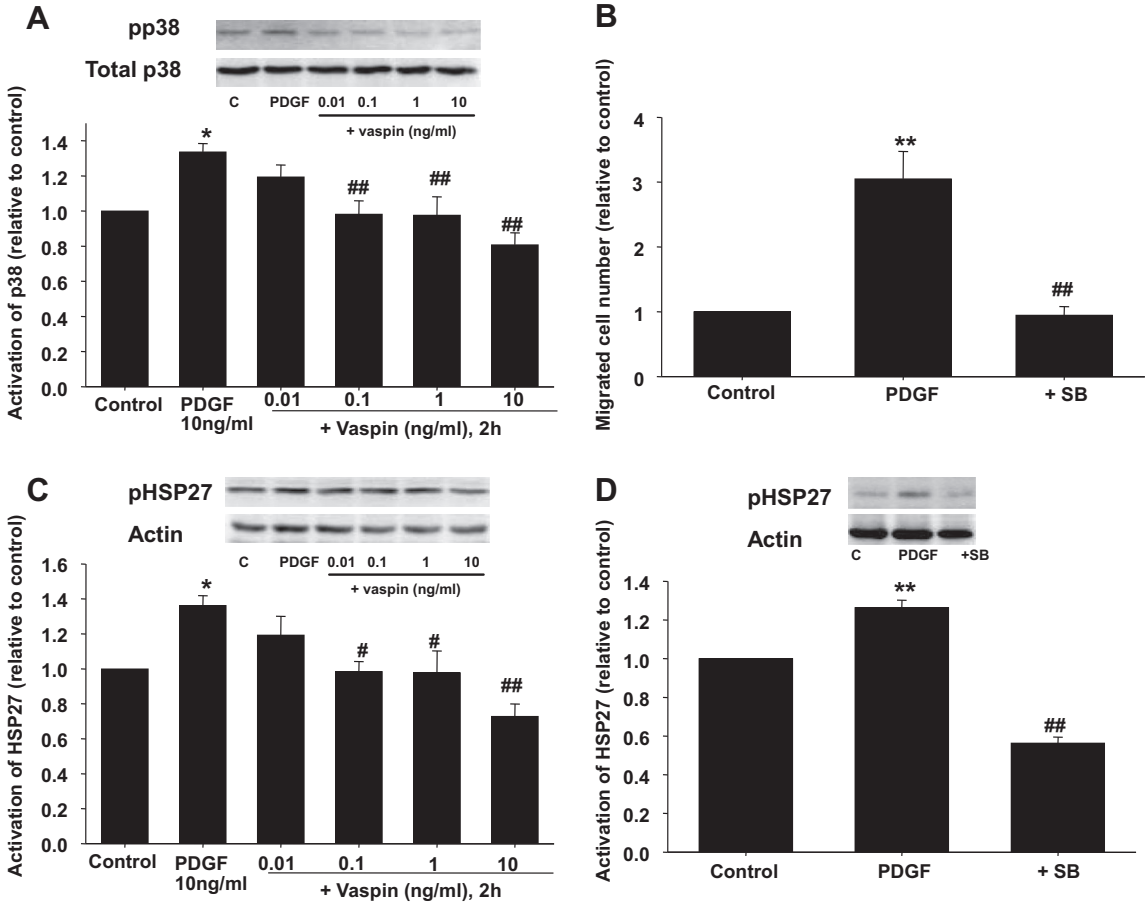

Fig. 2. (A, C) Effects of vaspin on PDGF-BB-induced phosphorylation of p38 and heat shock protein (HSP) 27 in SMCs. After SMCs were stimulated with PDGF-BB (10 ng/ml, 90 min) in the absence or presence of vaspin (0.01–10 ng/ml, pretreatment for 2 h), phosphorylation of p38 (A: $n = 7$) or HSP27 (C: $n = 6$) was determined by a Western blot analysis. (B) Effects of a p38 inhibitor, SB203580 on PDGF-BB-induced SMCs migration. After pretreated with SB203580 (30 μM, 2 h), SMCs were stimulated with PDGF-BB (10 ng/ml, 90 min). Migration of SMCs was determined by a Boyden chamber assay. The number of migrated cell is shown as fold increase relative to control ($n = 3$). (D) Effects of a p38 inhibitor on PDGF-BB-induced phosphorylation of HSP27. After SMCs were treated with PDGF-BB (10 ng/ml, 90 min) in the absence or presence of SB203580 (30 μM, pretreatment for 2 h), phosphorylation of HSP27 ($n = 3$) was determined by a Western blot analysis. Equal protein loading was confirmed using total p38 or α-smooth muscle actin antibody. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. PDGF-BB.

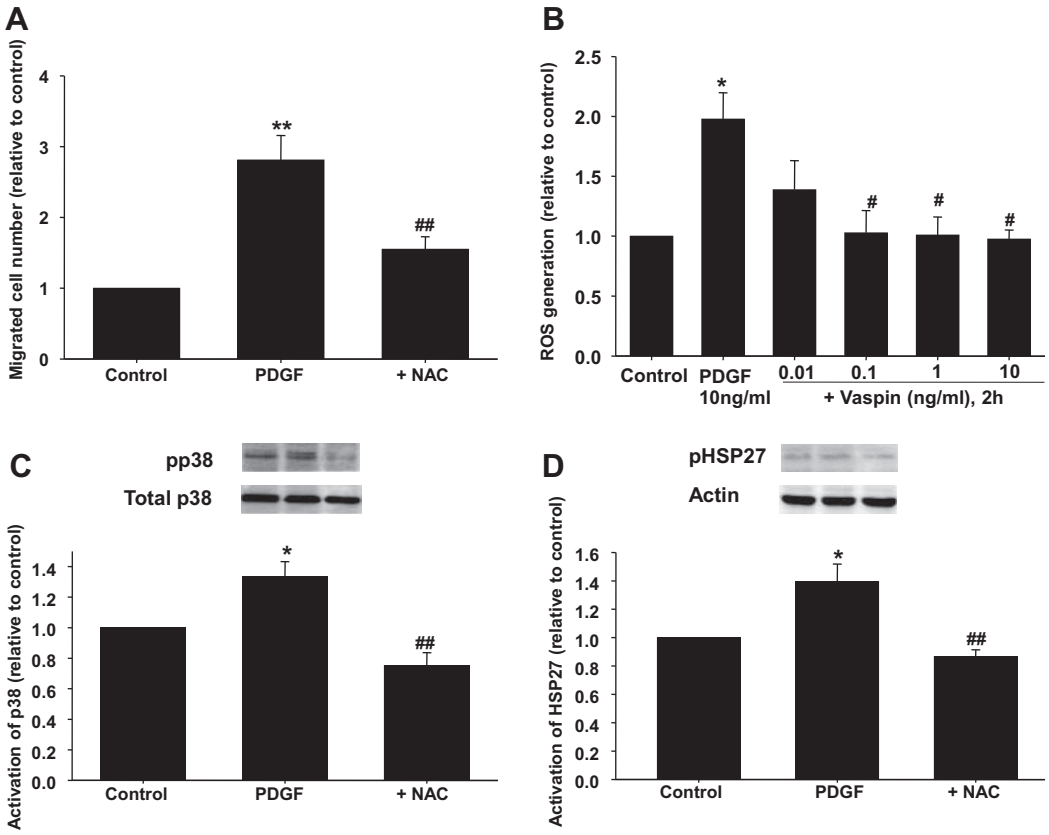

Fig. 3. (A) Effects of anti-oxidant drug, *N*-acetyl-L-cysteine (NAC) on PDGF-BB-induced SMCs migration. After pretreated with NAC (10 mM, 2 h), SMCs were stimulated with PDGF-BB (10 ng/ml, 90 min). Migration of SMCs was determined by a Boyden chamber assay. The number of migrated cell is shown as fold increase relative to control ($n = 5$). (B) Effects of vaspin on PDGF-BB-induced reactive oxygen species (ROS) generation in SMCs. After pretreated with vaspin (0.01–10 ng/ml, 2 h), SMCs were stimulated with PDGF-BB (10 ng/ml, 90 min). ROS production in SMCs was determined by a fluorescence staining using 2′,7′-dichlorofluorescein diacetate. Fluorescent intensity was measured using Image J software and is shown as fold increase relative to control ($n = 3$). (C, D) Effects of NAC on PDGF-BB-induced phosphorylation of p38 (C) and HSP27 (D). After SMCs were treated with PDGF-BB (10 ng/ml, 90 min) in the absence or presence of NAC (10 mM, pretreatment for 2 h), phosphorylation of p38 (C: $n = 3$) or HSP27 (D: $n = 3$) was determined by a Western blot analysis. Equal protein loading was confirmed using total p38 or α-smooth muscle actin antibody. The phosphorylation of p38 or HSP27 is shown as fold increase relative to control. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. PDGF-BB.

$P < 0.05$; PDGF-BB + vaspin (10 ng/ml): 0.73 ± 0.07-fold, $n = 6$, $P < 0.01$). To confirm phosphorylation of HSP27 is mediated via p38 activation, SMCs were pretreated with SB203580 (30 μM, 2 h) before PDGF-BB (10 ng/ml, 90 min) stimulation. SB203580 significantly inhibited the PDGF-BB-induced HSP27 phosphorylation (Fig. 2D, PDGF-BB: 1.26 ± 0.04-fold relative to control, $n = 3$ vs. PDGF-BB + SB203580: 0.56 ± 0.03-fold relative to control, $n = 3$, $P < 0.01$).

3.3. Effects of vaspin on PDGF-BB-induced ROS production

To further investigate the upstream mechanisms of vaspin to inhibit SMCs migration, we used anti-oxidant drug. As shown in Fig. 3A, pretreatment with an antioxidant drug, NAC (10 mM, 2 h) before PDGF-BB stimulation (10 ng/ml, 90 min) significantly reduced the migration of SMCs [PDGF-BB: 2.81 ± 0.35-fold relative to control, $n = 5$ vs. PDGF-BB + NAC: 1.55 ± 0.18-fold relative to control, $n = 5$, $P < 0.01$]. We next examined the effects of vaspin on ROS production by fluorescence staining using H_2DCFDA. Vaspin pretreatment (0.01–10 ng/ml, 2 h) before PDGF-BB stimulation (10 ng/ml, 90 min) significantly attenuated the increased fluorescence intensity by PDGF-BB (Fig. 3B, PDGF-BB: 1.98 ± 0.22-fold relative to control, $n = 3$ vs. PDGF-BB + vaspin (0.1 ng/ml): 1.03 ± 0.18-fold relative to control, $n = 3$, $P < 0.05$; PDGF-BB + vaspin (1 ng/ml): 1.01 ± 0.15-fold, $n = 3$, $P < 0.05$; PDGF-BB + vaspin (10 ng/ml): 0.98 ± 0.07-fold, $n = 3$, $P < 0.05$).

To further verify whether PDGF-BB-induced ROS generation mediates phosphorylation of p38 or HSP27, we pretreated SMCs with NAC (10 mM, 2 h) before PDGF-BB stimulation (10 ng/ml, 90 min). NAC significantly inhibited the PDGF-BB-induced phosphorylation of p38 (Fig. 3C, PDGF-BB: 1.33 ± 0.1-fold relative to control, $n = 3$ vs. PDGF-BB + NAC: 0.75 ± 0.08-fold relative to control, n = 3, $P < 0.01$) or HSP27 (Fig. 3D, PDGF-BB: 1.39 ± 0.12-fold relative to control, $n = 3$ vs. PDGF-BB + NAC: 0.87 ± 0.05-fold relative to control, n = 3, $P < 0.01$).

3.4. Effects of vaspin on PDGF-BB-induced reorganization of actin cytoskeleton

Cytoskeletal reorganization is essential for SMCs migration [14]. We then examined the effects of vaspin on reorganization of actin cytoskeleton in SMCs using a phalloidin staining. Vaspin pretreatment (10 ng/ml, 2 h) reduced lamellipodia formation induced by PDGF-BB (10 ng/ml. 90 min) (Fig. 4A, $n = 4$). To further confirm whether ROS participates in the PDGF-BB-induced reorganization of actin cytoskeleton, we pretreated SMCs with NAC (10 mM, 2 h) before PDGF-BB (10 ng/ml, 90 min) stimulation. As shown in Fig. 4B, NAC inhibited the PDGF-BB-induced lamellipodia formation. In addition, we confirmed that SB203580 (30 μM, 2 h) reduced the PDGF-BB (10 ng/ml, 90 min)-induced lamellipodia formation (Fig. 4C, $n = 3$).

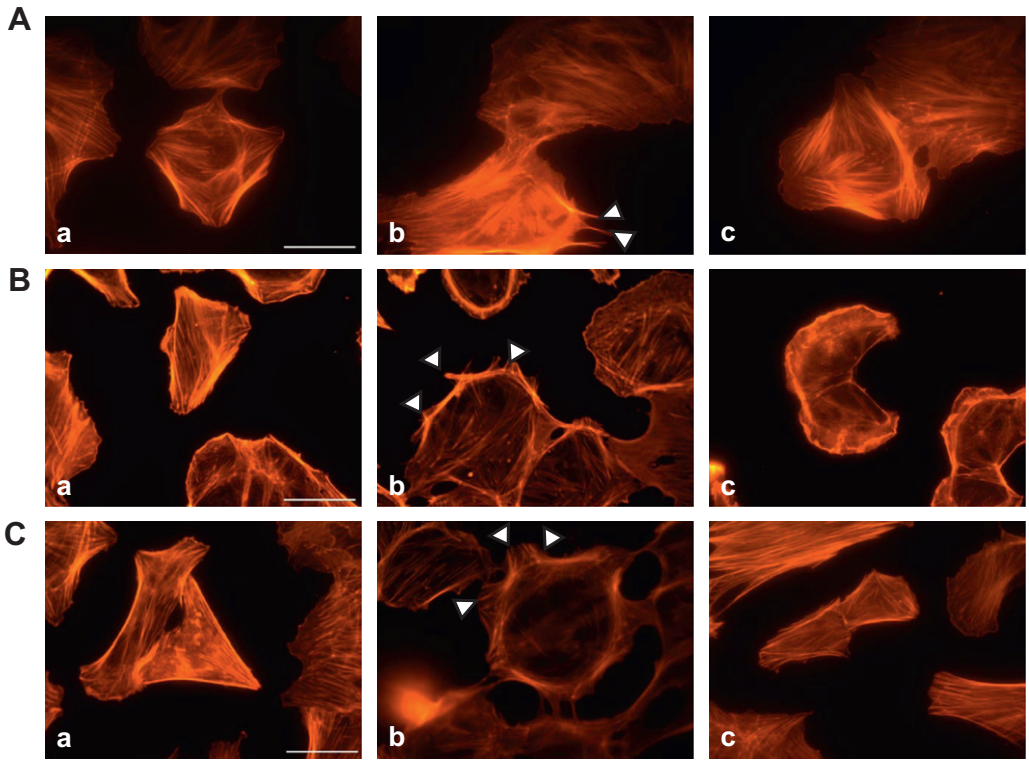

Fig. 4. (A) Effects of vaspin on PDGF-BB-induced cytoskeletal reorganization in SMCs. Actin cytoskeleton was examined with a rhodamine phalloidin staining. Representative photomicrographs of SMCs pretreated with vaspin (b: 0; c: 10 ng/ml, 2 h) before PDGF-BB (10 ng/ml, 90 min) stimulation were shown ($n = 4$). (B, C) Effects of NAC or SB203580 on PDGF-BB-induced cytoskeletal reorganization in SMCs. Representative photomicrographs of SMCs pretreated with NAC (b: 0; c: 10 mM, 2 h) (B: $n = 3$) or SB203580 (b: 0; c: 30 μM, 2 h) (C: $n = 3$) before PDGF-BB (10 ng/ml, 90 min) stimulation were shown. (a) shows the control non-stimulated cell. The lamellipodia was shown by the arrow heads. Scale bar: 50 μm.

4. Discussion

In the present study, we for the first time demonstrate that vaspin inhibits SMCs migration induced by PDGF-BB (Fig. 1). For further elucidating the effects of vaspin on intracellular signaling, the p38 pathways which regulate SMCs migration [13] were examined. We then found that PDGF-BB-induced activation of p38 was significantly suppressed by vaspin (Fig. 2A). To elucidate causal link between p38 activation and SMCs migration, we examined the effects of a p38 inhibitor, SB203580 on the PDGF-BB-induced SMCs migration and showed that SB203580 significantly inhibited SMCs migration (Fig. 2B), suggesting that the PDGF-BB-induced SMCs migration is actually mediated via activation of p38. Since p38 is one of the most critical targets for the prevention of the atherosclerosis in diabetic vasculature [15], the findings seem particularly important. Furthermore, the results from our previous study have shown that vaspin plays inhibitory roles on inflammatory states of vascular SMCs [10], which are involved in the process of atherosclerosis development. From these results, it is suggested that vaspin at least in part may play a protective role against atherosclerosis development.

We next investigated the downstream molecules in p38 signal pathways. Hedges et al. [13] reported that p38 regulates the activation of HSP27 in response to PDGF stimulation. As shown in Fig. 2C, vaspin pretreatment significantly inhibited the phosphorylation of HSP27 induced by PDGF-BB. The present study also provides evidence that p38 actually mediates HSP27 activation in response to PDGF-BB stimulation as revealed by the inhibition of the responses by SB203580 (Fig. 2D). We next evaluated the upstreams of PDGF-BB-induced activation of p38 and HSP27 in SMCs. ROS play important roles to modulate many signaling molecules leading to vascular migration [16]. Recent report suggested that PDGF mediates migration of vascular SMCs via ROS-dependent Syk tyrosine kinase activation, resulting in the phosphorylation of p38/HSP27 [17]. Thus we investigated whether vaspin affects PDGF-BB-induced ROS generation. The present study provided clear evidence that vaspin pretreatment significantly suppressed the PDGF-BB-induce ROS generation in SMCs (Fig. 3B). It has also been shown that antioxidant, NAC inhibited the PDGF-BB-induced SMCs migration (Fig. 3A). Furthermore, NAC blocked the PDGF-BB-induced phosphorylation of p38 (Fig. 3C) and HSP27 (Fig. 3D). These findings collectively indicate that vaspin inhibits PDGF-BB-induced SMCs migration via suppressing the ROS-dependent p38/HSP27 activation.

We further examined actin cytoskeletal reorganization (lamellipodia formation) using phalloidin staining. Matsumoto et al. [18] demonstrated that p38 activation modulated PDGF-induced cell motility and mediated actin reorganization in fibroblast cells. We found that vaspin pretreatment inhibited PDGF-BB-induced lamellipodia formation (Fig. 4A). Additionally, PDGF-BB-induced lamellipodia formation was blocked by SB203580 (Fig. 4C). Accordingly, previous report has shown that p38/HSP27 cascade regulates the actin cytoskeletal reorganization induced by PDGF [14]. Thus it is suggested that vaspin inhibits PDGF-BB-induced lamellipodia formation through preventing p38 activation. To further investigate the role of ROS on PDGF-BB-induced actin cytoskeletal reorganization, we used NAC. NAC significantly blocked the PDGF-BB-induced lamellipodia formation in SMCs (Fig. 4B). Several studies reported that ROS are mediators of PDGF-induced vascular SMCs migration and that activation of p38 mediates actin filament reorganization in response to oxidative stress [19,20]. Collectively, our present data for the first time revealed that vaspin inhibits PDGF-induced ROS and subsequent activation of p38/HSP27 pathways leading to actin cytoskeletal reorganization.

The signaling mechanisms of vaspin on vascular SMCs migration found in this study are similar to those of adiponectin, which exerts inhibitory effects on PDGF-BB-induced migration of vascular SMCs [21]. Moreover, Ishizawa et al. [22] have shown that adiponectin pretreatment significantly suppressed PDGF-induced rat mesangial cells migration via suppression of the big mitogen-activated protein kinase 1 and p38 pathways. On the other hand, the signaling mechanisms of another adipocytokine, resistin on migration were not similar to those of vaspin. Jung et al. [23] have reported that resistin enhanced vascular SMCs migration. Moreover, resistin induced vascular endothelial growth factor-induced human endothelial cell migration via activating p38 and ERK1/2 pathways [24].

In conclusion, the present study for the first time revealed that vaspin inhibits PDGF-BB-induced SMCs migration through inhibiting p38/HSP27 signals via preventing the ROS generation. Further investigations to elucidate the in vivo roles of vaspin on the development of atherosclerosis are warranted in order to contribute to develop the pharmaceutical therapeutics.

Acknowledgment

This study was supported in part by Grants for Scientific Research from the Kitasato University, School of Veterinary Medicine.

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