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RESEARCH PAPER

Endocannabinoid-like N-arachidonoyl serine is a novel pro-angiogenic mediator

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BACKGROUND AND PURPOSE

N-arachidonoyl serine (ARA-S) is a recently identified endocannabinoid-like lipid with weak affinity for the fully characterized cannabinoid receptors (CB_1 and CB_2) and the transient receptor potential vanilloid receptor 1 (TRPV-1). ARA-S induces vasodilatation and shows vasoprotective potential via activation of key signalling pathways in endothelial cells. Based on these findings, the effect of ARA-S on endothelial functions was further studied.

EXPERIMENTAL APPROACH

Primary human dermal microvascular endothelial cells (HMVEC) were used to investigate effects of ARA-S (0-10 μM) on certain endothelial functions, using cell proliferation, migration and wound repair models *in vitro*, and angiogenesis assays *in vitro* and *ex vivo*. Selective CB receptor antagonists and specific siRNAs were deployed to block individual CB receptors.

KEY RESULTS

We found that ARA-S stimulated angiogenesis and endothelial wound healing through induction of vascular endothelial growth factor C and its cognate receptor expression in primary HMVEC. Moreover, knock-down of G protein-coupled receptor 55 (GPR55) partly inhibited ARA-S-induced signal transduction and endothelial functions.

CONCLUSIONS AND IMPLICATIONS

Our results indicate that ARA-S is a pro-angiogenic factor in addition to a vessel dilator. The GPR55 receptor may serve as one target of ARA-S.

Abbreviations

2-AG, 2-arachidonoyl glycerol; AEA, anandamide; ARA-S, N-arachidonoyl serine; CAM, chick choriollantoic membrane; CB receptor, cannabinoid receptor; GPR55, G protein-coupled receptor 55; HMVEC, human dermal microvascular endothelial cells; TRPV-1, transient receptor potential vanilloid receptor 1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

Introduction

The endocannabinoid system is a ubiquitous lipid signalling network that regulates physiological functions throughout the body (Guzmán, 2003; Klein, 2005; Berghuis *et al.*, 2007; Murray *et al.*, 2007). The first endocannabinoid, anandamide (AEA), was found in 1992 (Devane *et al.*, 1992). To date, AEA and 2-arachidonoyl glycerol (2-AG) have been best characterized (Stella *et al.*, 1997; Maccarrone *et al.*, 2008). Several other endocannabinoid-like compounds including N-arachidonoyl serine (ARA-S) have also been reported but their functions are less well understood (Hanus *et al.*, 2001; Porter *et al.*, 2002; Milman *et al.*, 2006).

Endocannabinoids are synthesized from membrane phospholipids. Neurons and glial cells (Marsicano *et al.*, 2003; Stella, 2004), circulatory

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activated macrophages (Di Marzo *et al.*, 1999), platelets (Randall, 2007) and endothelial cells (Opitz *et al.*, 2007) are key sources of endocannabinoids. They are produced 'on demand' in response to a variety of patho-physiological stress, including ischaemia and wound damage (Pacher *et al.*, 2006, 2008; Bellocchio *et al.*, 2007). Once endocannabinoids are released into the extracellular milieu, they trigger G protein-coupled receptor (GPR)-mediated signal transduction and can modulate several biological processes, including pain (Agarwal *et al.*, 2007), appetite (Di Marzo and Matias, 2005), fertility (Wang *et al.*, 2003) and blood pressure (Pacher *et al.*, 2005).

Cannabinoid (CB) modulation of vascular tone appears to contribute to the pathophysiology of cardiovascular disorders (Bátkai *et al.*, 2004). Vasodilator effects of cannabinoids persist even in the absence of CB_1 and CB_2 receptors; therefore, other CB binding receptors are believed to exist on the endothelium (Járai *et al.*, 1999; receptor nomenclature follows Alexander *et al.*, 2009). One such receptor is the transient receptor potential vanilloid receptor 1 (TRPV-1), expressed on endothelium and capable of regulating vascular tone (Zygmunt *et al.*, 1999). Moreover, an endothelial CB receptor antagonized by O-1918 has been proposed (Mo *et al.*, 2004). Recently, the GPR55 receptor also has been considered as a novel CB receptor involved in this process. GPR55 shares low sequence identity with the CB_1 and CB_2 receptors (Nilsson *et al.*, 2007). It has been found in certain regions of the CNS and in other tissues including endothelium (Sawzdargo *et al.*, 1999; Baker *et al.*, 2006; Johns *et al.*, 2007; Ryberg *et al.*, 2007). The bioactive lipid L-α-lysophosphatidylinositol (LPI) and related lipids are likely to be endogenous ligands for GPR55 (Ryberg *et al.*, 2007; Henstridge *et al.*, 2009; Oka *et al.*, 2009).

In addition to modulation of vascular tone, cannabinoids also regulate vascular homeostasis. While cardioprotective properties of cannabinoids have been observed in some studies (Joyeux *et al.*, 2002; Lépicier *et al.*, 2006; Durst *et al.*, 2007), pro-apoptotic and anti-angiogenic activities of cannabinoids also have been described. AEA mainly behaves as an inhibitor of angiogenesis (Portella *et al.*, 2003; Pisanti *et al.*, 2007). 2-AG interacts with endothelin-1 and may play a role in microvascular function (Chen *et al.*, 2000).

N-arachidonoyl serine, a recently identified endocannabinoid-like lipid initially isolated from bovine brain, shows endothelial-specific characteristics but binds only weakly to CB_1, CB_2 and TRPV-1 receptors. However, ARA-S induces activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) and the phosphoinositide 3-kinase substrate, Akt in endothelial cells. Furthermore, ARA-S protects endothelial cells through suppression of reactive oxygen intermediates and inhibition of lipopolysaccharide (LPS)-induced tumour necrosis factor α (TNF-α) (Milman *et al.*, 2006). Recent studies indicate that ARA-S modulation of N-type Ca^{2+} channels and large conductance Ca^{2+}-activated K^+ channels is critical for its biological activities (Guo *et al.*, 2008; Godlewski *et al.*, 2009). These biological activities of ARA-S on endothelium prompted us to investigate its effects on vascular homeostasis. We found that ARA-S induced angiogenesis and endothelial wound healing, mediated in part through the GPR55 receptor pathway. Our results indicate that ARA-S may contribute to the maintenance of vascular integrity and angiogenesis.

Methods

Cell proliferation and migration assay

Primary human dermal microvascular endothelial cells (HMVEC; Adult) were purchased from Lonza Inc. and maintained in EBM-2 medium with EGM-2MV SingleQuots. Cell proliferation assay was assessed by the MTT proliferation assay kit (Roche Applied Science) and the CellTiter 96® AQueous One Solution cell proliferation assay kit (Promega Corporation). The proliferation index was defined by dividing the OD value after 48 h incubation in cultures with ARA-S by the OD value of cultures with vehicle control. Migration assays were performed over 2 h in triplicate using 24-well transwell plates (Corning Life Sciences) as described previously (Wang *et al.*, 2001). The migration index was calculated by dividing the number of cells migrated in response to stimulus by the number of spontaneously migrated cells.

In vitro angiogenesis assay

Tube formation assays were performed using the *in vitro* angiogenesis assay kit (Chemicon International Inc.). Digital images were captured to measure the total tube length in three random fields per well at low-power (×10) magnification using the IPLab software (Scanalytics, Inc.). The relative tube formation was calculated and shown.

Wound repair model

Endothelial wound models were prepared in 6-well plates as described previously (Albuquerque *et al.*, 2000; Wright *et al.*, 2005). The plates were washed three times with serum-free medium and incubated with stimuli in the EBM-2 medium containing 1%

BSA. Images were recorded just after wounding and 24 h later. The wound width was measured in ten sites from the top of wound to the bottom of the wound using the IPLab software. The percentage reduction in wound width was calculated using the following formula: [Average wound width (0 h) – Average wound width (24 h)]/Average wound width (0 h) × 100%.

The chick choriollantoic membrane (CAM) assay

Fertilized chicken embryos (Charles River Laboratories, Inc.) were cultured in a specialized incubator (Lyon Electric Company, Inc.) on day 1, cracked on day 3 into tissue culture Petri dishes, and incubated for three to four more days in 3% CO2 incubator. The choriollantoic membranes were fully developed on day 7. Sterile Whatman filter discs were prepared by cutting circular Whatman filter papers (diameter, 5 mm) into quarter circles, and impregnated with ARA-S, vascular endothelial growth factor (VEGF) or vehicle controls, were then placed on avascular areas of the CAMs. Neovascularization around the discs was photographed on day 10 using a dissecting microscope with a digital imaging system (West *et al.*, 2001). The total area of the vessels around each disc was normalized to the total area measured and measured by the IPLab software. The relative vascular area is shown.

Western blotting

Equal amounts of total proteins were resolved by SDS-PAGE and subjected to Western blot analysis using the enhanced chemiluminescence system (Amersham Pharmacia Biotech) (Zhang *et al.*, 2007). Anti-phospho-Akt and anti-GPR55 antibodies were purchased from Cell Signaling Technology and Abcam respectively. All other antibodies were purchased from Santa Cruz Biotechnology, Inc. For quantitative analysis of protein expression and phosphorylation, the ratio of expression or phosphorylation versus total protein in each lane was obtained by densitometry (The gel imaging system from Alpha Innotech Corp.). The phosphorylation index and expression index were determined by calculating the value of this ratio in each lane and presenting the ratio as the fold increase over the control value (control sample), which was designated as 1.

Cytokine analysis

The supernatant was collected and assayed for VEGF, VEGF-C and VEGF-D production using ELISA kits from R & D Systems and Immuno-Biological Laboratories, Inc.

The siRNAs

A specific GPR55 siRNA and a negative control siRNA were purchased from Qiagen, Inc. The target sequence of GPR55 siRNA was TGGGTCTACCAATGTGCTTAA. Transfection was performed using HiPerFect transfection reagent according to Qiagen's manual.

Statistical analysis

The results are expressed as the mean ± SD of data obtained from three experiments performed in triplicate. Statistical significance was determined by one-way analysis of variance (ANOVA) comparing the effects of ARA-S versus vehicle at $P < 0.05$.

Materials

N-arachidonoyl serine was purchased from Cayman Chemical Co. O-1918 [(-)-4-(3-3, 4-*trans*-p-menthadien-(1, 8)-yl)-orcinol] was synthesized as described and dissolved in ethanol (Offertáler *et al.*, 2003). Recombinant human VEGF was purchased from R & D Systems. *Pertussis* toxin (PTX) and LPI were purchased from Sigma Co. The selective antagonist for CB_1 receptors, AM251, was purchased from the Cayman Chemical Company. The TRPV-1 antagonist capsazepine was purchased from the Tocris Bioscience.

Results

ARA-S enhances endothelial wound healing

Previous studies showed that ARA-S induced significant phosphorylation of ERK1/2 and Akt in endothelial cells (Milman *et al.*, 2006). These changes suggested that ARA-S may modulate endothelial proliferation and migration. To this end, we examined its activity in HMVEC using a MTT assay and a transwell migration assay. We observed that ARA-S stimulated endothelial proliferation in a concentration-dependent manner, compared with vehicle controls (Figure 1A). We also found that ARA-S induced a concentration-dependent increase in endothelial cell migration (Figure 1B). The maximum effects were observed with 1 μM of ARA-S in both assays. Furthermore, we found that these effects were not blocked by either the CB_1 receptor antagonist AM251 or the TRPV-1 receptor antagonist capsazepine (data not shown).

We then sought to determine whether ARA-S can enhance endothelial wound repair. In order to mimic vascular wall damage, we created an artificial wound model *in vitro*. We photographed the wounds at 0 h and at 24 h after incubation with ARA-S or vehicle control, and then measured the

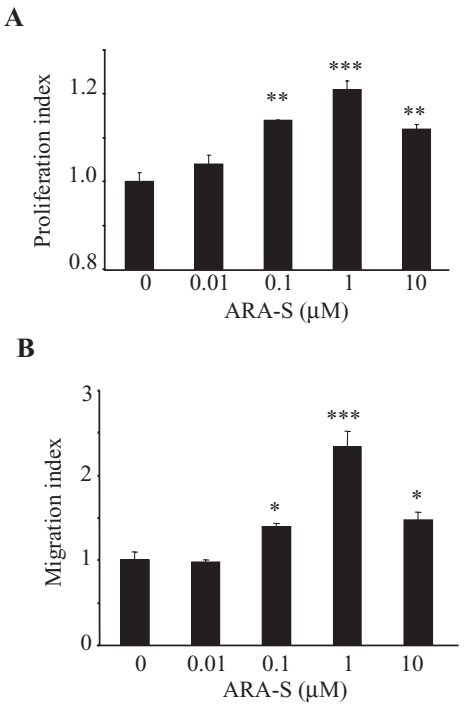

Figure 1

N-arachidonoyl serine (ARA-S) induces endothelial proliferation and migration. (A) ARA-S induced proliferation of HMVEC in a concentration-dependent manner. Proliferation indices show a significant effect of ARA-S. (B) ARA-S induced endothelial migration. Migrated cells were stained and counted. Data indicate the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for the treatment with ARA-S versus vehicle control. HMVEC, human dermal microvascular endothelial cells.

wound width using the IPLab imaging system. Morphological changes were apparent in cells treated with ARA-S as compared with the vehicle controls. Photographs shown are representative of three separate experiments (Figure 2A). The wound width was originally quantitated by the number of pixels. The percentage reduction was then calculated based on the number of pixels, shown in Figure 2B. VEGF served as a positive control. We found that ARA-S significantly reduced wound width as compared with the vehicle control. Again, the effect of ARA-S was concentration-dependent and peaked at 1 μM.

ARA-S enhances angiogenesis

In order to confirm that ARA-S can function as a pro-angiogenic lipid, we tested its activity on vascular tube formation *in vitro*. HMVEC were incubated with different concentrations of ARA-S (0, 0.1, 1, 10 μM) or vehicle controls during *in vitro* angiogenesis assays. We found that ARA-S enhanced tube formation in a concentration-dependent manner, as compared with the vehicle treatments (Figure 3A and B).

Next, we measured ARA-S activity on angiogenesis using a CAM assay, a standard method to detect the *ex vivo* angiogenic property of uncharacterized agents (West *et al.*, 2001). As we observed significant effects with 1 μM of ARA-S in the above experiments, we treated 7 day old choriollantoic membranes with discs soaked with 10 μL of ARA-S (1 μM) or vehicle control or 10 μL of VEGF (100 ng·mL^{-1}). On day 10, the level of vascularization was quantitated by calculating the total area of vascular structure using the IPLab imaging system, and presented as relative vascular area. A significant increase in vascularization was seen in the presence of ARA-S versus vehicle controls (Figure 4A and B).

ARA-S induces VEGF-C and its receptor expression in endothelial cells

In order to elucidate how ARA-S may affect key angiogenic pathways, we measured the production of VEGF, VEGF-C and VEGF-D in the cell culture supernatants after incubation with different concentrations of ARA-S or vehicle controls for 18 h. There was no significant change in VEGF or VEGF-D production (data not shown), but VEGF-C production increased after stimulation with ARA-S as compared with the treatment with vehicle control (Figure 5A). Lower concentrations of ARA-S (0.01–1 μM) showed greater effects on VEGF-C production. VEGF-C can bind to vascular endothelial growth factor receptor (VEGFR)-2 and 3, and regulate both angiogenesis and lymphangiogenesis (Adams and Alitalo, 2007).

We then assessed levels of expression of VEGFR-2 and 3 following ARA-S stimulation. A concentration-dependent increase in expression of both receptors was seen in Western blotting after overnight incubation with ARA-S (Figure 5B). As compared with the effects of ARA-S on VEGF-C production, higher concentrations of ARA-S (>1 μM) exhibited greater effects on expression of VEGF-C receptors.

Our results indicated that ARA-S may exert effects on endothelial growth and migration via modulation of VEGF-C and its cognate receptors.

ARA-S may target GPR55 on endothelial cells

To further investigate the targets of ARA-S on endothelial cells, we examined whether the GPR55 receptor was involved. GPR55 expression was knocked down using a specific GPR55 siRNA (Figure 6A). We then compared ARA-S-induced migration and *in vitro* tube formation in endothelial cells with normal versus reduced levels of GPR55. We found that the ARA-S-induced migration was significantly

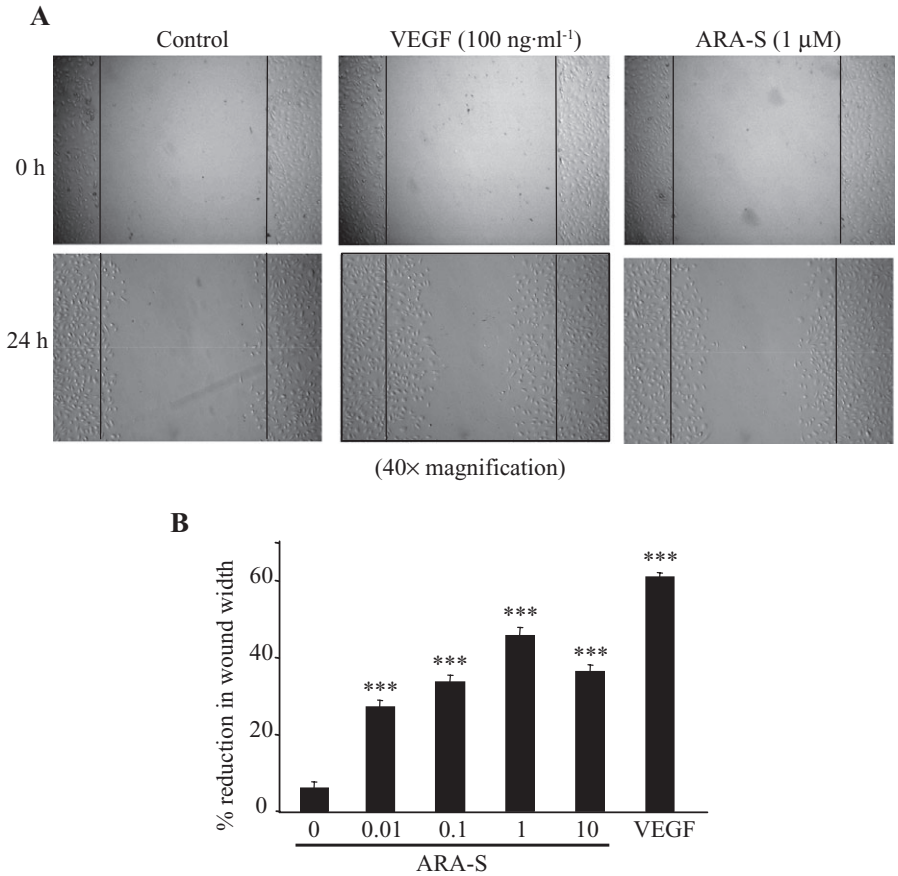

Figure 2

N-arachidonoyl serine (ARA-S) enhances endothelial wound healing. (A) ARA-S induced endothelial wound repair. Representative images at 0 h and 24 h are shown as indicated. The wounded cultures were recorded just after wounding and at 24 h after continued culturing in the presence or absence of ARA-S (0, 0.01, 0.1, 1, 10 μM), or VEGF (100 ng·mL^{-1}). (B) Quantitative data are shown as percent reduction in wound width. Data indicate the mean ± SD. ***P < 0.001 for the treatment with ARA-S versus vehicle control.

inhibited in the GPR55 siRNA-transfected cells as compared with that in the control siRNA-transfected cells (Figure 6B). Moreover, the ARA-S-induced tube formation also significantly decreased in the GPR55 siRNA-transfected cells, as compared with that in the control siRNA-transfected cells (Figure 6C). It should be noted that the control values, especially in the tube formation assay, were reduced by the GPR55 siRNA, suggesting that GPR55 is either constitutively active or maybe activated by unknown endogenous ligands.

To further explore how knock-down of GPR55 may cause these effects, we studied ARA-S-induced activation of two key downstream signalling molecules, Akt and ERK1/2, in cells with normal or reduced levels of GPR55. We found that activation of Akt and ERK1/2 upon stimulation with ARA-S was significantly inhibited in the GPR55 siRNA-transfected cells as compared with that in the control siRNA-transfected cells (Figure 7A). Moreover, pretreatment with PTX inhibited the ARA-S-induced Akt activation (Figure 7B). We also measured VEGF-C production in culture supernatant after 18 h incubation with ARA-S in these cells. The ARA-S-induced VEGF-C production was significantly decreased in the GPR55-knock-down cells as compared with that in the normal GPR55 cells (Figure 7C).

Based on above results, the GPR55 signalling pathway seems play a role in endothelial functions. To support this conclusion, we further studied whether the GPR55 agonist, LPI, may have similar effects like ARA-S on endothelial functions. We found that low concentrations of LPI moderately stimulated endothelial proliferation (Figure 8). These results strongly imply the participation of GPR55 in endothelial functions.

The atypical CB O-1918 inhibits ARA-S-induced tube formation

The atypical CB O-1918 was found to block the putative endothelial CB receptor in previous studies

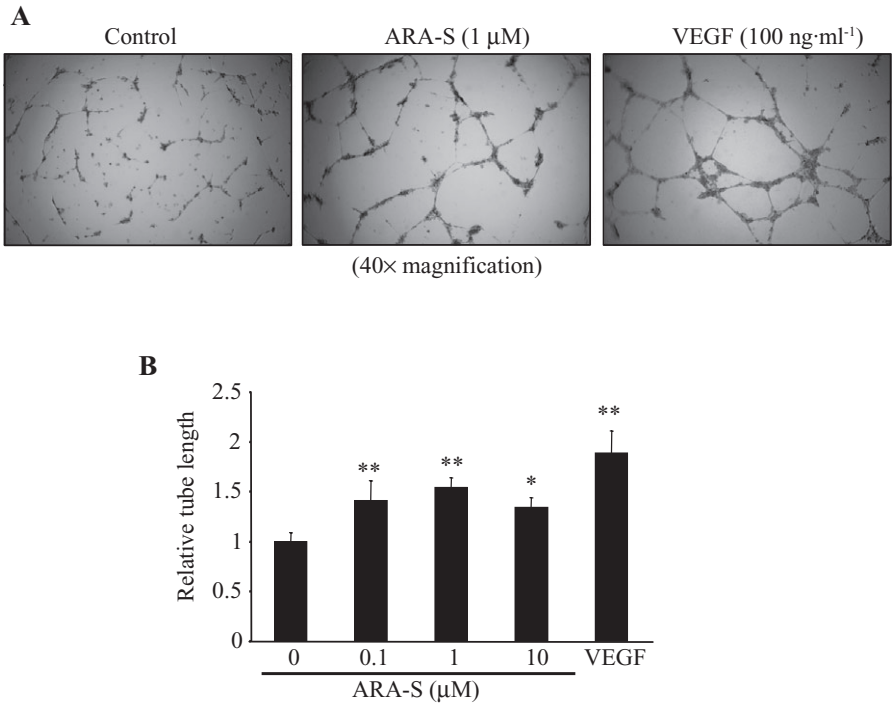

Figure 3

N-arachidonoyl serine (ARA-S) induces *in vitro* angiogenesis. A Matrigel assay was used to measure ARA-S activity as described. (A) Representative images and average length of tube per view field are shown. (B) The tube length was measured and the relative tube length is presented. Data indicate the mean ± SD. *$P < 0.05$, **$P < 0.01$, for the treatment with ARA-S or VEGF versus vehicle control. VEGF, vascular endothelial growth factor.

(Begg *et al.*, 2003; Offertáler *et al.*, 2003). To address whether O-1918 modulates ARA-S-induced functions, we pretreated endothelial cells with O-1918 or vehicle control before ARA-S stimulation. ARA-S alone induced significant effects on tube formation ($P < 0.05$), and pretreatment with O-1918 (2 μM for 30 min) significantly inhibited the effects of ARA-S (Figure 9), suggesting that the receptor binding O-1918 was critical for ARA-S-induced tube formation. Moreover, we performed experiments to test whether O-1918 displays similar effects in cells after knock-down of the GPR55 receptor. We found that O-1918 had no further inhibitory activity after knock-down of the GPR55 receptor (data not shown).

Discussion

The vascular endothelium serves as a major target of endocannabinoids, modulating vasodilatation. Less is known about how such endocannabinoids may affect vasculogenesis and angiogenesis. Classical endocannabinoids like AEA and 2-AG predominantly target the CNS (the CB_1 receptor) and peripheral immune system (the CB_2 receptor), respectively, and exert biological effects on vascular endothelium (Chen *et al.*, 2000; Portella *et al.*, 2003; Pisanti *et al.*, 2007). As compared with these endocannabinoids, ARA-S shows a unique biological profile (Milman *et al.*, 2006; Guo *et al.*, 2008; McHugh *et al.*, 2008; Godlewski *et al.*, 2009). Our findings expand on the properties of ARA-S, and strongly support that ARA-S can modulate angiogenesis and endothelial wound repair. Furthermore, the GPR55 receptor appears to be involved in these effects, as knock-down of GPR55 significantly inhibited angiogenesis.

Endothelial-specific cannabinoids and their targets have been pharmacologically predicted for decades. Considerable evidence exists that surface receptors other than CB_1 and CB_2 receptors are expressed on endothelial cells (Begg *et al.*, 2005; Brown, 2007). The GPR55 receptor is one such receptor. GPR55 ligands activate the small GTPase family, induce phosphorylation of ERKs and increase intracellular Ca^{2+} levels (Oka *et al.*, 2007; Lauckner *et al.*, 2008; Henstridge *et al.*, 2009). Although several exogenous cannabinoids and endocannabinoids have been reported to interact with GPR55 (Waldeck-Weiermair *et al.*, 2008; Ross, 2009), LPI and related lipids are most likely to be the

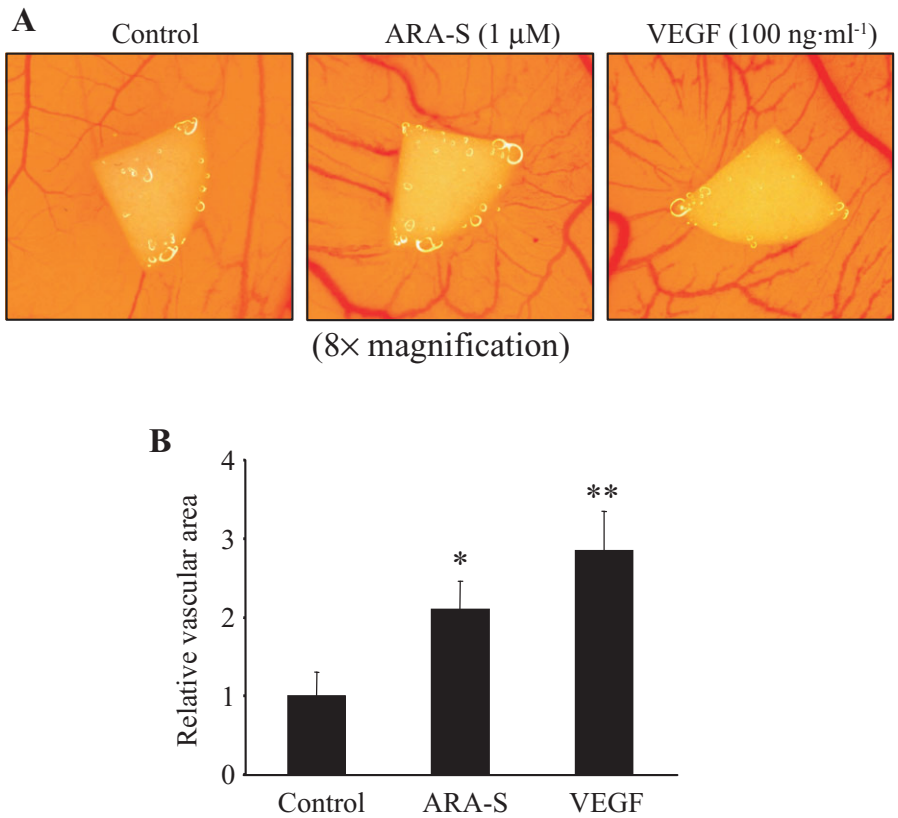

Figure 4

N-arachidonoyl serine (ARA-S) induces *in vivo* angiogenesis. (A) A typical CAM assay was employed to test effect of ARA-S on angiogenesis *in vivo*. VEGF served as a positive control. Representative images are shown. (B) Relative vascular areas are quantitated and shown. Data indicate the mean ± SD. **P* < 0.05, ***P* < 0.01 for the treatment with ARA-S or VEGF versus vehicle control. CAM, chick choriollantoic membrane; VEGF, vascular endothelial growth factor.

cognate ligands but their activity on endothelial functions is not well characterized (Ryberg *et al.*, 2007; Henstridge *et al.*, 2009; Oka *et al.*, 2009). We observed the effects of GPR55 knock-down on basal activities of endothelial cells, also suggesting existence of endogenous agonists of GPR55 like LPI. Our study links ARA-S and GPR55, and provides new evidence for endothelial-selective endocannabinoids and receptors. However, GPR55 may be not the only target for ARA-S due to only the partial inhibition observed with the GPR55 siRNA.

In addition to GPR55, the atypical CB receptor binding O-1918 is also believed to be endothelial-selective. In published reports, GPR55 and the receptor binding O-1918 were found to act differently in modulating certain CB-mediated effects (Offertáler *et al.*, 2003; Ryberg *et al.*, 2007). In our study, O-1918 alone inhibited the effects of ARA-S. However, O-1918 had no further inhibitory activity after knock-down of GPR55 (data not shown). These results suggest an overlapping effect between the receptor binding O-1918 and GPR55 upon stimulation with ARA-S in endothelial cells. Our findings differ from a previous report that O-1918 antagonized abnormal cannabidiol vasodilator responses in the GPR55 knockout animal model (Ryberg *et al.*, 2007). Further investigation is needed to clarify vasodilatory modulation of CB effects on endothelium.

Lipid-mediated vascular protection, angiogenesis and wound healing have been noted in patients after surgery (Wakamatsu *et al.*, 1990; Mitchell *et al.*, 1996). Considerable evidence indicates a strong correlation between cardioprotection and the endocannabinoid system. However, specific targets on relevant tissues have not been characterized. Endocannabinoid-like lipids such as ARA-S may exert protective properties through stimulation of VEGF-C production and its cognate receptors (VEGFR-2 and 3). VEGF-C stimulates angiogenesis via VEGFR-2, and induces lymphangiogenesis through VEGFR-3 (Adams and Alitalo, 2007). Activation of calcium channels, Akt and ERK1/2 may contribute to effects of ARA-S on VEGF-C/VEGFR-2/VEGFR-3 as such downstream signalling changes

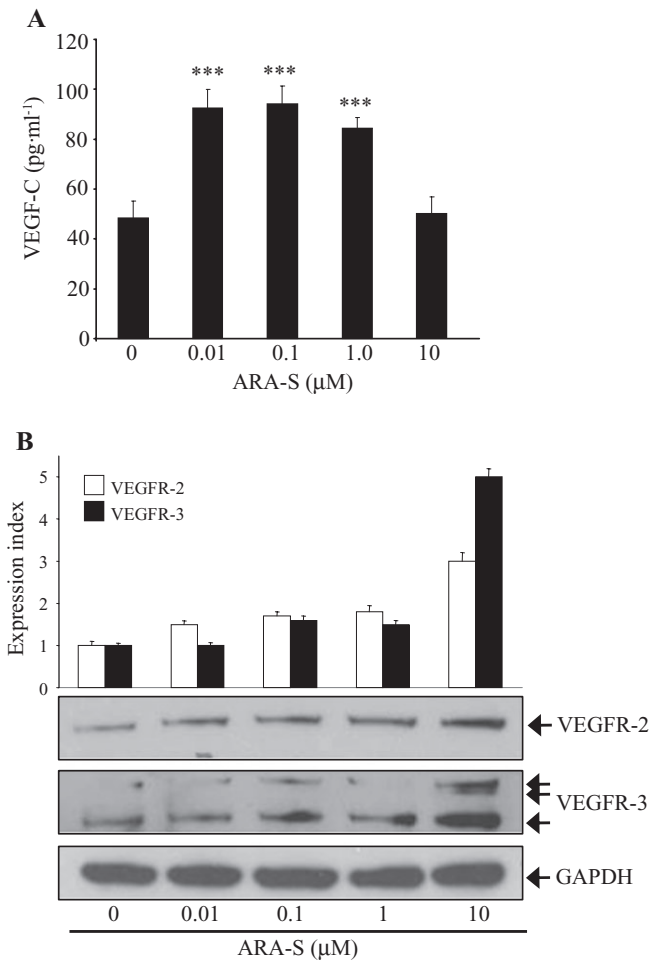

Figure 5

N-arachidonoyl serine (ARA-S) induces VEGF-C production and its receptor expression. HMVEC were incubated with different concentrations of ARA-S or vehicle controls for 18 h, as indicated. (A) The culture supernatant was collected to measure VEGF-C production using an ELISA assay. Data indicate the mean ± SD. ***$P < 0.001$ for the treatment with ARA-S versus vehicle control. (B) The total cell lysates were collected and used to detect expression of VEGFR-2 and 3 in Western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control. The expression indices are shown in the upper panel. HMVEC, human dermal microvascular endothelial cells; VEGFR, vascular endothelial growth factor receptor.

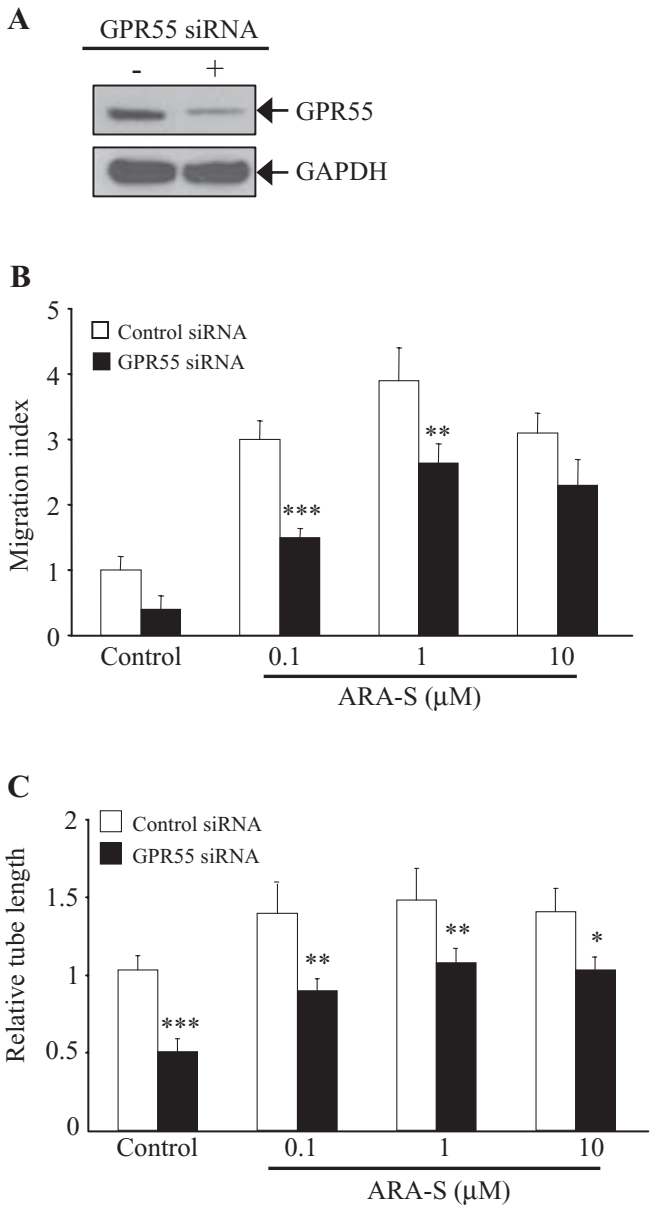

Figure 6

Knock-down of GPR55 inhibits the ARA-S-induced endothelial migration and *in vitro* tube formation. (A) HMVEC were transfected with a specifc GPR55 siRNA or a negative control siRNA for 72 h respectively. Total cell lysates were collected to detect expression of GPR55 in Western blotting. The blot was probed again with anti-GAPDH antibody to assure uniformity in the protein loading. (B) The transfected cells were used to test ARA-S activity on endothelial migration after GPR55 reduction. The number of migrated cells was counted. The migration index was calculated and shown. (C) The transfected HMVEC were also used to perform *in vitro* angiogenesis assays as described above. The effects of ARA-S were significant in the control siRNA-transfected cells ($P < 0.05$). Quantitative data are presented as the relative tube length. Data indicate the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for treatment with the GPR55 siRNA versus the negative control siRNA. ARA-S, N-arachidonoyl serine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPR55, G protein-coupled receptor 55; HMVEC, human dermal microvascular endothelial cells.

can initiate the synthesis of VEGF and augment expression of VEGF receptors (Berra *et al.*, 2000; Pore *et al.*, 2004; Man *et al.*, 2008).

In summary, an activated endocannabinoid system may modulate vascular repair and angiogenesis. Endocannabinoid-like ARA-S may activate the GPR55 receptor and its downstream signalling cascade, including Akt and ERK1/2, thereby protecting stressed endothelial cells and/or fostering vascular repair. These functions would be important in the maintenance of vascular integrity. Specific targeting of the endocannabinoid system provides the

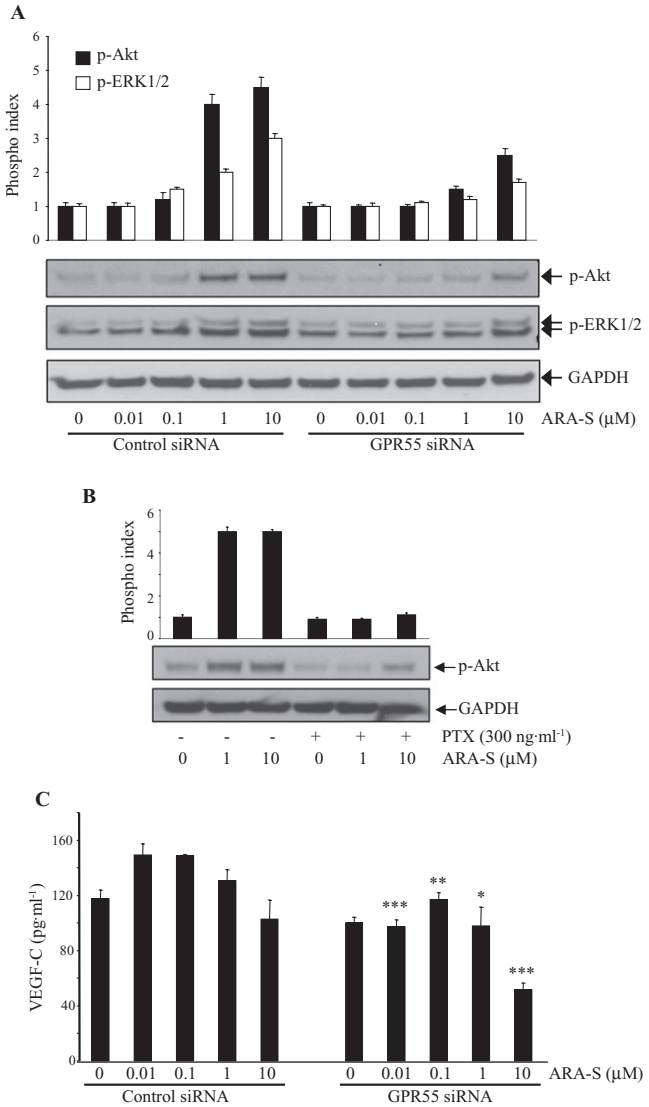

Figure 7

Knock-down of GPR55 blocks the ARA-S-induced effects. (A) The transfected cells described in Figure 6 were serum-starved for 2 h and stimulated with different concentrations of ARA-S for 15 min as indicated. Phosphorylation of Akt and ERK1/2 were examined in Western blotting. GAPDH shows equal protein loadings. Fold increase was calculated as described above. (B) Treatment with *Pertussis* toxin (PTX) inhibits ARA-S-induced phosphorylation of Akt. HMVEC were incubated with PTX (300 ng·mL^{-1}) or vehicle control for 4–6 h. Cells were then stimulated with ARA-S for 15 min as indicated. Phosphorylation of Akt was detected by Western blotting. GAPDH serves as a loading control. The phosphorylation indices (Phospho index) of the respective blots are shown in the upper panels. (C) Knock-down of GPR55 inhibits the ARA-S-induced VEGF-C production. VEGF-C production was measured in the transfected cells treated as described above. Data indicate the mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ for treatment with the GPR55 siRNA versus the control siRNA. ARA-S, N-arachidonoyl serine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPR55, G protein-coupled receptor 55; HMVEC, human dermal microvascular endothelial cells; VEGF, vascular endothelial growth factor.

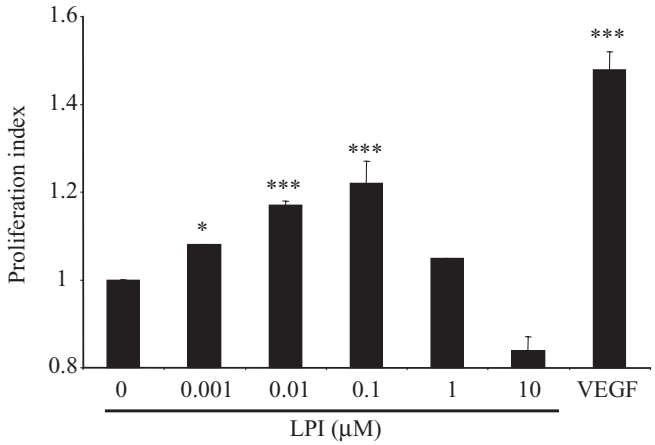

Figure 8

The GPR55 agonist LPI stimulates endothelial proliferation. Proliferation assay was performed as described above. Proliferation indices were calculated and shown. Data indicate the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for the treatment with LPI or VEGF versus vehicle controls. GPR55, G protein-coupled receptor 55; LPI, lysophosphatidylinositol; VEGF, vascular endothelial growth factor.

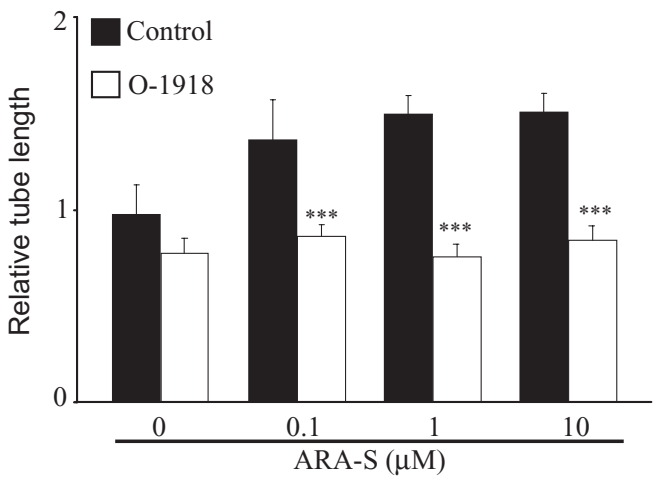

Figure 9

O-1918 inhibits ARA-S-induced tube formation. HMVEC were pretreated with O-1918 (2 μM) or vehicle control for 30 min and then used for *in vitro* tube formation assays in the presence or absence of ARA-S. Quantitative data are presented as the relative tube length. Data indicate the mean ± SD. ***$P < 0.001$ for treatment with ARA-S versus vehicle controls. ARA-S, N-arachidonoyl serine; HMVEC, human dermal microvascular endothelial cells.

opportunity for novel pharmacological approaches to limit vascular damage.

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Conflict of interest

None.

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