의학박사 학위논문

The Role of Adenylyl Cyclase-Associated Protein1 (CAP1) in Transendothelial Migration of Monocytes to Promote Chronic Inflammation

만성염증을 촉진하는 단핵구 경내피세포 이동에서의 CAP1 의 역할 규명

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ABSTRACT

Background

Resistin, originally described as an adipocyte-specific hormone, was first discovered in obese mice and named for its ability to resist insulin action. Resistin has been proposed to be an important mediator between obesity and diabetes. Although it is mainly secreted from adipocytes in rodents, interestingly, significant levels of resistin expression in humans are mainly found in mononuclear leukocytes, macrophages and bone marrow cells. Recently, various studies indicate that resistin plays important regulatory roles in a variety of human diseases, such as altered glucose homeostasis, atherosclerosis, cardiovascular disease, non-alcoholic fatty liver disease and chronic low-grade inflammation. Even though there is growing evidence that links resistin-mediated chronic inflammation, the connection between the inflammatory function of human resistin and its role in metabolic disease has not been clarified yet, due to lack of information about its corresponding receptor and signaling mechanism. Recently, our group identified adenylyl cyclase-associated protein 1 (CAP1), as a novel functional

receptor for human resistin, and clarified its intracellular signaling pathway to modulate inflammation of human monocytes. In the present study, I investigated the physiological role of CAP1, as a functional receptor of human resistin in endothelial cells with the mechanism being distinguished from monocytes. I found that resistin bound to CAP1, activated adenylyl cyclase, and increased cell adhesion molecules such as hICAM-1 and hVCAM-1 through the cAMP-PKA-NF-κB signaling pathway in endothelial cells. As a result, Resistin promoted the transendothelial migration (TEM) of monocytes.

Methods

To examine whether CAP1 is a functional receptor of human resistin in endothelial cells *in vitro*, I cloned adenovirus expressing human CAP1. Also, knockdown of CAP1 was achieved by using siRNA. For *in vivo* loss of function studies, I generated the CAP1 heterozygous deficient mice ($CAP1^{+-}$) using gene editing system TALENs (TAL effector nuclease).

Results

In the present study, I newly identified that CAP1 serves as a functional receptor of human resistin and plays a role as a

critical regulator of inflammation in endothelial cells. Resistin binding to CAP1 dissociates CAP1-AC3-CAV1 complex, results in activation of adenylyl cyclase, and stimulates cAMP-PKA-NF-κB pathway followed by overexpression of ICAM-1 and VCAM-1 expression. Finally, these results facilitate the transendothelial migration (TEM) of monocytes.

Conclusions

CAP1 of endothelial cells is an important regulator of the infiltration of mononuclear cells into inflamed tissues as a functional receptor for human resistin.

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Keywords: Resistin, Inflammation, Transendothelial migration (TEM), Adenylyl cyclase-associated protein 1 (CAP1), hICAM-1, hVCAM-1, Caveolin-1, Lipid Raft, Endothelial cell

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LIST OF ABBREVIATIONS

CAP1 : adenylyl cyclase-associated protein 1

TEM : transendothelial migration

cAMP : cyclic adenosine monophosphate

PKA : protein kinase A

NF-κB : nuclear factor kappa B

AC : adenylyl cyclase

THP-1 : human monocytic leukemia cell

HUVEC : human umbilical vein endothelial cell

mFc-hResistin : mouse Fc conjugated human resistin

rhResistin : recombinant human resistin

hICAM-1 : human intercellular adhesion molecule-1

hVCAM-1 : human vascular cell adhesion molecule-1

PECAM-1 : platelet endothelial cell adhesion molecule-1

VE-Cadherin : vascular endothelial cadherin

ZO-2 : zonula occuludens-2

CAV1 : caveolin-1

siRNA : small interfering RNA

TALEN : transcription activator-like effector nucleases

INTRODUCTION

Resistin is one of the adipocyte-derived cytokines, which was first identified as a potential mediator linking obesity and insulin resistance in rodents[1]. Resistin, with a molecular mass 12.5kDa, also belongs to a family of cysteine-rich proteins that have been associated with the regulation of inflammatory processes[2]. Although murine resistin has been implicated in the pathogenesis of obesity-mediated insulin resistance and type 2 diabetes[1], the human physiology of resistin is still under debate[3,4]. Also, human resistin is distinguished from murine resistin, in terms of sequence homology and types of cells expressing resistin. In fact, there is an only approximately 60% homology of amino acid sequences between murine and human resistin[5] and human resistin is mainly produced by peripheral blood mononuclear cells (PBMCs), macrophages and bone marrow cells[6], while in rodents, resistin is primarily secreted from mature adipocytes[1]. Several *in vitro* studies described that resistin induces the activation of p38, Erk and phosphatidylinositol 3-kinase (PI3K), and seems to be involved in the recruitment of other immune cells and the secretion of

inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12 in macrophages via a nuclear factor-κB (NF-κB)-dependent pathway[7]. Resistin also upregulates the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in human endothelial cells[8,9,10,11], increases smooth muscle cell proliferation[12,13] and migration[14], and accelerates foam cell formation[15]. Much evidence shows that resistin is associated with inflammatory diseases and atherogenesis, whether associated with insulin resistance or not[14]. Resistin, which is present in both murine and human atherosclerotic lesions[10,14], was suggested to be an inflammatory marker of atherosclerosis in humans[16] and known to promote atherogenesis by activating monocytes. I previously reported that resistin is a key player to modulate monocytes, endothelial cells, and smooth muscle cells, leading to progression of atherosclerosis in rabbit carotid artery[13].

Despite much research into the link between human resistin and chronic inflammation, because the receptor for resistin has not been clearly elucidated, so there has been much controversy about the definitive biological mechanism of resistin. Recently,

our group identified adenylyl cyclase-associated protein 1 (CAP1) as a novel functional receptor for human resistin and clarified its intracellular signaling pathway to modulate inflammatory action of human monocytes[17]. CAP1, with a molecular mass of 52kDa, was first isolated as a component of adenylyl cyclase complex and was known to be involved in Ras / cAMP signaling pathway in yeast[18,19,20]. CAP1 is conserved in all eukaryotes studied, including yeast, fungus, plants, and mammals. CAP1 shares three well conserved structural domains in the N-terminus, proline-rich SH3 (Src homology 3) binding middle domain and C-terminus[21]. In yeast, the N-terminus of CAP1 binds adenylyl cyclase and is sufficient for mediating Ras signaling[21,22,23,24]. The centrally located proline-rich domain interacts with SH3 domain of specific proteins[25,26]. Finally, the highly conserved C- terminus domain of CAP1 binds to monomeric actin and is necessary for normal cellular morphology[27,28]. For the first time, our group demonstrated that human resistin directly binds to CAP1 in human monocytes and upregulates intracellular cAMP concentration, PKA activity and NF-κB-related transcription of inflammatory cytokines such as IL-6, TNFα and IL-1β. In contrast, suppression of CAP1

by small interfering RNA abrogated the resistin-mediated inflammation[17]. In the present study, I demonstrated that CAP1 in endothelial cells can act as a functional receptor for resistin. I found that CAP1 knockdown in endothelial cells, like as resistin treatment, activates cAMP-PKA-NF-κB signaling pathway, increases of hICAM-1 and hVCAM-1 expression, and consequently, facilitates transendothelial migration (TEM) of monocytes. These data suggest that CAP1 can serve as a physiological receptor of human resistin in endothelial cells and play a role as a critical regulator of monocytes infiltration.

MATERIALS AND METHODS

1. Cloning of Adenovirus Expressing Human CAP1

The human CAP1 plasmid based on a pOTB7 vector was purchased from ImaGenes Bio (Germany). The human CAP1 construct was amplified by PCR and then cloned into the *KpnI* and *XhoI* site of a shuttle vector (pAdTrack-CMV) containing green fluorescence protein (GFP). The primers are as follow : forward, 5'-GCGGTACCACCATGGCTGACATGCAAAATC-3',reverse,5'-GCCTCGAGTTATCCAGCAATTTCTGTCAC-3'. The recombinant shuttle vector was co-transfected with adenoviral genome (pAdEasy-1) into *E. coli* (BJ5183), where homologous recombination occurred. The recombinant adenoviral vectors expressing both human CAP1 and GFP simultaneously (Ad-hCAP1/GFP) were transfected into HEK 293A cells to get the viral particles, which were purified by CsCl (Sigma) ultracentrifugation and dialysis for titration. The construct of Ad.hCAP1/GFP was confirmed by DNA sequencing. Adenovirus encoding GFP (Ad-GFP) was used as a control.

2. RNA Interference for CAP1 Knockdown

For knockdown of CAP1, siRNA molecule was synthesized by QIAGEN. The target sequence was 5'-AAACCGAGTCCTCA AAGAGTA-3'. As a control, negative control siRNA (non-silencing siRNA) was purchase from QIGEN. Transfection of CAP1 siRNA was achieved with HiPerFect transfection reagent (QIAGEN).

3. Cloning of mFc-conjugated hResistin

The construct of mFc-conjugated hResistin was amplified by PCR and cloned into the *HindIII* and *XhoI* site of pcDNA3.1/V5-His-TOPO vector (Invitrogen). The human Resistin plasmid based on a pCR4-TOPO vector was purchased from Openbiosystems and mouse Fc was obtained from mFc-Fusion Construct, kindly provided by Dr. Jun-Ho Jung (Seoul National University College of Medicine, Korea). The primer used for mouse Fc is as follow : forward, 5'-CCCAAGCTTA TGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGG GTTCCAGGTTCCACTGGTGACGAGCCCAAATCTAGCGACAA -3'; reverse, 5'-CGAGCCACCGCCACCCGAGCCACCGCCA CCCGAGCCACCGCCACCTTTACCAGGAGAGTGGGAGA-3'. The primer used for human resistin was as follow : forward,

5'-GGTGGCGGTGGCTCGGGTGGCGGTGGCTCGGCCCAGGC GGCCAAGACCCTGTGCTCCATGG-3'; reverse, 5'-CCGCT CGAGGGCCGGCCTGGCCTCAGGGCTGCACACGACA-3'. The clones with the correct size of interested DNA were sequenced (SolGent co., the Republic of Korea). After transfection of mFc-conjugated hResistin DNA into HEK 293F cells, the mFc fusion protein to N-terminal of human resistin was purified using CaptureSelected Multi Species Fc matrix (the Bio Affinity Company).

4. Immunoprecipitation

To identify hCAP1 and hResistin interaction on cell membrane, HUVECs were incubated with recombinant human resistin protein(1ug/ml) for 12hr at 37℃. Following incubation, the membrane extracts of the cells were prepared using Qproteome Cell Compartment kit (QIGEN, Cat. No. : 37502). Lysates were incubated for 24hr at 4℃ with anti-human CAP1 antibody (Santa Cruz Biotechnology, sc-100917) or normal mouse IgG (Santa Cruz Biotechnology, sc-2025). After incubation with the antibody, the lysates were incubated with protein A/G agarose beads (Santa Cruz Biotechnology, sc-2003) for 3hr at 4℃.

After washing 3 times with cold PBS, the beads were resuspended in 2X reducing sample buffer and heated for 5min at 95℃ to dissociate captured antigen from beads. Beads were removed by centrifugation at 2,500rpm, immunoprecipitates from supernatants were separated on a 10% SDS-PAGE Tris-Glycine gel, and transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 5% fat-free milk and was immunoblotted with anti-human CAP1 antibody or anti-human resistin antibody (Adipogen, Cat# AG-25A-0013).

5. Western Blot

Cells were washed in cold PBS and harvested by scraping in a lysis buffer (50mM Tris pH7.2, 250mM NaCl, 1% NP-40, 0.05% SDS, 2mM EDTA, 0.5% deoxycholic acid, 10mM β-glycerolphoshoate and 1mM sodium orthovanadate) containing protease inhibitor cocktail (Roche) and 100mM NaF. Protein samples were separated on 8~10% SDS-PAGE gels and transferred to PVDF membrane (Millipore). Primary antibodies used in this study were as follows : anti-human CAP1 antibody (Sigma, cat# WH0010487M1), anti-human Resistin (Adipogen,

cat# AG-25A-0013), anti-NFκB (p65) (SantaCruz, sc-109), anti-NFκB (p50) (SantaCruz, sc-114), anti-phospho-VASP(Ser157) (Cell Signaling, cat# 3111), anti-His tag (Cell Signaling, cat# 2365), anti-mFc-HRP (Sigma, cat# A0168), anti-Lamin A/C (SantaCruz, sc-6215), anti-phospho-CREB (SantaCruz, sc-101663), anti-AC3 (SantaCruz, sc-558), anti-AC4 (Abcam, ab103484), anti-AC5/6 (SantaCruz, sc-590), anti-AC7 (Abcam, ab102751), anti-AC9 (Abcam, ab110159), anti-Caveolin1 (SantaCruz, sc-894), anti-Caveolin2 (SantaCruz, sc-7942), anti-Caveolin3 (SantaCruz, sc-5310), anti-ICAM-1 (SantaCruz, sc-7891), anti-VCAM-1 (Dr. Junho Chung kindly provided), anti-GAPDH (Sigma Aldrich, G9545), and anti-α-tubulin (Oncogene, cat# CP06). As secondary antibody, anti-mouse IgG HRP (Promega), anti-goat IgG HRP (Promega), and anti-rabbit IgG HRP (Promega) were used. ECL or ECL-PLUS (Amersham) was used for detection.

6. Cell culture

Pooled human umbilical vein endothelial cells (HUVECs) were purchased at Lonza and cultured in EGM-PLUS SingleQuots Kit

(Lonza, CC-4542) supplemented with 10% fetal bovine serum (FBS). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and all used HUVECs were passage 6 to 7.

7. RNA preparation and Real-Time PCR

Total RNA was extracted using RNeasy Mini Kit (QIAGEN, Cat. No. : 74104) according to the manufacturer instructions. One microgram of total RNA was used for reverse transcription. The first strand cDNA was synthesized using the PrimeScript 1st strand cDNA Syntehsis Kit (TaKaRa, Cat. No. : 6110A). Quantitative real time PCR was performed using the ABI 7500 Thermal Cycler apparatus with PRISM® 7500 Sequence Detection System that was programmed with ABI standard cycling program of 50℃ for 2 minutes, denaturation at 95℃ for 10 minutes, 40 cycles of primer annealing at 95℃ for 15 seconds, and primer extension at 60℃ for 1minute. cDNA quantification was performed using FastStart universal SYBR Green Master (Roche, 2X conc.) according to the manufacturer' s instructions. The results were expressed as mRNA level normalized to standard housekeeping gene (human GAPDH or mouse 18S RNA) using the ΔΔCt method.

8. Cyclic AMP Assay

Before ELISA, HUVECs were starved at 1% FBS of EBM for 2 hours. Cells were stimulated with recombinant human Resistin at 100ng/ml concentration for 5 minutes to 2 hours and were treated with cAMP-elevating agonist, forskolin (50Mm for 30minutes), as a positive control. For the effect of CAP1 knockdown, cells were transfected with siRNA. Cyclic adenosine monophosphate (cAMP) production were measured using cAMP Assay kit (R&D Systems, cat.# KGE002B) according to the manufacturer's instructions.

9. Cytosol and Nuclear Fractionation

To prepare cytosolic extracts, cells were carefully collected by using cytosol lysis buffer (10mM HEPES pH7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 0.1mM DTT, 1mM Na3VO4). Samples have been lysed by adding 0.6% NP40 for a minute and quickly centrifuged at 13,000rpm. Supernatant was obtained as cytosolic extracts and remained pellets were lysed in nuclear lysis buffer(25mM HEPES pH7.9, 0.4M NaCl, 0.5mM EDTA, 0.5mM EGTA, 0.1mM DTT, 1mM Na_3VO_4) for 30 minutes incubation at 4° C.

10. Transwell Migration Assays

Monocyte chemotaxis was measured using a 24-well Micro Transwell Permeable Supports (Corning). For CPA1 knockdown, HUVECs were transfected by using siRNA. Then HUVECs were seeded on the upper chamber coated with fibronectin (BD, #354008), and cultured for 48 hours until full confluence. THP1 cells stimulated with resistin were transferred onto an upper chamber. The chemoattractant, recombinant human resistin (100ng/ml), was added to the lower chamber. The lower and upper chambers were separated by a polycarbonate membrane (5㎛ pore size). Transmigration was performed for 6hr to 12 hr at 37C in a humidified atmosphere with 5% CO_2. All cells migrating through the polycarbonate membrane to lower chamber were counted.

11. Lipid Raft Isolation

Lipid raft fractionation was performed as described in reference[30,31]. HUVECs were incubated with DTSSP (ThermoFisher Scientific, #21578) for 2 hours at 4° C. Then cells were collected in cold PBS and centrifuged to isolate pellets. Pellets were frozen at −80° C for 16 hours and lysed in

membrane isolation lysis buffer (0.1% Triton X-100, 50mM Tris-HCl [pH7.4], 150mM NaCl, 5mM EDTA, 0.1% Triton X-100, 1X protease inhibitor cocktail). Next, cells were additionally lysed by forcing through 23-gauge needle for 20 times and then centrifuged for 20 minutes at 100x g at 4° C. 1ml Opti-Prep (60% iodixanol) was added to 500ul of post-nuclear supernatant (resulting in a 40% iodixanol solution). Equal amount of 40% iodixanol solution and sample mixtures, 30% iodixanol and 5% iodixanol solution was transferred into ultracentrifuge tube (Beckman) in consecutive order. After centrifugation at 132,000x g at 4° C for 5 hours, five fractions of equal volume (0.8ml) were collected.

12. In vivo analysis using CAP1$^{+/-}$ knockout mice

All animal experiments were performed after receiving approval from the Institutional Animal Care and Use Committee (IACUC) of Clinical Research Institute in Seoul National University Hospital, Korea (approval number : SNU-140528-1-5). Age-matched (9 to 10 week old) CAP1$^{+/-}$ knockout mice were used in this study. Wild type mice with an intact CAP1 were used as a control. As the TEM animal model, I used the carrageenan-

induced edema formation and the mustard oil-induced vascular leakage model. Firstly, to induce hyper-resistinemia, resistin adenovirus ($1X10^9$ pfu/ul) was intracardiac injected into wild type mice and $CAP1^{+/-}$ mice for four weeks, respectively. Then, after isolating the monocytes with intact CAP1 from wild type mice, DiI labeled monocytes were intracardiac injected, and 50ul of λ-carrageenan (1%, w/v), an inflammatory substance, was injected in a hind paw of mice. As a control, normal saline was injected on the opposite paw. Paws were harvested 18 hours later, I measured the edema depth, paw volume and monocyte infiltration in the edema regions. To examine the vascular leakiness, mustard oil, an inflammatory agent, was applied to the ear for 1 minute after injection of Evans blue dye. Mustard oil was diluted to 5% in mineral oil, applied to the dorsal and ventral surfaces of the ear, and it was reapplied 15 minutes later. Nothing was done to the other ear of mice treated with mustard oil, as a baseline control. Thirty minutes after the stimulus, ears were removed, Evans blue was extracted from the ear with 600ul of formamide overnight at 55℃ and measured spectrophotometrically at 610nm.

13. Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). The differences of continuous variables between experimental groups were analyzed by Student's t-test or one-way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA), and $p < 0.05$ was considered statistically significant.

RESULTS

1. Identification of a human resistin receptor

Recently, I reported adenylyl cyclase-associated protein 1 (CAP1) as a novel functional receptor for human resistin in monocytes[17]. Human resistin directly bound to CAP1 of monocyte and regulated the inflammatory cytokine production of monocytes. In brief, the process of finding CAP1, a new receptor for resistin, was as follows. To identify a putative resistin receptor, I cloned the mFc-conjugated hResistin fusion protein (mFc-hResistin). Human resistin was conjugated with the Fc region of mouse immunoglobulin to detect potential receptor proteins using immunoprecipitation (Figure 1A, B). After transfecting mFc-hResistin DNA into human embryonic kidney 293F (HEK293F) cells, the mFc-hResistin fusion protein was expressed and purified using mFc-specific beads. The expression and purification of mFc-hResistin were confirmed by western blotting with an anti-mFc-HRP antibody and by Coomassie staining, respectively (Figure 1C). Through western blots using the mFc-hResistin as a primary antibody to human monocytic leukemia (THP-1) cell lysate, I was able to identify a protein with a molecular mass of about 55 kDa (Figure

1D). This protein nearly disappeared, after blocking the membrane with an abundant non-Fc-conjugated recombinant human resistin protein (rhResistin) (Figure 1E). These findings suggested that THP-1 cells expressed a 55 kDa sized protein capable of specifically binding with human resistin. To identify this protein presumed to be a receptor for human resisitin, I incubated the mFc-hResistin with THP-1 cell lysates and performed immunoprecipitation using mFc-specific beads. As a result, a specific band corresponding to a protein of approximately 55 kDa was detected (Figure 1F). Then, I excised the gel band and performed MALDI-TOF (Figure 1G). This analysis identified the binding protein as CAP1. Figure H depicted the full amino acid sequence of human CAP, and the matched sequences by MALDI-TOF analysis indicated the bold and underlined part.

2. Human resistin binds to CAP1 in endothelial cells

In the previous study, I have confirmed that CAP1 of monocytes directly binds to resistin[17] (Supplemental Figure 1). Therefore, I examined whether CAP1 also could act as a functional receptor for resistin in endothelial cells. First, to

identify whether CAP1 and resistin bind each other, immunoprecipitation was performed after his-tagged CAP1 plasmid transfection and myc-tagged resistin overexpression using adenovirus in HUVEC. As expected, CAP1 and resistin bound to each other in endothelial cells. (Figure 2A, B). Resistin is known as an inflammatory cytokine that increases the expression of ICAM-1 and VCAM-1 in endothelial cells[8,11]. Similarly, I confirm that hICAM-1 and hVCAM-1 significantly were increased in HUVEC treated with rhResistin and resistin adenovirus (Figure 2C-E). Next, I tested the neutralizing effect of CAP1 on resistin in order to confirm whether resistin binds to CAP 1 as a true ligand of CAP 1. Fluorescence-activated cell sorting (FACS), western blot, and quantitative PCR showed that when resistin was treated in HUVEC, the expression level of hVCAM-1 was increased about two fold compared to vehicle, but when five-fold concentration of recombinant CAP1 protein was pre-incubated with resistin, the expression of hVCAM-1 was reduced significantly (Figure 2F-H). It suggests that CAP1 bind to resistin and successfully neutralize the effect of resistin. The above results imply that CAP1 can serve as a functional receptor for human resistin in endothelial cells.

3. CAP1 knockdown augments the expression of hICAM-1 and hVCAM-1, like as a resistin

The depletion of CAP1 in monocytes significantly abrogated the inflammatory action of resistin, and while overexpression of CAP1 augmented it[17] (Supplemental Figure 2). In other words, the effect of resistin was dependent on CAP1 in monocytes. Thus, I examined the effect of CAP1 knockdown on the expression of adhesion molecules including hICAM-1 and hVCAM-1 in endothelial cells. When rhResistin was treated in HUVEC, the expression level of hICAM-1 and hVCAM-1 was increased. However, in contrast to monocytes, CAP1 knockdown in endothelial cells rather significantly augmented the expression of hICAM-1 and hVCAM-1 both mRNA and protein level (Figure 3A-C). In addition, CAP1 complementation using adnovirus effectively returned the increased hVCAM-1 expression to a normal level (Figure 3D). These inverted results imply that although CAP1 functions as a receptor for human resistin both in endothelial cells and monocytes, there are considerable differences in mechanism of action along with the cell types.

4. Adenylyl cyclase isoforms are differentially expressed in endothelial cells and monocytes

Unlike in monocytes[17], CAP1 knockdown in endothelial cells rather increased the hICAM-1 and hVCAM-1 expression. This implies that CAP functions may vary depending on the cell contexts. To clarify the inverted relationship in endothelial cells, I focused on adenylyl cyclase (AC) and cAMP level, and investigated the differences of adenylyl cyclase isoform between monocytes and endothelial cells. This was based on the fact that cAMP was a key second messenger to transmit signaling by resistin and CAP1 interaction[17]. Interestingly, there are ten different isoforms of adenylyl cyclase and these isoforms have a tissue specific distributions[32]. Therefore, I hypothesized that the isoforms of adenylyl cyclase might be differently regulated by CAP1 depending on cell contexts. Indeed, HUVEC and THP1 showed different profiles of adenylyl cyclase isoforms. At mRNA level, AC3, AC4, AC6 and AC9 showed relatively high expressions in HUVEC, while AC3, AC7 and AC9 were predominant in THP1 (Figure 4A, B). However, there was no difference in expression of AC isoforms by

resistin treatment. Even though AC3 and AC9 were overlapped, a definitive predominance was distinguished at a protein level. Through western blot, I concluded that the protein level of AC3 was consistent with mRNA data in HUVEC, and AC7 was in THP-1, respectively (Figure 4C). Next, I investigated that whether AC3 and AC7 were localized at caveolae or not using OptiPrep Density gradient centrifugation (Figure 4D)[30,31]. The localization of specific cell membrane compartments is one of the critical characteristics to adenylyl cyclase. Indeed, many previous studies reported that AC1, AC3, AC5/6 and AC8 were lipid raft-localizing isoforms, whereas AC2, AC4 and AC7 were non-lipid raft localizing isoforms[32]. Caveolae, a subset of membrane lipid rafts, has a unique marker protein known as the caveolin[33] and has important roles in regulating signaling molecules including adenylyl cyclase, G_{α}, $G_{\beta\gamma}$, PKA, PKC, Src and Erk through scaffolding domain of caveolin[34]. Thus, I guessed that adenylyl cyclase and CAP1 distribution on caveolae could make critical differences in inflammatory action by resistin because caveolae was enriched in endothelial cells compared to other cell types. Mammalian cells have three isoforms of caveolin, namely, caveolin-1 (CAV1), caveolin-2

(CAV2), and caveolin-3 (CAV3). CAV1 and CAV2 are abundant in caveolae rich non-muscle cells, whereas CAV3 is found in skeletal muscle and in some smooth muscle cells[35]. Thus, I examined more precise localization of adenylyl cyclase and CAP1 in each two different cells. OptiPrep Density gradient centrifugation was an efficient method to fractionate lipid raft and non-lipid raft as well as cytosol (Figure 4D)[30,31]. By using this method, I readily identified that AC3 and AC6 were localized at lipid rafts in HUVEC (Figure 4E) and AC7 was localized at non-lipid rafts in THP1 (Figure 4F). Furthermore, interestingly, I observed that CAP1 was partly co-localized with CAV1 in lipid raft. Taken together, AC7, localized at non-lipid rafts, is a dominant isoform of THP1 and AC3 and AC6, localized at lipid raft, are a dominant isoform of HUVEC. Intriguingly, CAP1 is partly distributed at lipid raft with AC3, AC6 and CAV1 (Figure 4G).

5. Resistin dissociates the CAP1-AC3-CAV1 complex in lipid raft and results in the activation of adenylyl cyclase

Caveolin scaffolding domain was known to have an inhibitory effect of adenylyl cyclase, but the relationship between caveolin

and adenylyl cyclase was still controversial[36]. I hypothesized that AC3 might be arrested in CAP1-CAV1 complex in lipid raft, but resistin binding to CAP1 could dissociate this suppressive assembly and result in activation of adenylyl cyclase (Figure 5A). First, I confirmed that whether CAP1, AC3 and CAV1 were interacted each other or independently existed at the cell membrane. As a result of immunoprecipitation using anti-CAV1 antibody, AC3 and CAP1 were bound to CAV1 at the cell membrane of HUVEC (Figure 5B). To prove the hypothesis, I examined that whether resistin induces the translocation of CAP1 from lipid raft to non-lipid raft by OptiPrep Density gradient centrifugation. Interestingly, CAP1 was disappeared from lipid raft just after 30 minutes of resistin treatment, and finally vanished within 2 hours. At the same time, Gαs was recruited to lipid rafts, indicating activation of adenylyl cyclase (Figure 5C). For more clear explanation, I performed the immunoprecipitation of AC3 and CAP1. The assembly of CAP1-AC3-CAV1 was dissociated by resistin. After resistin treatment, CAP1 and AC3 were dissociated from each other, and CAV1 was also separated from CAP1. But Gαs binding to AC3 was increased by resistin (Figure 5D). Taken together,

when resistin binds to CAP1, CAP1 is expelled from lipid raft to non-lipid raft or cytosol and it results in disassembly of CAP1-AC3-CAV1 complex. Subsequently, the liberated AC3 from CAP1-AC3-CAV1 suppressive complex is activated by Gαs binding. Next, I wondered if CAP1 bind to resistin, why CAP1 couldn't interact with AC3 or CAV1. In the previous study[17], our group reported that proline rich-SH3 binding domain (BD) of CAP1 was a putative binding motif to human resistin. I speculated that the binding motif of CAP1 to AC3, CAV1 and resistin might be proximal enough to influence on each other. Proline rich SH3 BD of CAP1 is structurally located at the middle, unlike adenylyl cyclase BD and actin BD. Because of this structural characteristic, resistin binding to SH3 BD could induce conformational change for CAP1 and dissociate CAP1 from AC3 or CAV1. Thus, to identify CAV1 binding domain of CAP1, I performed *in vitro* binding assays using each CAP1 deletion mutant and CAV1. I cloned Flag-tagged three deletion mutants of human CAP1 : an adenylyl cyclase BD deletion mutant (ΔAC BD deletion), a mutant in which both the SH3 BD and the Actin BD were deleted (ΔSH3ΔActin BD deletion), and an Actin BD deletion mutant (ΔActin BD deletion)

(Figure 5E). After the overexpression of Flag-tagged CAP1 deletion mutants and GFP-tagged CAV1 in HEK 293T cells, whole cell lysates were immunoprecipitated with Flag antibody. Then, samples were immunoblotted by using antibodies against both Flag and GFP. As a result, CAV1 band was detectable slightly in the ⊿AC BD deletion mutant, whereas it was detected significantly in the ⊿SH3⊿Actin BD deletion mutant (Figure 5F). These observations imply that CAV1 seems to bind CAP1 via the AC BD. In summary, AC3 is suppressed through the interaction CAP1 and CAV1 at the basal status. But, resistin binding to SH3 BD of CAP1 induces the conformational change for CAP1 and results in the dissociation of CAP1 and CAV1 from AC3. Finally, AC3 liberated from CAP1-AC3-CAV1 suppressive complex is activated by Gαs binding (Figure 5G).

6. CAP1 knockdown activates the cAMP-PKA-NF-κB signaling pathway, increases the expression of hICAM-1 and hVCAM-1, and facilitates the TEM of monocytes.

Because CAP1 is known to play a role in adenylyl cyclase activation[19,20], I tested the effect of cAMP synthesis by resistin

or CAP1 knockdown in endothelial cells. As expected, resistin significantly increased cAMP levels in HUVEC (Figure 6A). In addition, CAP1 knockdown itself stimulated cAMP synthesis by forskolin, the direct agonist of AC (Figure 6B). Maybe it implies that CAP1 can have an inhibitory effect of adenylyl cyclase activation in endothelial cells. Next, I investigated which signaling pathways are involved in hICAM-1 and hVCAM-1 expression by CAP1 knockdown. To determine the intracellular signaling of hICAM-1 and hVACM-1 by CAP1 depletion, CAP1 was reduced with specific small interfering RNAs (siRNAs) in HVUEC. Because cAMP typically is a transducer to PKA, I confirmed PKA activation by detecting VASP phosphorylation (Ser157). CAP1 knockdown increased p-VASP (Ser157) and the expression of hICAM-1 and hVCAM-1 in cytosol fraction, like as resistin. Also, NF-κB nuclear translocation, the transcription factor of hICAM-1 and hVCAM-1, was increased by CAP1 depletion (Figure 6C). Interestingly, CAV1 knockdown by siRNA increased hICAM-1 and hVCAM-1 expression, like as a CAP1 knockdown (Figure 6D). Lastly, I explored whether the increased hICAM-1 and hVCAM-1 can enhance TEM of monocytes. To examine transmigration, I performed Transwell

assay using HUVECs and THP-1 cells. HUVECs were manipulated with CAP1 siRNA for 24 hours, and THP1 cells were pre-activated by rhResistin for an effective transmigration. Indeed, resistin facilitated TEM of monocytes and interestingly, CAP1 knockdown itself drastically increased monocyte transmigration (Figure 6E). The above results was consistent with our previous paper that resistin increased monocyte adhesion to the endothelial cells[13]. In summary, resistin treatment or CAP1 depletion upregulates cAMP synthesis, PKA activity and NF-κB-related transcription of hICAM-1 and hVCAM-1. Consequently, these results increase TEM of monocytes (Figure 6F).

7. Generation of TALEN-mediated CAP1$^{+/-}$ knockout mice

Next, I evaluated whether alteration of CAP1 expression *in vivo* affects TEM of monocyte. To test this, our group constructed a CAP1 knockout mouse using the gene editing system, TALENs (TAL effector nuclease). After preparing TALEN which targets exon3 of mouse CAP1, each TALEN mRNA pair was injected into the cytoplasm of mouse pronuclear stage embryos (Figure 7A, B). We obtained heterozygous CAP1 knockout mice

($CAP1^{+/-}$), but homozygous CAP1 knockout mice ($CAP1^{-/-}$) were not born. It is presumed that homozygous CAP1 knockout mice seem to be lethal because CAP1 is a multifunctional molecule essential for actin cytoskeleton maintenance, morphogenesis, cytokinesis, and cell migration. However, it is thought that further studies are needed to clarify the lethality of homozygous CAP1 knockout mice. First, when gross phenotype of $CAP1^{+/-}$ mice was observed, no abnormal abnormalities were found compared to wild type mice (Figure 7C, D). In addition, the expression of CAP1 in various organs of $CAP1^{+/-}$ mice was reduced by half compared to wild type mice (Figure 7E, F). Since homozygous CAP1 knockout mice ($CAP1^{-/-}$) could not be obtained, future *in vivo* experiments were carried out using heterozygous CAP1 knockout mice ($CAP1^{+/-}$).

8. In Vivo analysis using $CAP1^{+/-}$ knockout mice

Lastly, I confirmed whether CAP1 down-regulation in endothelial cells of $CAP1^{+/-}$ mice could increase the TEM of monocytes, likewise *in vitro* experiments. As the TEM animal model, I used the carrageenan-induced edema formation model. Before starting animal experiments using $CAP1^{+/-}$ mice, I

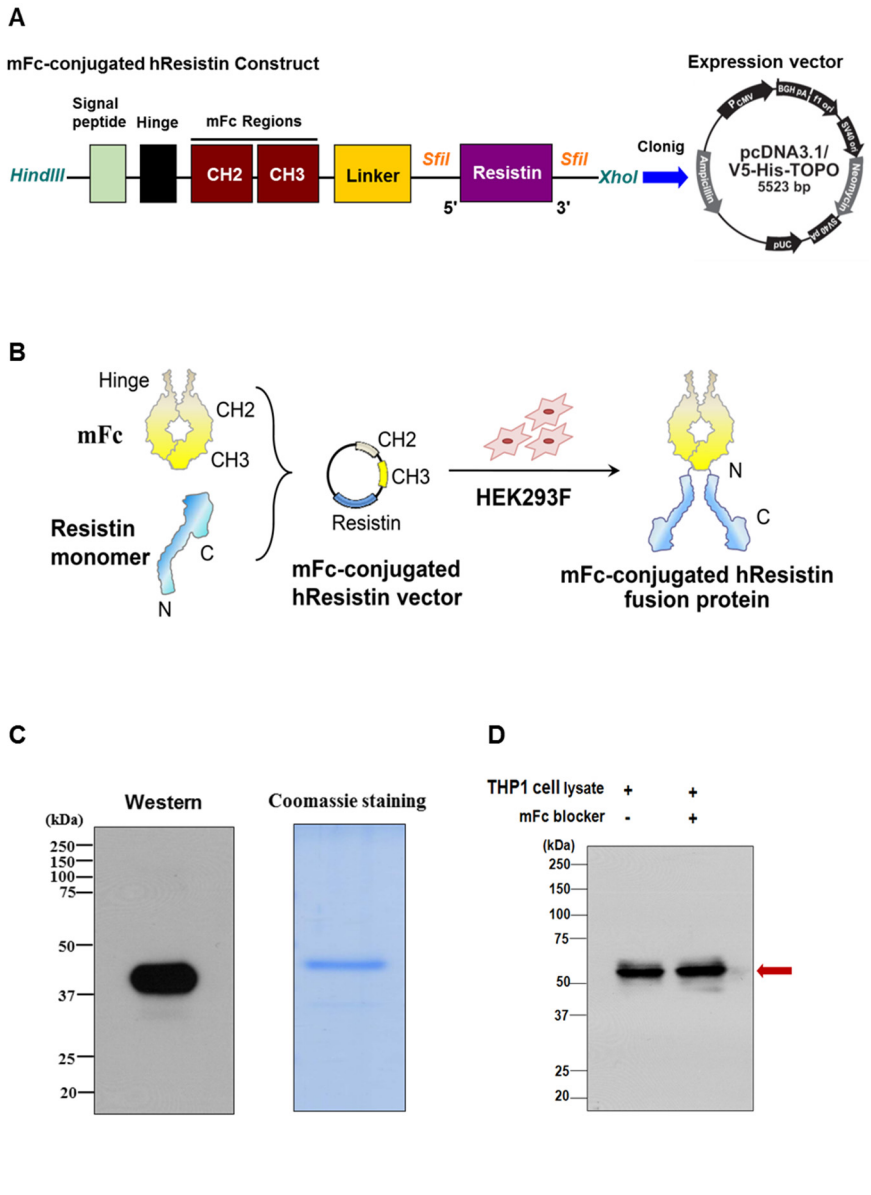
examined that CAP1 expression was decreased in the monocytes of CAP1$^{+/-}$ mice (Figure 8A) and the edema was well induced by carrageenan (Figure 8B). In addition, I identified that hyper-resistinemia was induced by resistin adenovirus delivery *in vivo* (Figure 8C). Because the endothelial cell specific CAP1 knockout mice were not available, I made the endothelial cell specific CAP1 knockout condition *in vivo,* similarly *in vitro*. Firstly, to induce adenovirus-mediated chronic hyper-resistinemia, resistin virus was intracardiac injected into wild type mice and CAP1$^{+/-}$ mice for four weeks, respectively. And, after isolating the monocytes with intact CAP1 from wild type mice, DiI labeled monocytes were intracardiac injected and 50ul of λ-carrageenan (1%, w/v), an inflammatory substance, was administrated in a hind paw of mice. Paws were harvested 18 hours later, I measured edema depth, paw volume and monocyte infiltration in wild type mice and CAP1$^{+/-}$ mice, respectively (Figure 8D). As a result, the edema depth of CAP1$^{+/-}$mice was less than that of wild type mice (Figure 8E). Also, when adenovirus-mediated chronic hyper-resistinemia was induced, the edema depth was decreased in the CAP1$^{+/-}$ mice compared to wild type mice

(Figure 8F). In addition, the paw volume by carrageenan was significantly reduced about a half in the CAP1$^{+/-}$ mice compared to wild type mice (Figure 8G). On the other hand, the tissue invasion of monocytes with intact CAP1 was increased about twice in the CAP1$^{+/-}$ mice compared to wild type mice (Figure 8H, I). In summary, the inflammation of CAP1$^{+/-}$ mice themselves were decreased overall. That is, the edema depth and paw volume by carrageenan were generally reduced. However, the tissue infiltration of monocytes with intact CAP1 was increased under the condition that only CAP1 of endothelial cells was depleted. These results were consistent with *in vitro* transwell assay which the monocyte infiltration was increased in CAP1 knockdown endothelial cells. Next, I wondered whether the increased infiltration of monocytes with intact CAP1 in the CAP1$^{+/-}$ mice was related to the dysfunction of endothelial junction. Thus, I examined the vascular leakage by mustard oil, an inflammatory agent that induces plasma leakage and inflammation. After the intracardiac injection of Evans blue dye, mustard oil was applied to the ear of wild type mice and CAP1$^{+/-}$ mice, and the mouse ear was harvested 30 minutes later. The leakage of Evans blue dye was measured

spectrophotometrically at 610 nm. Vascular leakage by mustard oil in the ear of CAP1$^{+/-}$mice was twice as high as that of wild type mice (Figure 8J, K). Lastly, I tested whether the increase of vascular leakiness in CAP1$^{+/-}$ mice was due to changes in junctional proteins. After the knockdown of CAP1 using siRNA in HUVEC, I confirmed changes in junctional proteins such as PECAM-1, VE-Cadherin and ZO-2. Unlike the expectation that junctional proteins would be reduced, PECAM-1 was decreased, and VE-cadherin and ZO-2 were increased by CAP1 knockdown or resistin treatment (Figure 8L). PECAM-1 is known to be an important regulator of leukocyte trafficking and the maintenance of endothelial cell junctional integrity. It has been reported that the reduction of PECAM-1 and VE-cadherin by Erk activation destabilizes the intercellular junctions and makes more migratory phenotype[37]. Therefore, I confirmed the increase of Erk phosphorylation by CAP1 knockdown. As a result, Erk phosphorylation was significantly increased in HUVEC by CAP1 depletion using siRNA (Figure 8M). These results suggest that a decrease in PECAM-1 by CAP1 knockdown is associated with Erk activation. However, there are conflicting reports that in the case of leukocyte

transmigration, an increase in PECAM-1 and a decrease in VE-Cadherin promote transmigration and create a diapedesis-inducing state[38]. The relationship between the integrity of endothelial cell junction and the tissue infiltration of leukocytes has not been elucidated yet, and there are still many conflicting reports. Therefore, it is considered that further investigations are needed to clarify the relationship between junctional integrity and leukocyte transmigration by CAP1 alteration in endothelial cells.

Figure 1.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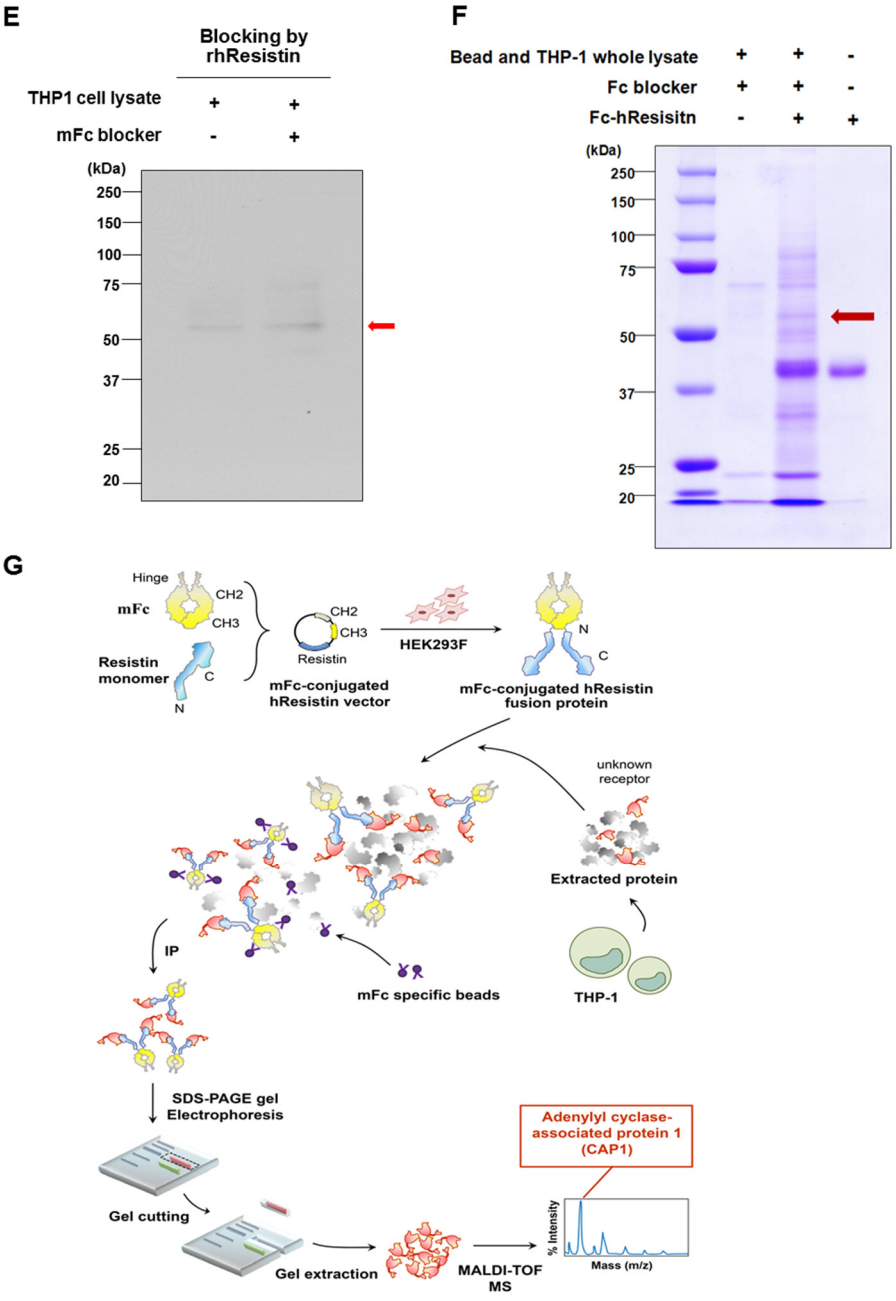
mFc-conjugated hResistin Construct
Signal peptide
Hinge
mFc Regions
HindIII
CH2
CH3
Linker
SfiI
Resistin
SfiI
XhoI
5'
3'
Clonig
Expression vector
pcDNA3.1/
V5-His-TOPO
5523 bp
B
Hinge
CH2
mFc
CH3
Resistin monomer
C
N
CH2
CH3
Resistin
mFc-conjugated hResistin vector
HEK293F
N
C
mFc-conjugated hResistin fusion protein
C
Western
Coomassie staining
(kDa)
250
150
100
75
50
37
25
20
D
THP1 cell lysate + +
mFc blocker - +
(kDa)
250
150
100
75
50
37
25
20


E
Blocking by rhResistin
THP1 cell lysate + +
mFc blocker - +
(kDa)
250
150
100
75
50
37
25
20
F
Bead and THP-1 whole lysate + + -
Fc blocker + + -
Fc-hResisitn - + +
(kDa)
250
150
100
75
50
37
25
20
G
Hinge
mFc
CH2
CH3
Resistin monomer
C
N
CH2
CH3
Resistin
mFc-conjugated hResistin vector
HEK293F
N
C
mFc-conjugated hResistin fusion protein
unknown receptor
Extracted protein
THP-1
mFc specific beads
IP
SDS-PAGE gel Electrophoresis
Gel cutting
Gel extraction
MALDI-TOF MS
Adenylyl cyclase-associated protein 1 (CAP1)
% Intensity
Mass (m/z)

H

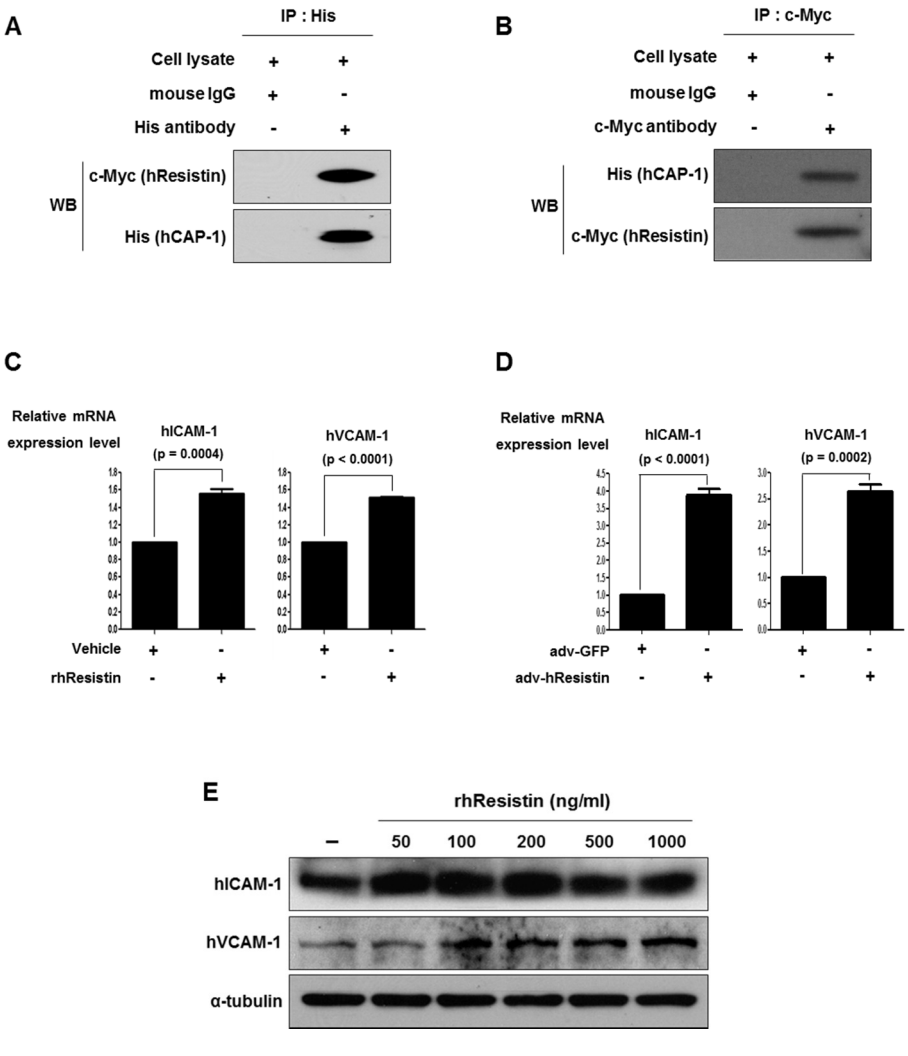
1	MADMQNLVER	LERAVGRLEA	VSHTSDMHRG	YADSPSKAGA	APYVQAFDSL
51	LAGPVAEYLK	ISKEIGGDVQ	KHAEMVHTGL	KLERALLVTA	SQCQQPAENK
101	LSDLLAPISE	QIKEVITFRE	KNRGSKLFNH	LSAVSESIQA	LGWVAMAPKP
151	GPYVKEMNDA	AMFYTNRVLK	EYKDVDKKHV	DWVKAYLSIW	TELQAYIKEF
201	HTTGLAWSKT	GPVAKELSGL	PSGPSAGSCP	PPPPPCPPPP	PVSTISCSYE
251	SASRSSLFAQ	INQGESITHA	LKHVSDDMKT	HKNPALKAQS	GPVRSGPKPF
301	SAPKPQTSPS	PKRATKKEPA	VLELEGKKWR	VENQENVSNL	VIEDTELKQV
351	AYIYKCVNTT	LQIKGKINSI	TVDNCKKLGL	VFDDVVGIVE	IINSKDVKVQ
401	VMGKVPTISI	NKTDGCHAYL	SKNSLDCEIV	SAKSSEMNVL	IPTEGGDFNE
451	FPVPEQFKTL	WNGQKLVTTV	TEIAG		

Figure 1. Identification of a human resistin receptor.

(A, B) Schematic figures of the mFc-hResistin construct. To detect a putative resistin receptor, I cloned the mFc-conjugated hResistin fusion protein. The mFc region was conjugated at the N-terminus of human resistin. (C) The expression and purification of mFc-hResistin were confirmed by western blotting with an anti-mFc-HRP antibody and by coomassie staining, respectively. (D, E) Western blot. I applied mFc-hResistin as a primary antibody to THP-1 cell extracts and identified an approximately 55 kDa protein that bound specifically to mFc-hResistin (red arrow) (D), which was not detectable after blocking the membrane with rhResistin (E). (F) Immunoprecipitation using mFc-hResitin. The incubation mixture of mFc-hResistin and THP-1 cell extracts was

precipitated using mFc-specific beads. The red arrow indicates a putative human resistin receptor at approximately 55 kDa. (G) Schematic figure of the methodology used to identify the human resistin receptor. After the immunoprecipitation and gel electrophoresis, I excised the gel band and performed MLDI-TOF analysis to identify the receptor protein. (H) Amino acid sequences of human CAP1. The sequences in bold and underlined part indicate the matched peptide sequences profiled by mass spectrometric analysis.

Figure 2.


A
IP : His
Cell lysate + +
mouse IgG + -
His antibody - +
WB
c-Myc (hResistin)
His (hCAP-1)
B
IP : c-Myc
Cell lysate + +
mouse IgG + -
c-Myc antibody - +
WB
His (hCAP-1)
c-Myc (hResistin)
C
Relative mRNA expression level
hICAM-1
(p = 0.0004)
hVCAM-1
(p < 0.0001)
Vehicle + - + -
rhResistin - + - +
D
Relative mRNA expression level
hICAM-1
(p < 0.0001)
hVCAM-1
(p = 0.0002)
adv-GFP + - + -
adv-hResistin - + - +
E
rhResistin (ng/ml)
– 50 100 200 500 1000
hICAM-1
hVCAM-1
α-tubulin

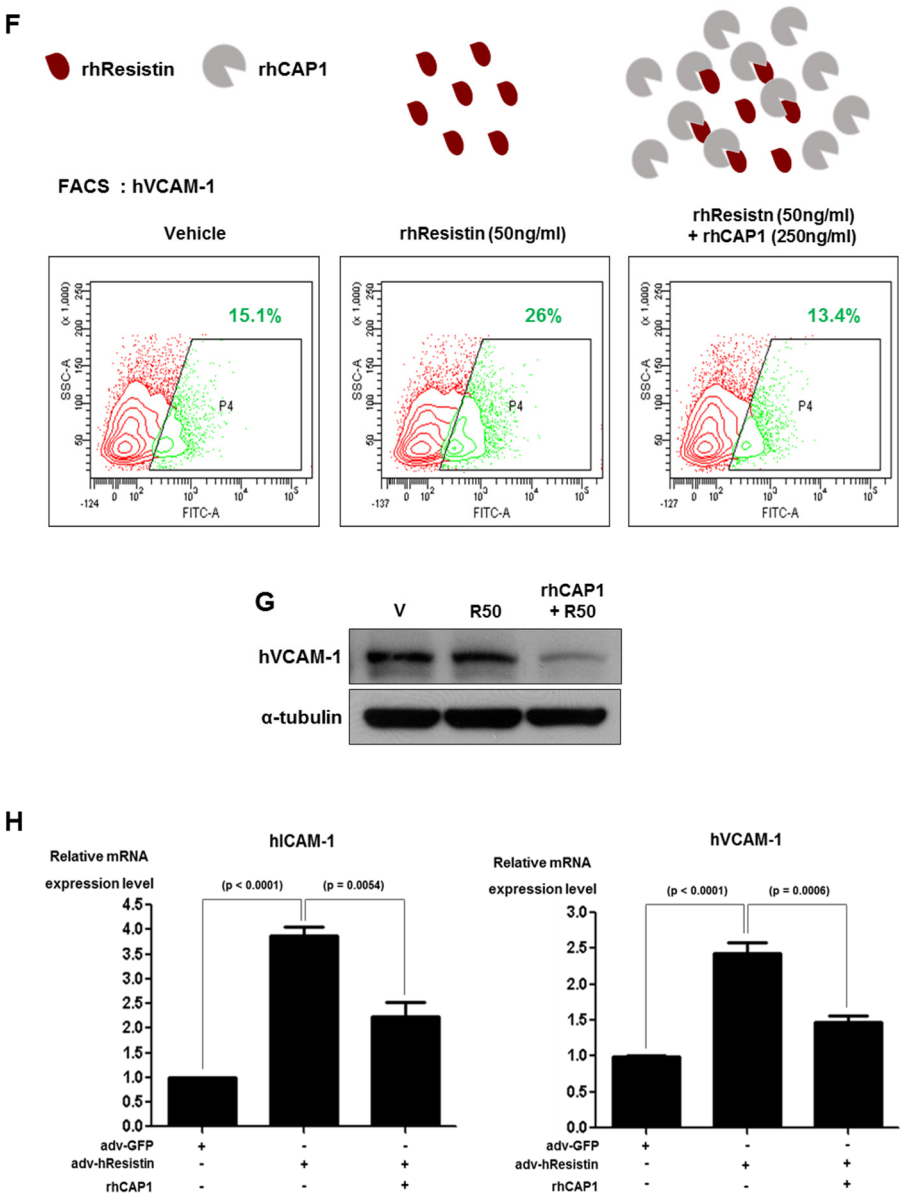


Figure 2. Human resistin binds to CAP1 in endothelial cells.

(A, B) Immunoprecipitation of human resistin and CAP1 in HUVEC. His-tagged CAP1 plasmid was transfected (A) and resistin was also overexpressed using myc-tagged adenovirus

for 48 hours (B). Cell lysates were immunoprecipitated with anti-His antibody and anti-Myc antibody, and detected by western blot. **(C, D)** The mRNA expression of hICAM-1 and hVCAM-1 by Quantitative PCR in HUVEC. The gene expression of hICAM-1 and hVCAM-1 was increased by rhResistin (50ng/ml) and resistin adenoviral transfection (n=3). **(E)** The protein expression of hICAM-1 and hVCAM-1 by western blot in HUVEC. rhResistin dose-dependently increased hICAM-1 and hVCAM-1. **(F-H)** The surface flow cytometric analyses (F), western blot (G), and quantitative PCR (H) demonstrated that rhCAP1 protein successfully neutralized the effect of resistin. The expression of hVCAM-1 on the cell surface was increased after the stimulation with rhResistin (50ng/ml), however the pre-incubation of rhResistin (50ng/ml) and rhCAP1 (250ng/ml) diminished hVCAM-1 expression by resistin (F, G). rhCAP1 also neutralized the mRNA expression of hICAM-1 and hVCAM-1 by resistin adenovirus (H).

Figure 3.

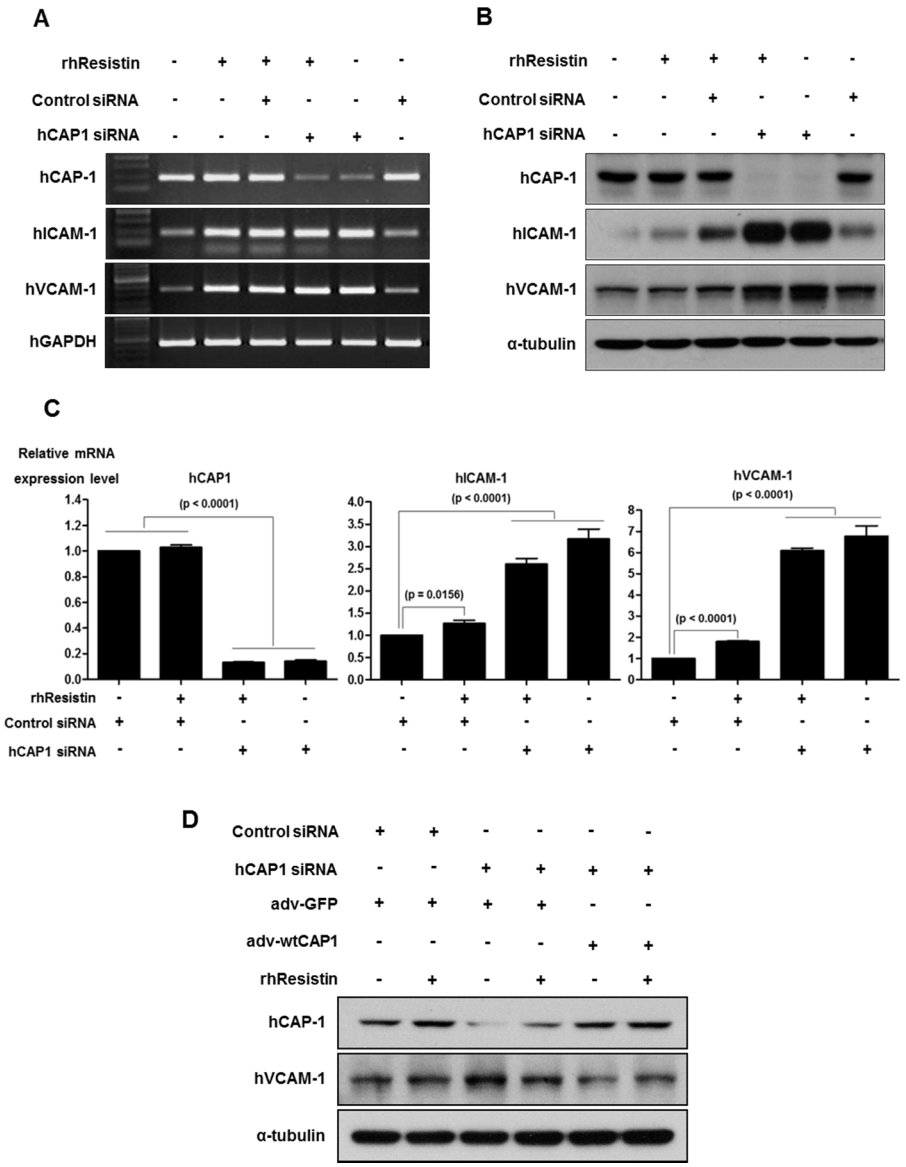
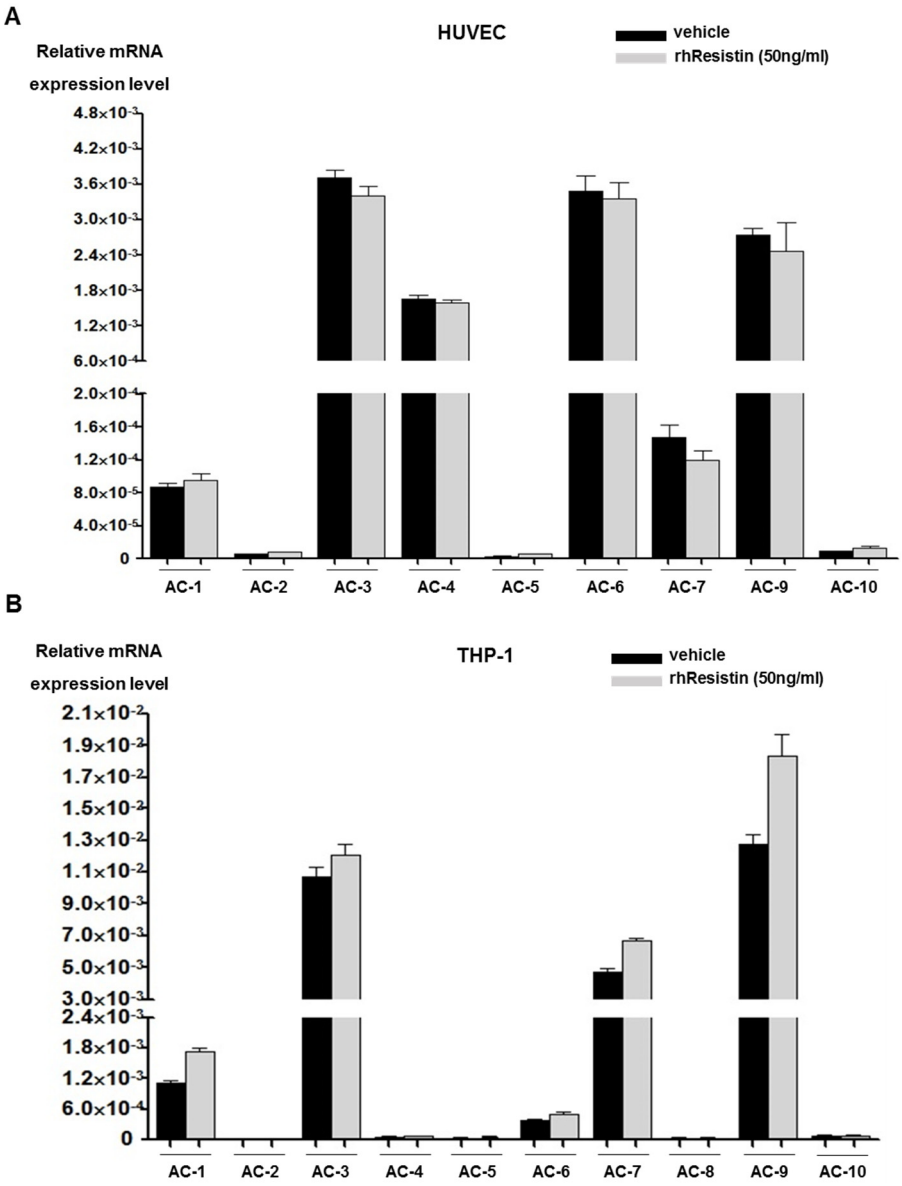


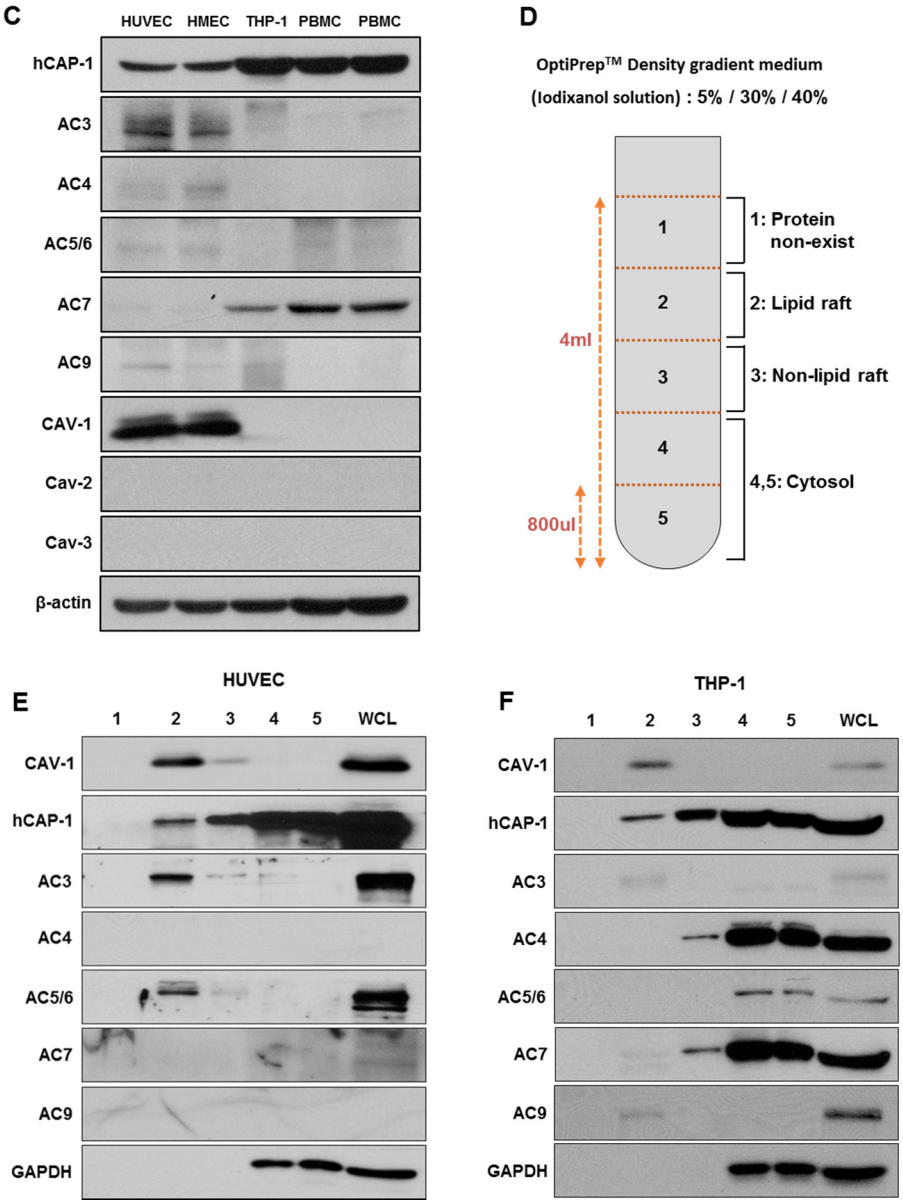
Figure 3. CAP1 knockdown augments the expression of hICAM-1 and hVCAM-1, like as a resistin.

(A-C) CAP1 knockdown increased the hICAM-1 and hVCAM-

1 expression in both mRNA (A, C, n=3) and protein level (B). CAP1 knockdown was conducted for 24 hours using siRNA and then rhResistin (100ng/ml) was treated for 24 hours. (D) CAP1 complementation returned the increased hVCAM-1 expression to a normal level. CAP1 knockdown has been achieved by siRNA for 24 hours and then CAP1 adenovirus was transfected for 24 hours. Lastly, rhResistin (100ng/ml) was treated for 12 hours. Figure 3D is quoted from unpublished our group data.

Figure 4.


A
HUVEC
vehicle
rhResistin (50ng/ml)
Relative mRNA
expression level
4.8×10-3
4.2×10-3
3.6×10-3
3.0×10-3
2.4×10-3
1.8×10-3
1.2×10-3
6.0×10-4
2.0×10-4
1.6×10-4
1.2×10-4
8.0×10-5
4.0×10-5
0
AC-1
AC-2
AC-3
AC-4
AC-5
AC-6
AC-7
AC-9
AC-10
B
THP-1
vehicle
rhResistin (50ng/ml)
Relative mRNA
expression level
2.1×10-2
1.9×10-2
1.7×10-2
1.5×10-2
1.3×10-2
1.1×10-2
9.0×10-3
7.0×10-3
5.0×10-3
3.0×10-3
2.4×10-3
1.8×10-3
1.2×10-3
6.0×10-4
0
AC-1
AC-2
AC-3
AC-4
AC-5
AC-6
AC-7
AC-8
AC-9
AC-10


C
HUVEC
HMEC
THP-1
PBMC
PBMC
hCAP-1
AC3
AC4
AC5/6
AC7
AC9
CAV-1
Cav-2
Cav-3
β-actin
D
OptiPrep™ Density gradient medium
(Iodixanol solution) : 5% / 30% / 40%
4ml
800ul
1
2
3
4
5
1: Protein non-exist
2: Lipid raft
3: Non-lipid raft
4,5: Cytosol
E
HUVEC
1
2
3
4
5
WCL
CAV-1
hCAP-1
AC3
AC4
AC5/6
AC7
AC9
GAPDH
F
THP-1
1
2
3
4
5
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AC3
AC4
AC5/6
AC7
AC9
GAPDH

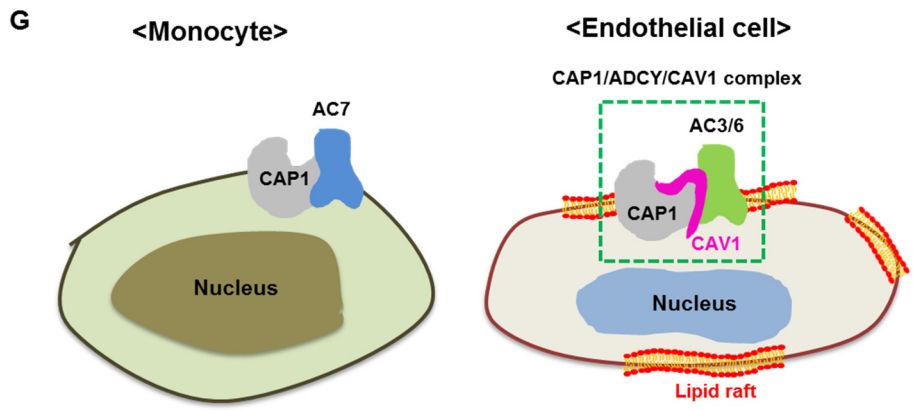
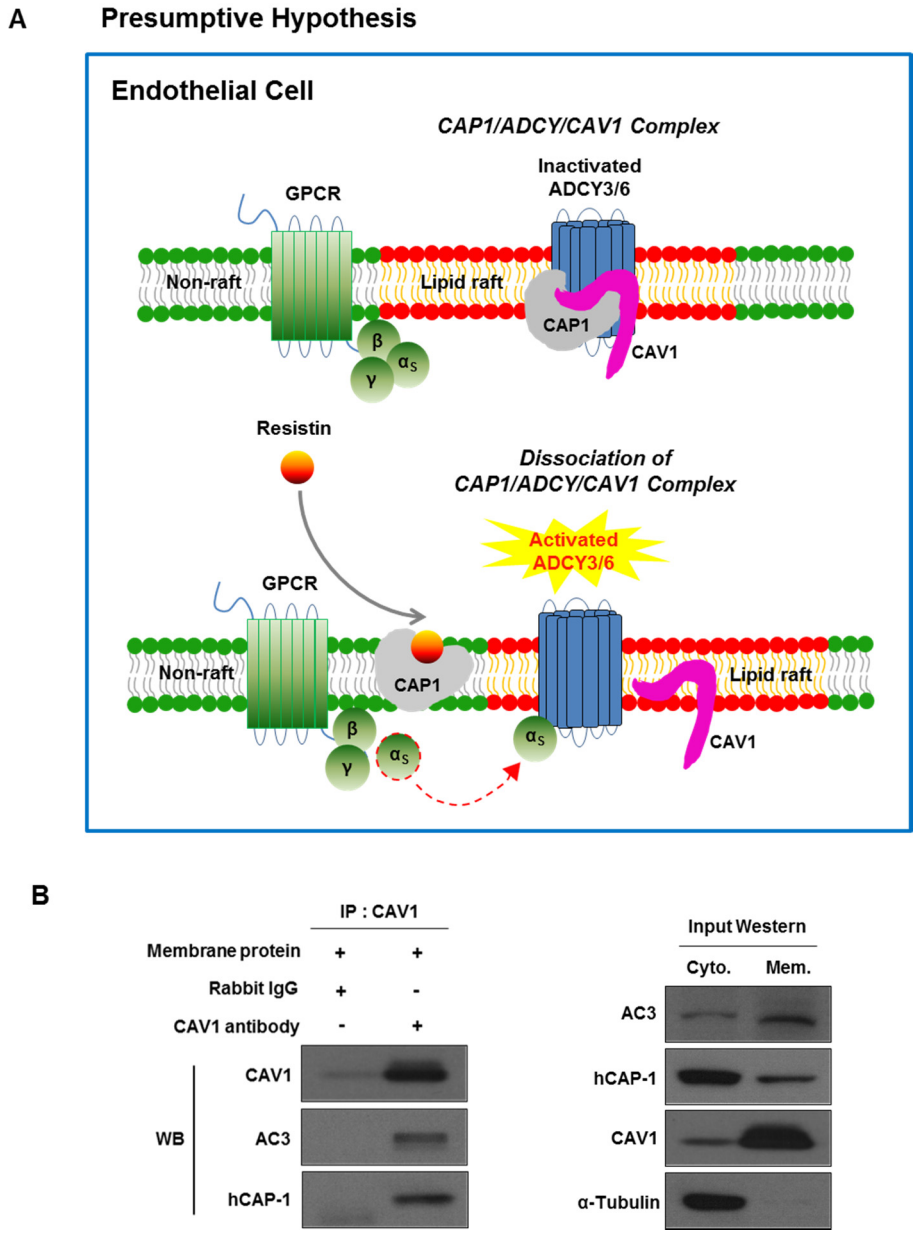


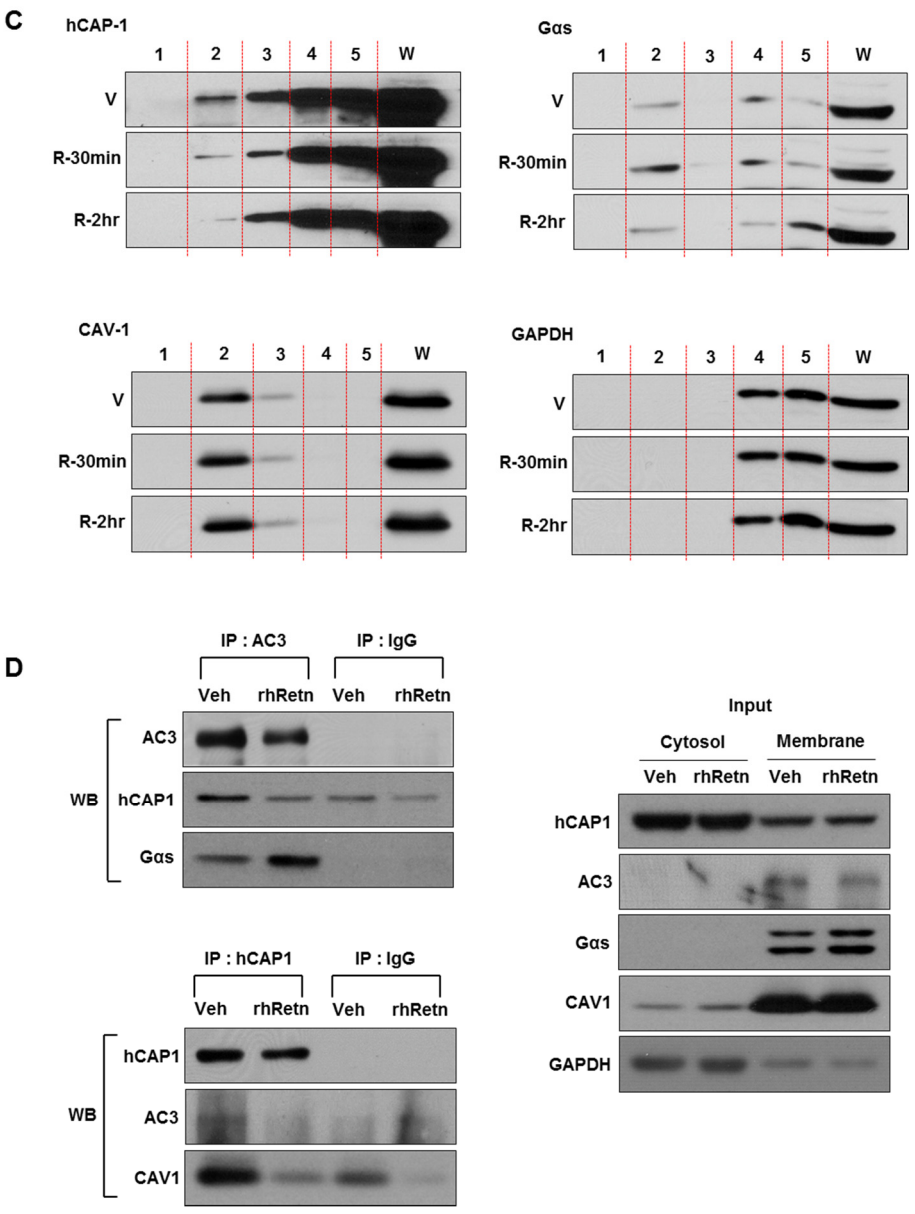
Figure 4. Adenylyl cyclase isoforms are differentially expressed in endothelial cells and monocytes.

(A, B) Expression profiles of adenylyl cyclase isoforms by quantitative PCR. HUVEC and THP1 showed different profiles of adenylyl cyclase isoforms. At mRNA level, AC3, AC4, AC6, and AC9 showed relatively high expressions in HUVEC (A), while AC3, AC7, and AC9 were predominant in THP1 (B). Relative mRNA expression levels were normalized to GAPDH. (C) Western bot of adenylyl cyclase isoforms. At protein level, AC3 was dominant in endothelial cells, and AC7 was dominant in monocytes. Also, CAV1 was enriched in only endothelial cells. (D) Illustration of OptiPrep Density gradient method (1 : protein non-exist, 2 : lipid raft, 3 : non-lipid raft, 4, 5 : cytosol). (E, F) A localization of adenylyl cyclase by OptiPrep

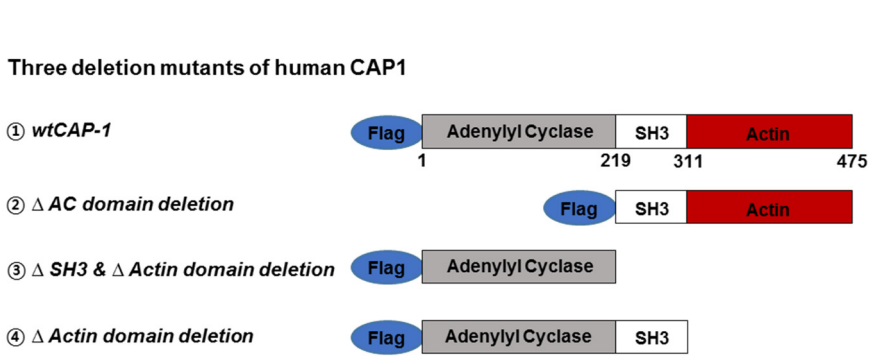
Density gradient centrifugation. AC3 and AC6 were localized at lipid rafts in HUVEC (E) and AC7 was localized at non-lipid rafts in THP1 (F). Furthermore, CAP1 was partly co-localized with CAV1, AC3, and AC6 at lipid raft in HUVEC. (G) A Schematic figure of membrane complexes that bind to CAP1 in monocyte and endothelial cell. Figure 4E and F are quoted from unpublished our group data.

Figure 5.

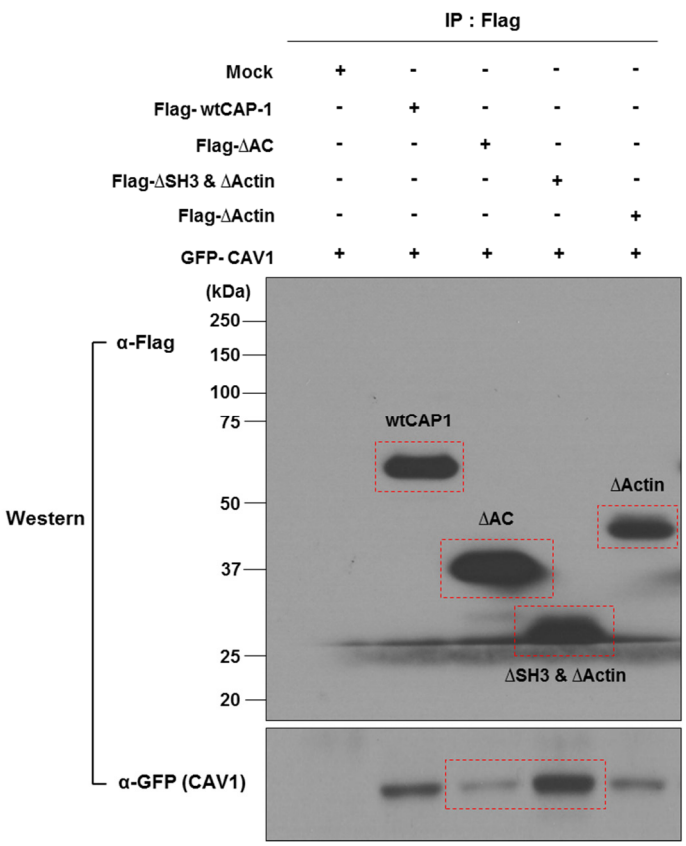

A
Presumptive Hypothesis
Endothelial Cell
CAP1/ADCY/CAV1 Complex
Inactivated
ADCY3/6
GPCR
Non-raft
Lipid raft
CAP1
CAV1
β
γ
αS
Resistin
Dissociation of
CAP1/ADCY/CAV1 Complex
Activated
ADCY3/6
GPCR
Non-raft
CAP1
Lipid raft
CAV1
β
γ
αS
αS
B
IP : CAV1
Membrane protein + +
Rabbit IgG + -
CAV1 antibody - +
WB
CAV1
AC3
hCAP-1
Input Western
Cyto. Mem.
AC3
hCAP-1
CAV1
α-Tubulin


C
hCAP-1
1 2 3 4 5 W
V
R-30min
R-2hr
Gαs
1 2 3 4 5 W
V
R-30min
R-2hr
CAV-1
1 2 3 4 5 W
V
R-30min
R-2hr
GAPDH
1 2 3 4 5 W
V
R-30min
R-2hr
D
IP : AC3
IP : IgG
Veh rhRetn Veh rhRetn
WB
AC3
hCAP1
Gαs
IP : hCAP1
IP : IgG
Veh rhRetn Veh rhRetn
WB
hCAP1
AC3
CAV1
Input
Cytosol
Membrane
Veh rhRetn Veh rhRetn
hCAP1
AC3
Gαs
CAV1
GAPDH

E


Three deletion mutants of human CAP1
① wtCAP-1
Flag
Adenylyl Cyclase
SH3
Actin
1
219
311
475
② Δ AC domain deletion
Flag
SH3
Actin
③ Δ SH3 & Δ Actin domain deletion
Flag
Adenylyl Cyclase
④ Δ Actin domain deletion
Flag
Adenylyl Cyclase
SH3

F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Flag- wtCAP-1 - + - - -
Flag-ΔAC - - + - -
Flag-ΔSH3 & ΔActin - - - + -
Flag-ΔActin - - - - +
GFP- CAV1 + + + + +
(kDa)
250
150
100
75
50
37
25
20
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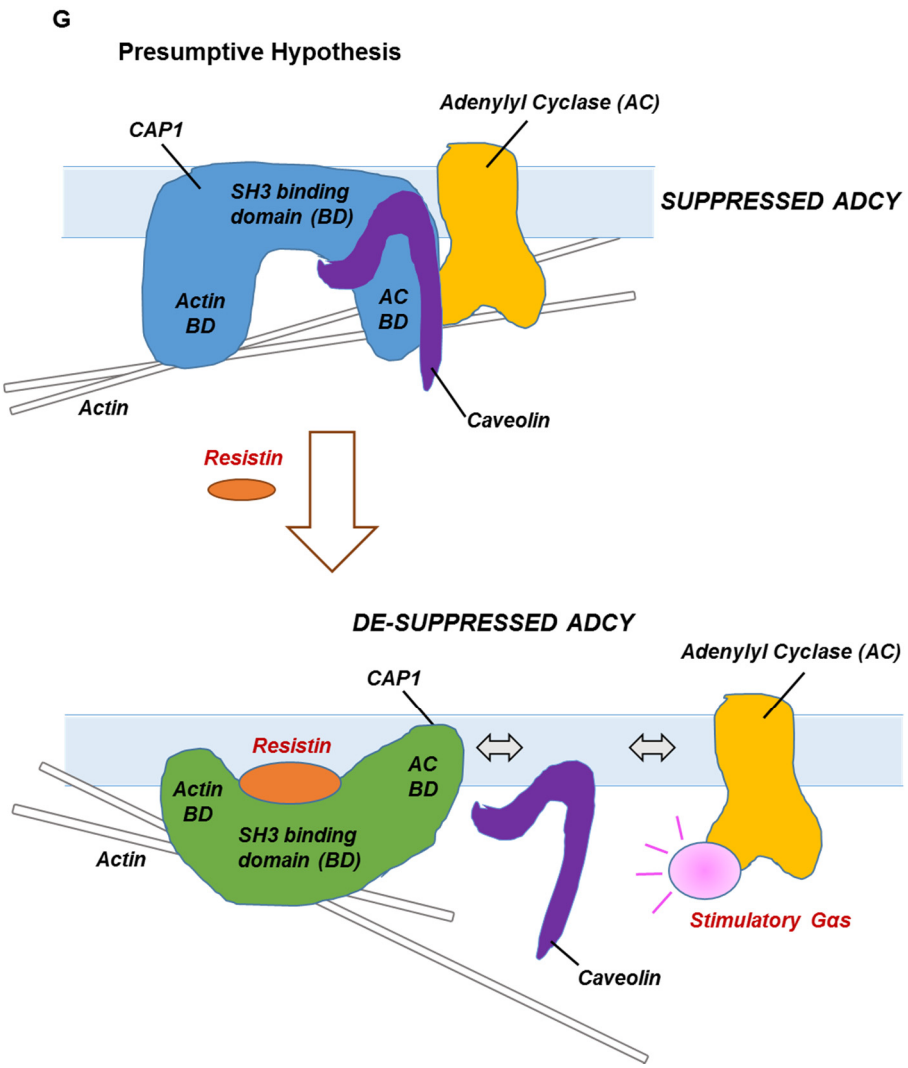
α-GFP (CAV1)
wtCAP1
ΔAC
ΔActin
ΔSH3 & ΔActin



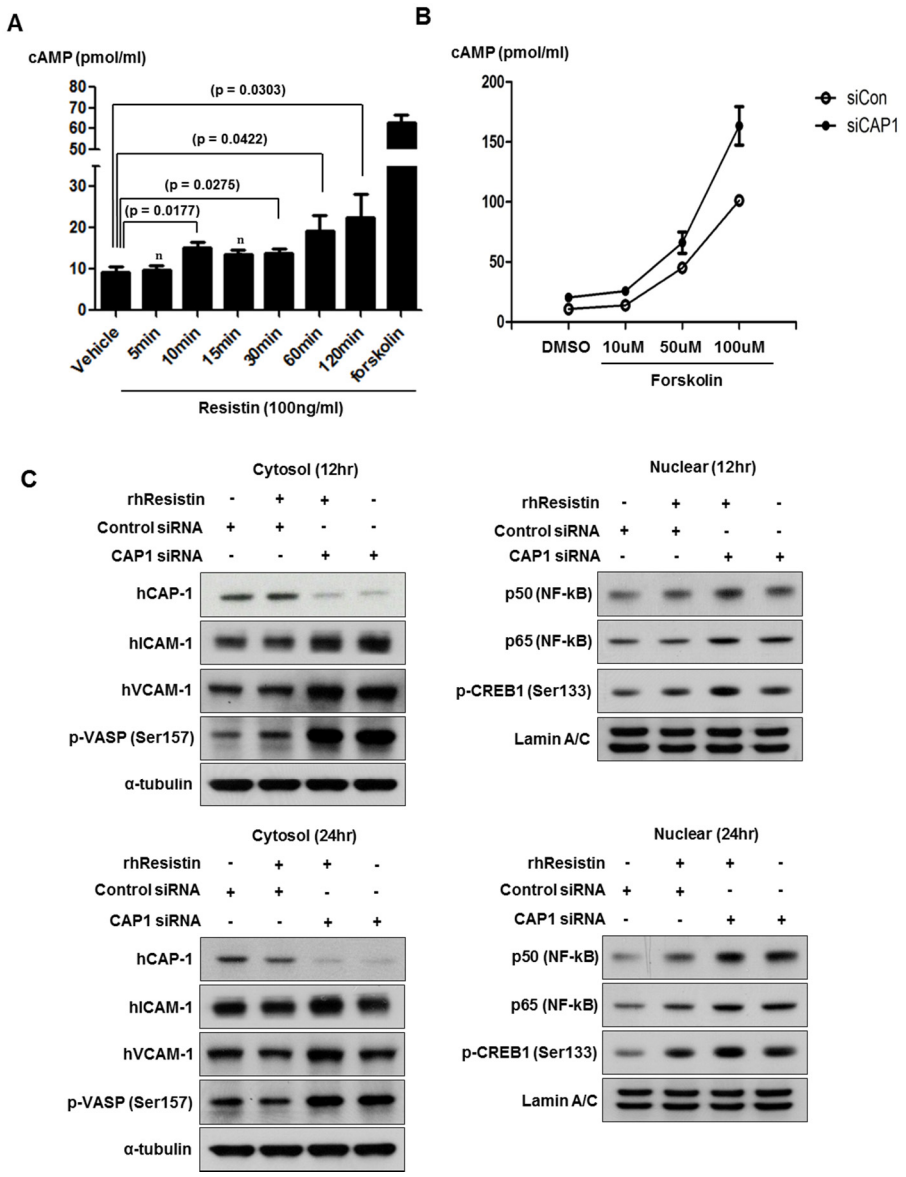
Fiuge 5. Resistin dissociates the CAP1-AC3-CAV1 complex in lipid raft and results in the activation of adenylyl cyclase.

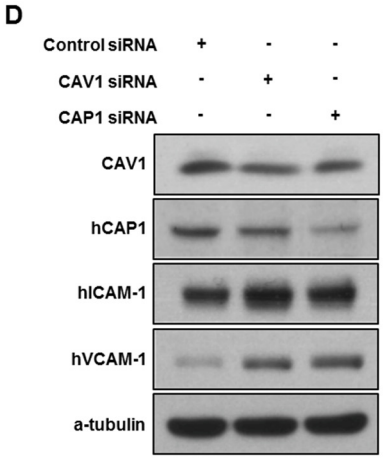
(A) The presumptive schematic figure of CAP1-AC3-CAV1 dissociation. AC3 might be arrested in CAP1-CAV1 complex in lipid raft, but resistin binding to CAP1 could dissociate this suppressive assembly and result in activation of adenylyl

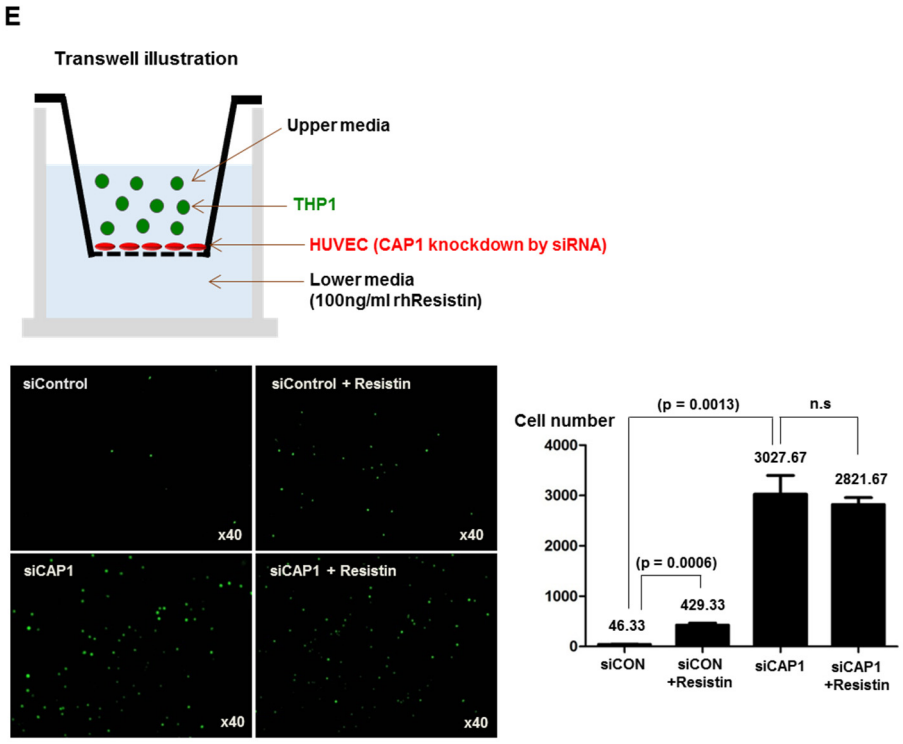
cyclase. **(B)** CAP1, AC3 and CAV1 interact each other in the plasma membrane of HUVEC. Through immunoprecipitation using anti-CAV1 antibody, AC3 and CAP1 were bound to CAV1 in the cell membrane. **(C)** Resistin induces the translocation of CAP1 from lipid raft to non-lipid raft. CAP1 was disappeared from lipid raft just after 30 minutes of resistin treatment, and finally vanished within 2 hours. At the same time, Gas was recruited to lipid rafts which AC3 was located. Resistin (1ug/ml) was treated for 30 minutes. Lipid raft was isolated by OptiPrep Density gradient centrifugation. **(D)** Immunoprecipitation of AC3 and CAP1. The assembly of CAP1-AC3-CAV1 was dissociated by resistin. After resistin treatment, CAP1 and AC3 were dissociated from each other, and CAV1 was also separated from CAP1. But Gαs binding to AC3 was increased by resistin. **(E)** Schematic illustration of the three deletion mutants of human CAP1. **(F)** *In vitro* binding assay using each CAP1 deletion mutant and CAV1. After the overexpression of Flag-tagged CAP1 mutants and GFP-tagged CAV1 in HEK 293T cells, whole cell lysates were immunoprecipitated with Flag antibody. CAV1 band was detectable slightly in the ΔAC BD deletion mutant, whereas it

was detected significantly in the ⊿SH3⊿Actin BD deletion mutant. These observations imply that CAV1 seems to bind CAP1 via the AC BD. (G) The putative schematic figure of CAP1 conformational change. AC3 is suppressed through the interaction CAP1 and CAV1 at the basal status. But, resistin binding to SH3 BD of CAP1 induces the conformational change of CAP1, and results in the dissociation of CAP1 and CAV1 from AC3. Finally, AC3 liberated from CAP1-AC3-CAV1 suppressive complex is activated by Gαs binding. Figure 5C is quoted from unpublished our group data.

Figure 6.


A
cAMP (pmol/ml)
(p = 0.0303)
(p = 0.0422)
(p = 0.0275)
(p = 0.0177)
Vehicle
5min
10min
15min
30min
60min
120min
forskolin
Resistin (100ng/ml)
B
cAMP (pmol/ml)
siCon
siCAP1
DMSO
10uM
50uM
100uM
Forskolin
C
Cytosol (12hr)
rhResistin
Control siRNA
CAP1 siRNA
hCAP-1
hICAM-1
hVCAM-1
p-VASP (Ser157)
α-tubulin
Nuclear (12hr)
p50 (NF-kB)
p65 (NF-kB)
p-CREB1 (Ser133)
Lamin A/C
Cytosol (24hr)
Nuclear (24hr)


D
Control siRNA + - -
CAV1 siRNA - + -
CAP1 siRNA - - +
CAV1
hCAP1
hICAM-1
hVCAM-1
a-tubulin


E
Transwell illustration
Upper media
THP1
HUVEC (CAP1 knockdown by siRNA)
Lower media
(100ng/ml rhResistin)
siControl
x40
siControl + Resistin
x40
siCAP1
x40
siCAP1 + Resistin
x40
Cell number
(p = 0.0013)
n.s
(p = 0.0006)
4000
3000
2000
1000
0
46.33
429.33
3027.67
2821.67
siCON
siCON +Resistin
siCAP1
siCAP1 +Resistin

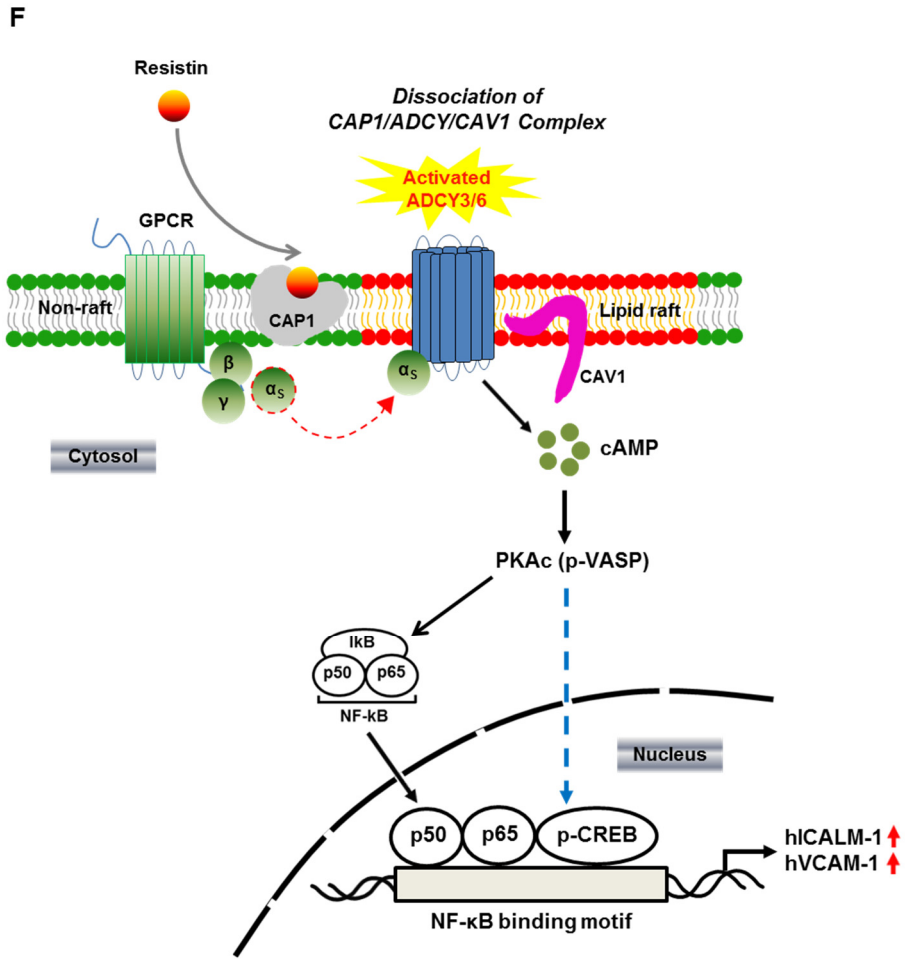
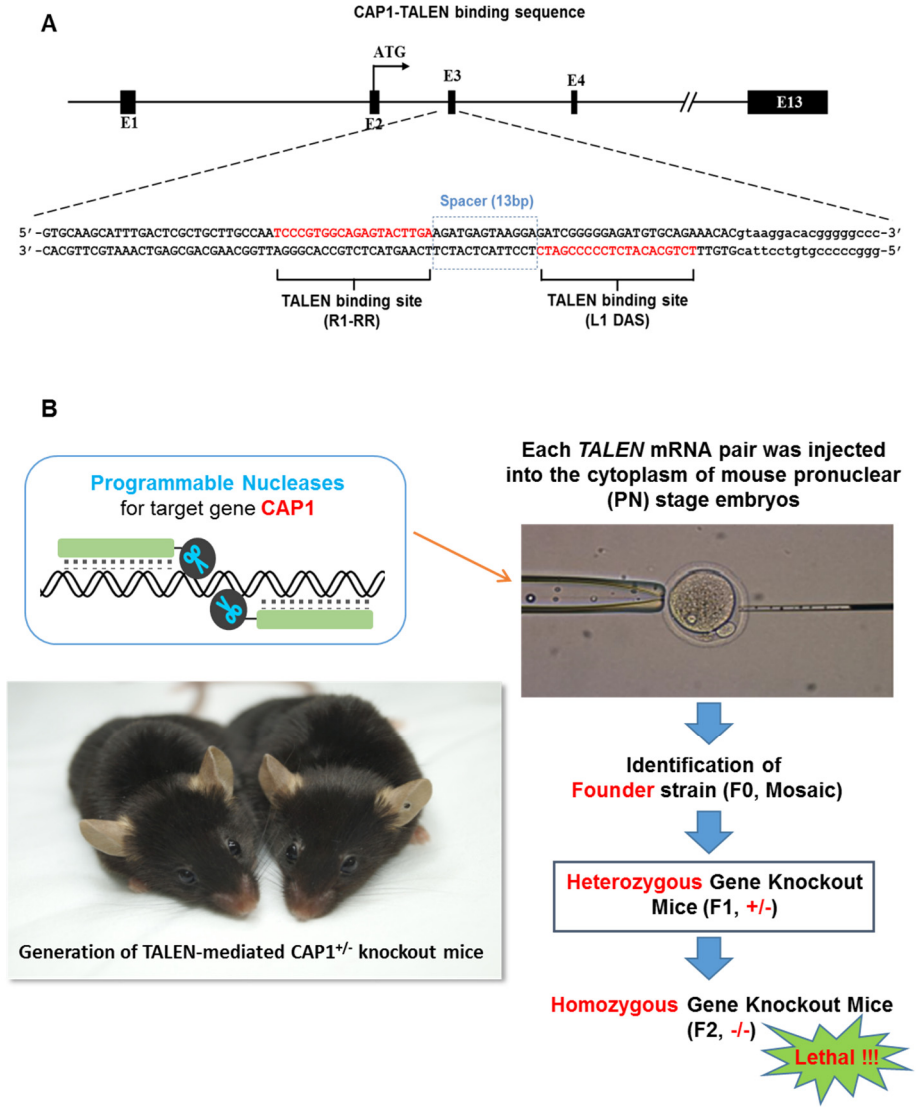


Figure 6. CAP1 knockdown activates the cAMP-PKA-NF-κB signaling pathway, increases the expression of hICAM-1 and hVCAM-1, and facilitates the TEM of monocytes.

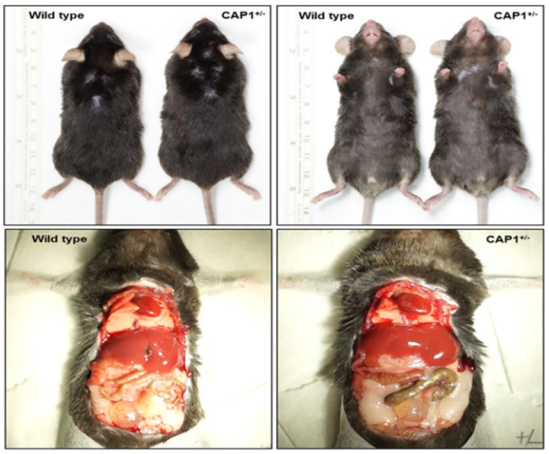
(A, B) cAMP synthesis by resistin treatment or CAP1 knockdown in HUVEC. Resistin significantly increased cAMP levels (A). Also, CAP1 knockdown stimulated cAMP synthesis and was more sensitive to forskolin, the direct agonist of AC

(B). **(C)** CAP1 knockdown increased p-VASP (Ser157), hICAM-1 and hVCAM-1 in cytosol fraction. In addition, NF-κB nuclear translocation (p50 and p65) and CREP phosphorylation (Ser133) were increased by CAP1 depletion. **(D)** CAV1 knockdown by siRNA also increased hICAM-1 and hVCAM-1, like as CAP1 knockdown. **(E)** Transwell assay of monocyte transmigration. HUVECs were manipulated with siRNA for 24 hours, and THP1 cells were pre-activated by rhResistin (100ng/ml) for an effective transmigration. Resistin treatment and CAP1 knockdown drastically facilitated TEM of monocytes. **(F)** The schematic signaling figure of hICAM-1 and hVCAM-1 expression by CAP1 depletion. Figure 6A, B, C and E are quoted from unpublished our group data.

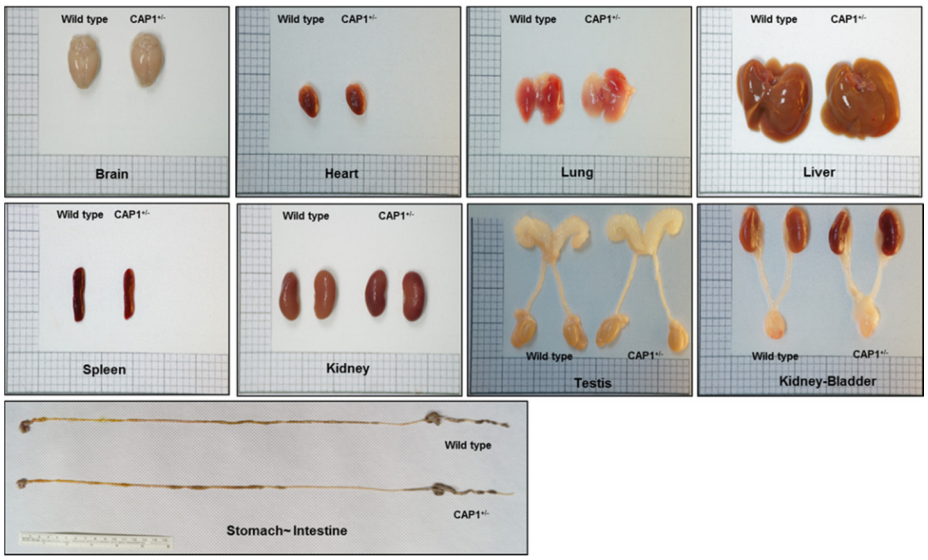
Figure 7.


A
CAP1-TALEN binding sequence
ATG
E1
E2
E3
E4
E13
Spacer (13bp)
5′-GTGCAAGCATTTGACTCGCTGCTTGCCAATCCCGTGGCAGAGTACTTGAAGATGAGTAAGGAGATCGGGGGAGATGTGCAGAAACACgtaaggacacgggggccc-3′
3′-CACGTTCGTAAACTGAGCGACGAACGGTTAGGGCACCGTCTCATGAACTTCTACTCATTCCTCTAGCCCCCTCTACACGTCTTTGTGcattcctgtgcccccggg-5′
TALEN binding site
(R1-RR)
TALEN binding site
(L1 DAS)
B
Programmable Nucleases
for target gene CAP1
Each TALEN mRNA pair was injected into the cytoplasm of mouse pronuclear (PN) stage embryos
Generation of TALEN-mediated CAP1+/- knockout mice
Identification of
Founder strain (F0, Mosaic)
Heterozygous Gene Knockout
Mice (F1, +/-)
Homozygous Gene Knockout Mice
(F2, -/-)
Lethal !!!

C


Wild type
CAP1+/-
Wild type
CAP1+/-
Wild type
CAP1+/-

D


Wild type
CAP1+/-
Brain
Heart
Lung
Liver
Spleen
Kidney
Testis
Kidney-Bladder
Wild type
CAP1+/-
Stomach~Intestine

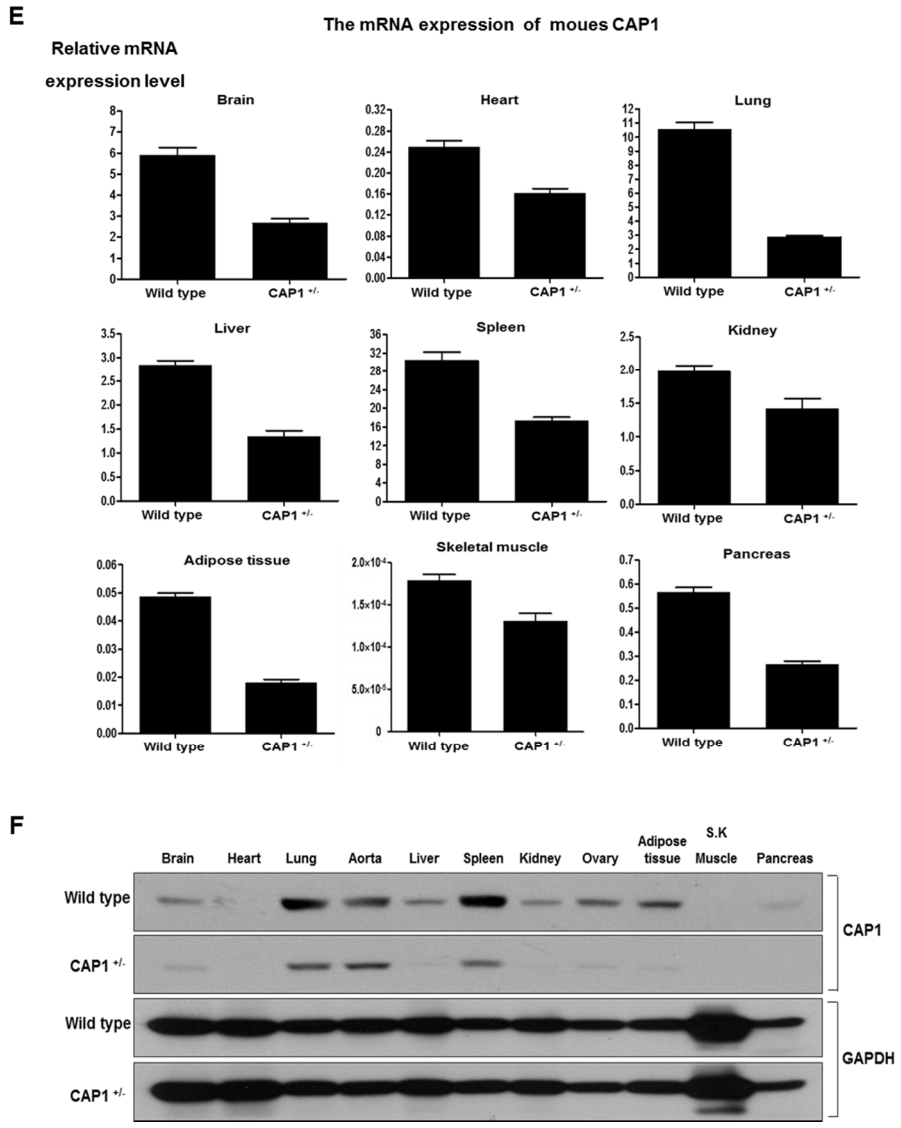
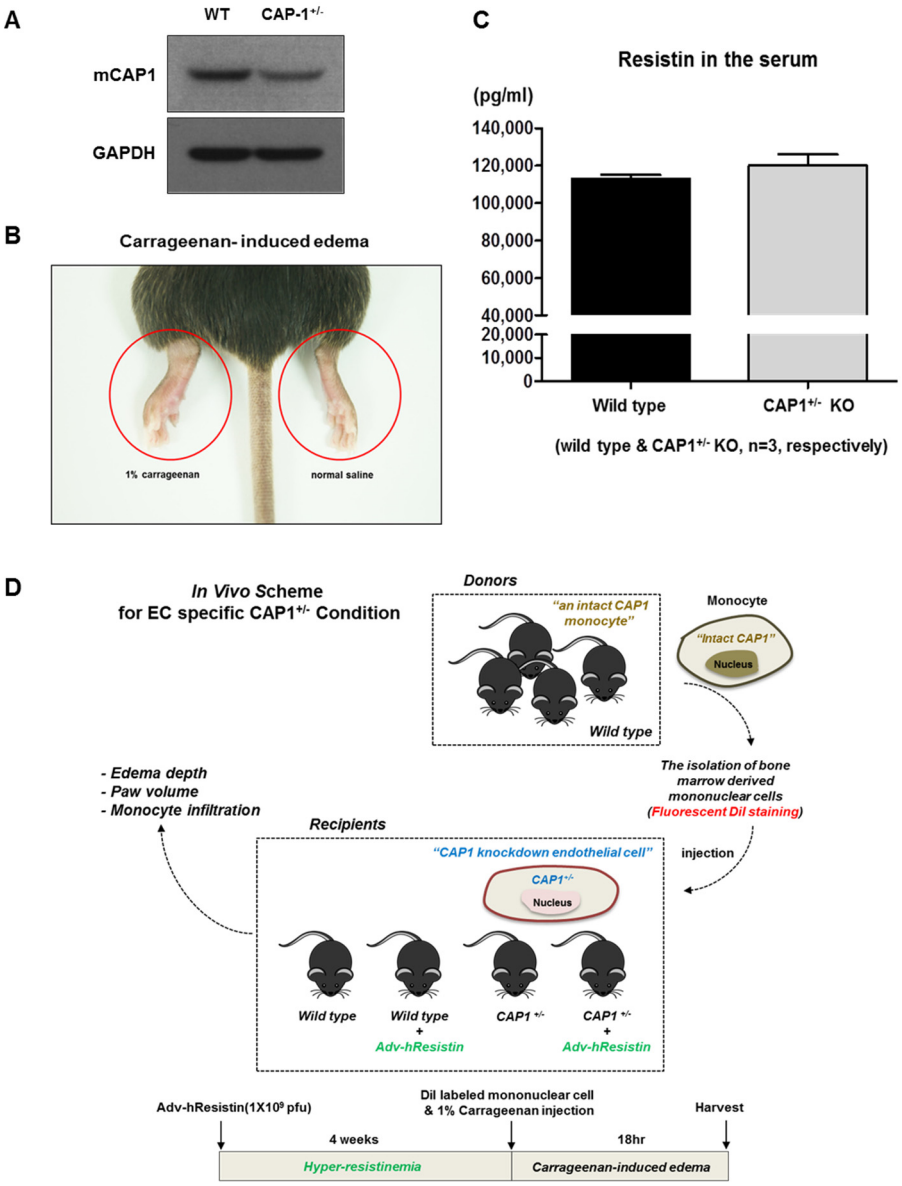


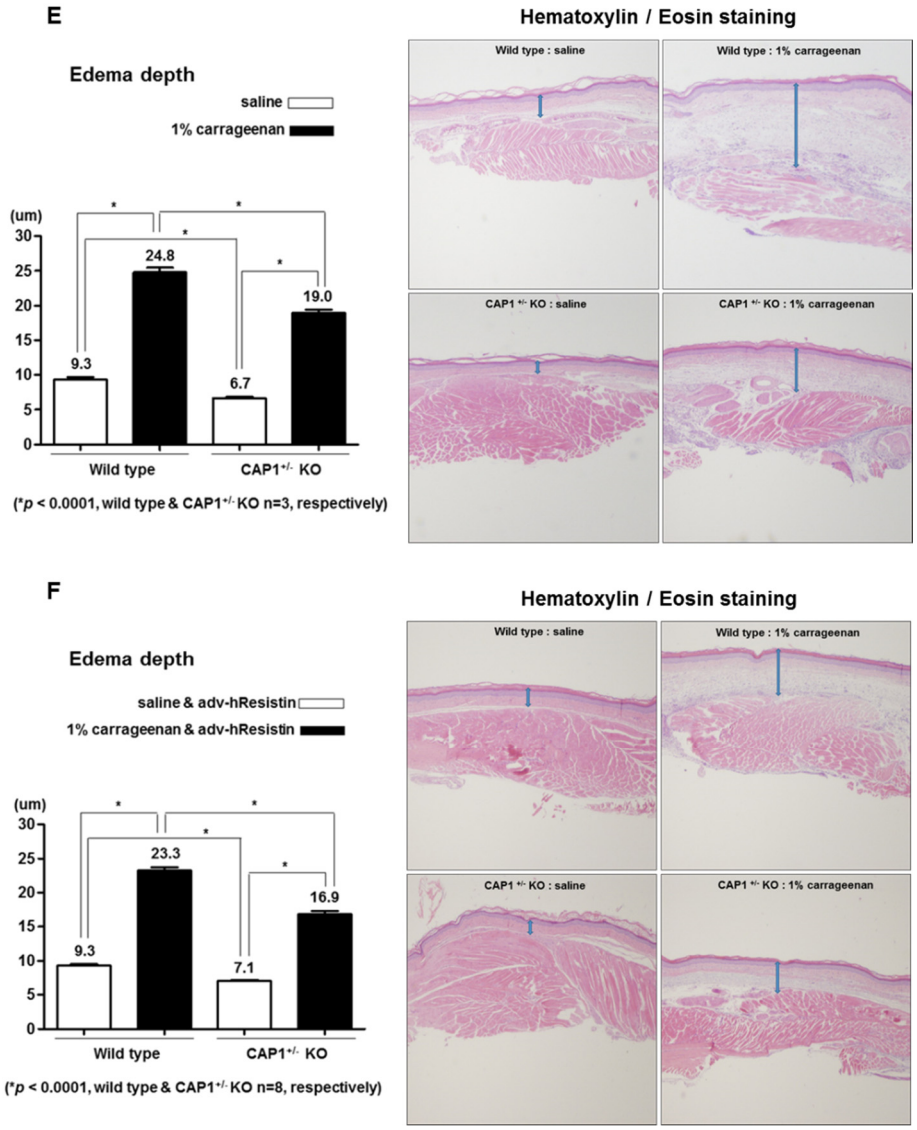
Figure 7. Generation of TALEN-mediated CAP1$^{+/-}$ knockout mice.

(A) Schematic figure of the highly specific TALENs which targets exon3 of mouse CAP1. (B) Each TALEN mRNA pair was injected into the cytoplasm of mouse pronuclear stage

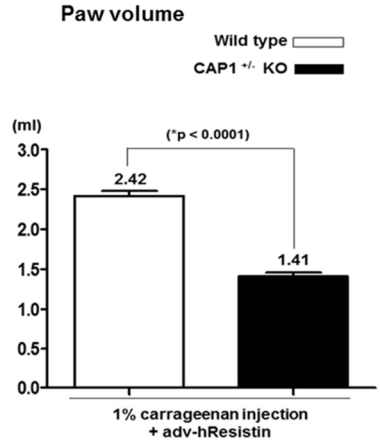
embryos. Heterozygous CAP1 knockout mice ($CAP1^{+/-}$) were obtained, but homozygous CAP1 knockout mice ($CAP1^{-/-}$) were not born. It is presumed that homozygous CAP1 knockout mice seem to be lethal. **(C, D)** In gross phenotype of $CAP1^{+/-}$ mice, no abnormal abnormalities were found compared to wild type mice. **(E, F)** The mRNA and protein expression of $CAP1^{+/-}$ mice. The mouse CAP1 in various organs of $CAP1^{+/-}$ mice was reduced by half compared to wild type mice.

Figure 8.


A
WT
CAP-1+/-
mCAP1
GAPDH
B
Carrageenan- induced edema
1% carrageenan
normal saline
C
Resistin in the serum
(pg/ml)
140,000
120,000
100,000
80,000
60,000
40,000
20,000
10,000
0
Wild type
CAP1+/- KO
(wild type & CAP1+/- KO, n=3, respectively)
D
In Vivo Scheme
for EC specific CAP1+/- Condition
Donors
"an intact CAP1 monocyte"
Wild type
Monocyte
"Intact CAP1"
Nucleus
The isolation of bone marrow derived mononuclear cells (Fluorescent DiI staining)
injection
- Edema depth
- Paw volume
- Monocyte infiltration
Recipients
"CAP1 knockdown endothelial cell"
CAP1+/-
Nucleus
Wild type
Wild type + Adv-hResistin
CAP1 +/-
CAP1 +/- + Adv-hResistin
Adv-hResistin(1X10⁹ pfu)
Dil labeled mononuclear cell & 1% Carrageenan injection
Harvest
4 weeks
18hr
Hyper-resistinemia
Carrageenan-induced edema

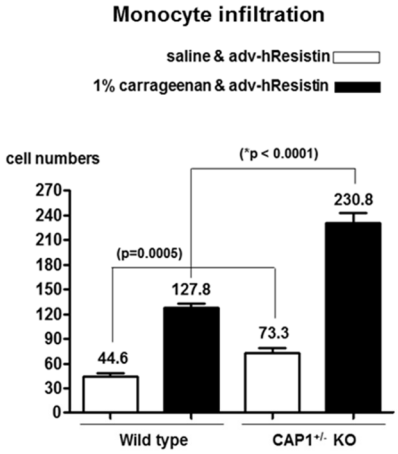

E
Edema depth
saline
1% carrageenan
(um)
30
25
20
15
10
5
0
24.8
19.0
9.3
6.7
Wild type
CAP1+/- KO
(*p < 0.0001, wild type & CAP1+/- KO n=3, respectively)
Hematoxylin / Eosin staining
Wild type : saline
Wild type : 1% carrageenan
CAP1 +/- KO : saline
CAP1 +/- KO : 1% carrageenan
F
Edema depth
saline & adv-hResistin
1% carrageenan & adv-hResistin
(um)
30
25
20
15
10
5
0
23.3
16.9
9.3
7.1
Wild type
CAP1+/- KO
(*p < 0.0001, wild type & CAP1+/- KO n=8, respectively)
Hematoxylin / Eosin staining
Wild type : saline
Wild type : 1% carrageenan
CAP1 +/- KO : saline
CAP1 +/- KO : 1% carrageenan

G



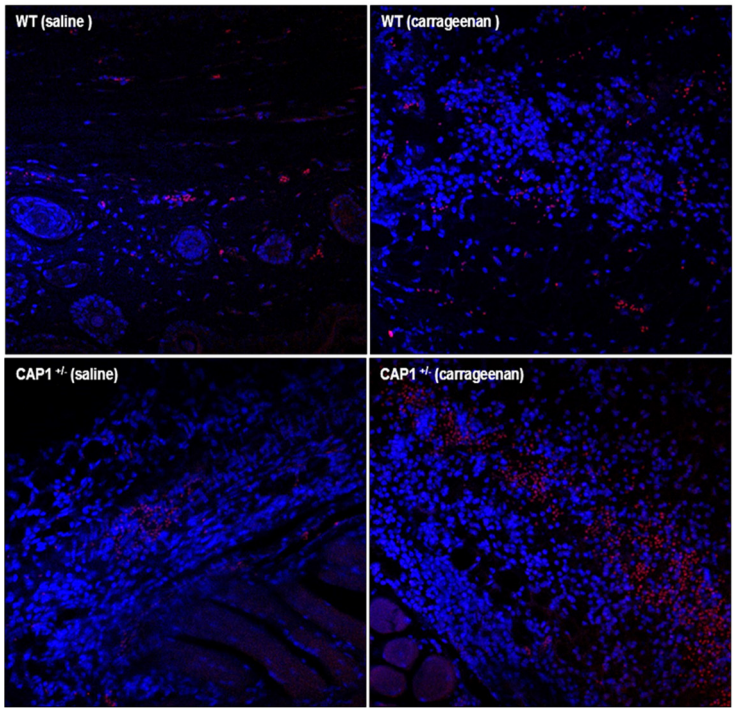
(*p < 0.0001, wild type & CAP1$^{+/-}$ KO n=8, respectively)

H

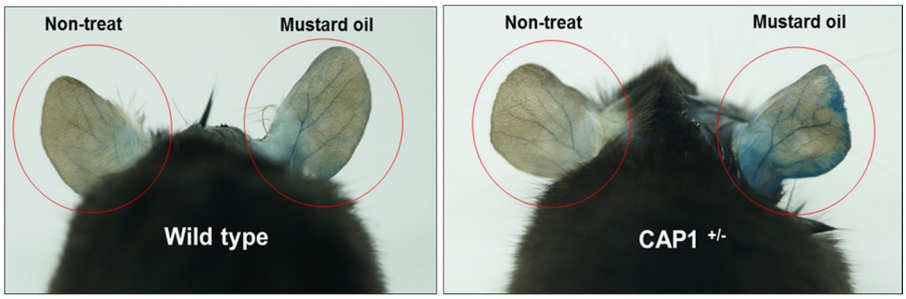


(*p < 0.0001, wild type & CAP1$^{+/-}$ KO n=4, respectively)

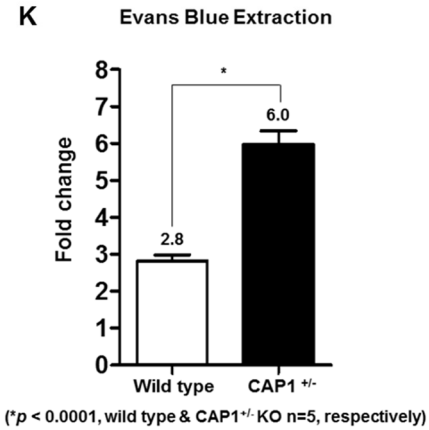
I



J

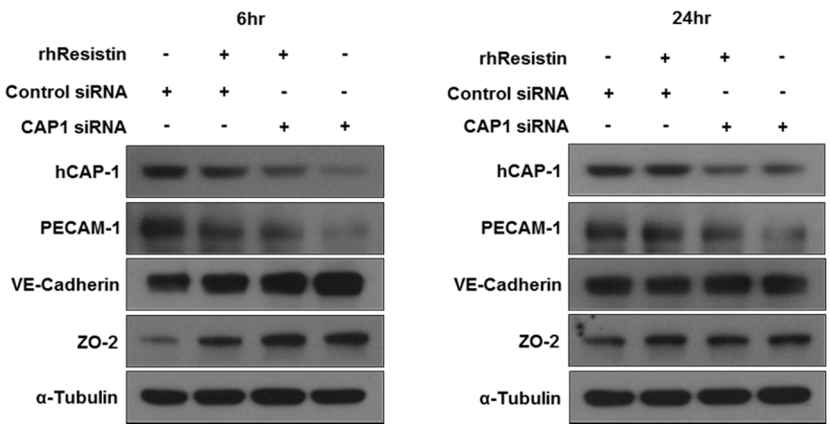


K



(*p < 0.0001, wild type & CAP1$^{+/-}$ KO n=5, respectively)

L



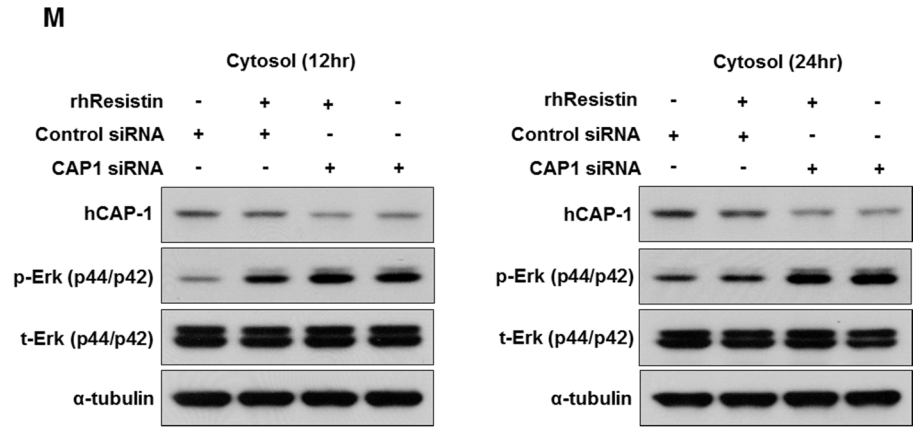


Figure 8. In Vivo analysis using CAP1$^{+/-}$ knockout mice.

As the TEM animal model, the carrageenan-induced edema formation and the mustard oil-induced vascular leakage model were used. (A) The CAP1 expression was decreased in the monocytes of CAP1$^{+/-}$ mice. (B) The representative figure of carrageenan-induced edema formation. (C) *In vivo*, the chronic hyper-resistinemia was induced by resistin adenovirus delivery for four weeks. Resistin level in serum was measured by using ELISA. (D) *In Vivo* Scheme for endothelial cell specific CAP1$^{+/-}$ condition. Edema depth, paw volume and monocyte infiltration were measured in wild type mice and CAP1$^{+/-}$ mice, respectively. (E) The edema depth of CAP1$^{+/-}$ mice was less than that of wild type mice. (F) Also, when adenovirus-mediated chronic hyper-resistinemia was induced,

the edema depth was decreased in the CAP1$^{+/-}$ mice compared to wild type mice. **(G)** The paw volume by carrageenan was significantly reduced about a half in the CAP1$^{+/-}$ mice compared to wild type mice. **(H, I)** On the other hand, the tissue invasion of monocytes with intact CAP1 was increased about twice in the CAP1$^{+/-}$ mice compared to wild type mice. Figure I indicates the representative confocal image of infiltrated DiI positive monocytes. **(J)** Representative pictures of mice subjected to mustard oil-induced vascular leakage. **(K)** The leakage of evans blue dye in the ear of CAP1$^{+/-}$mice was twice as high as that of wild type mice. **(L, M)** The change of junctional protein by CAP knockdown. PECAM-1 was decreased, and VE-cadherin and ZO-2 were increased in HUVEC by CAP1 depletion using siRNA. Erk phosphorylation was significantly increased by CAP1 knockdown.

Figure 9.

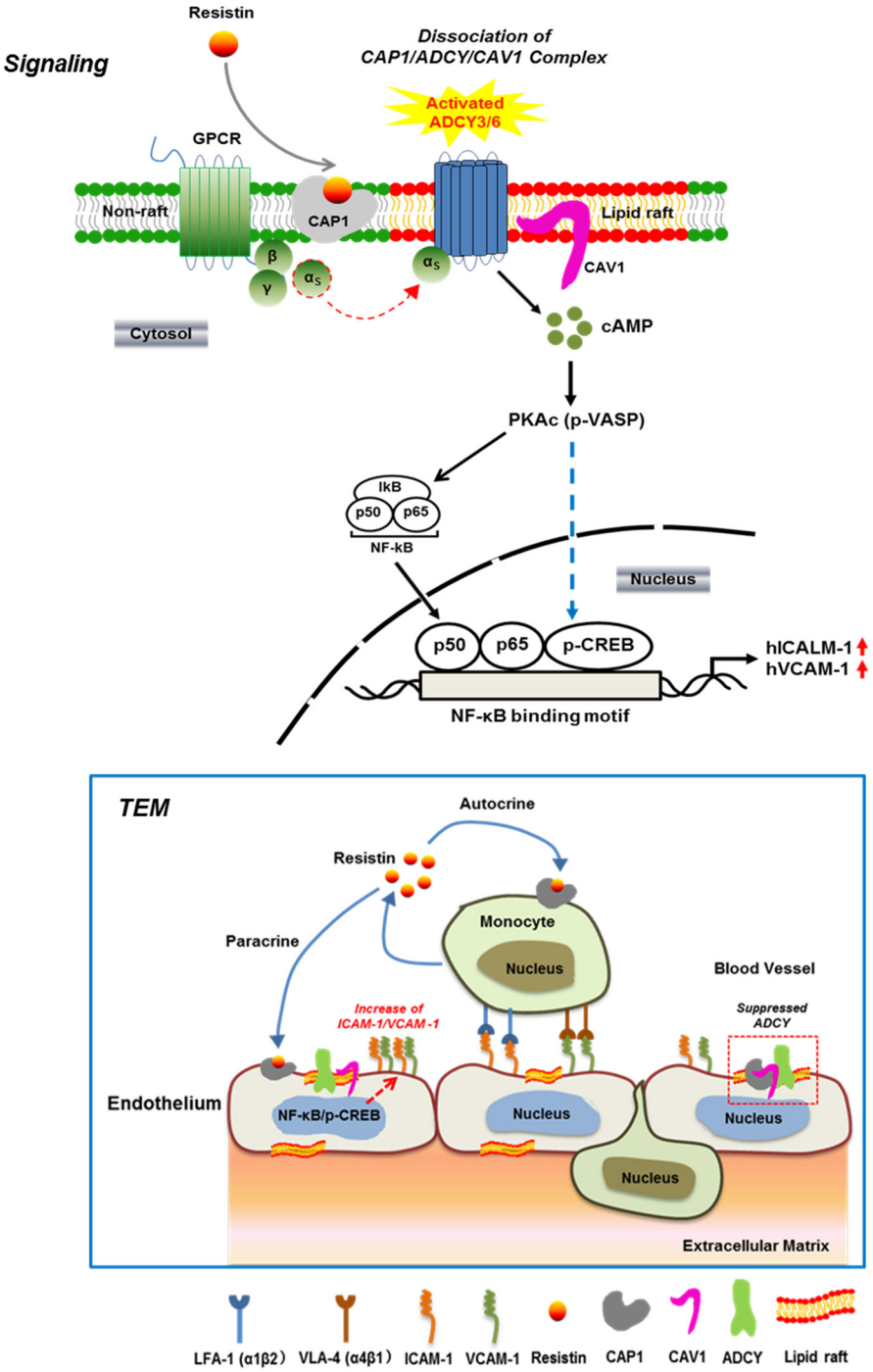

Signaling
Resistin
Dissociation of
CAP1/ADCY/CAV1 Complex
Activated
ADCY3/6
GPCR
Non-raft
CAP1
Lipid raft
β
γ
αS
αS
CAV1
Cytosol
cAMP
PKAc (p-VASP)
IkB
p50
p65
NF-kB
Nucleus
p50
p65
p-CREB
hICALM-1
hVCAM-1
NF-κB binding motif
TEM
Autocrine
Resistin
Paracrine
Monocyte
Nucleus
Blood Vessel
Increase of
ICAM-1/VCAM-1
Suppressed
ADCY
Endothelium
NF-κB/p-CREB
Nucleus
Nucleus
Nucleus
Extracellular Matrix
LFA-1 (α1β2)
VLA-4 (α4β1)
ICAM-1
VCAM-1
Resistin
CAP1
CAV1
ADCY
Lipid raft

Figure 9. Diagrammatic representation of TEM of monocytes mediated by CAP1 in endothelial cells.

AC3 is arrested in the CAP1-CAV1 complex in lipid raft, but when resistin binding to CAP1 dissociates this suppressive assembly and activates adenylyl cyclase. As a result, cAMP, hICAM-1 and hVCAM-1 are increased through PKA and NF-κB pathway, and consequently, facilitated the TEM of monocytes.

DISCUSSION

In the present study, I demonstrated that CAP1 serves as a functional receptor of human resistin in endothelial cells and plays a role as a critical regulator of monocyte transendothelial migration (TEM). I also found that the action mechanism of CAP1 between endothelial cells and monocytes was different. In the previous study, I reported that the depletion of CAP1 in monocytes significantly inhibited the inflammatory action of resistin, and while the overexpression of CAP1 exacerbated it[17]. However, interestingly, CAP1 knockdown in endothelial cells markedly increased the expression of the inflammatory adhesion molecules, such as hICAM-1 and hVCAM-1 (Figure 3). This difference is probably due to the different profiles of adenylyl cyclase (AC) isoforms that interact with CAP1, the specific localization of CAP1 in lipid raft or non-raft, and the complexity of molecules that bind to CAP1 according to the cell contexts. In fact, AC3 and AC6 were major isoforms in endothelial cells, and AC7 was a dominant isoform in monocytes. Particularly, AC3 and AC6 were present in lipid raft together with CAV1 and CAP1 in endothelial cells (Figure 4). Caveolin has important roles to regulate various signaling molecules

including adenylyl cyclase, G_{α}, $G_{\beta\gamma}$, PKA, PKC, Src and Erk through its scaffolding domain[34]. Through the immunoprecipitation, I confirmed that CAP1, AC3 and CAV1 form a complex in the membrane of endothelial cell (Figure 5B). Interestingly, this complex was dissociated by resistin-CAP1 binding. That is to say, CAP1 moves away from AC to non-raft, and CAV1 also falls away from CAP1 (Figure 5C, D). As a result, AC was activated, the expression of hICAM-1 and hVCAM-1 increased through the cAMP-PKA-NF-κB signaling pathway, and consequently, the TEM of monocytes was facilitated by resistin treatment and CAP1 knockdown (Figure 6).

In the previous study, I demonstrated that the proline-rich SH3 binding domain (BD) of CAP1 was a presumptive binding motif to resistin[17]. However, in the present study, it was not clear how the combination of resistin and CAP1 interferes with the binding of AC3 and CAV1 in endothelial cells. One presumptive hypothesis is that reistsin, AC3, and CAV1 can affect binding to each other when they bind to CAP1, as the motif that bind to CAP1 is located closely. In fact, the proline-rich SH3 BD is located in the center of the structure of CAP1, and AC BD and

actin BD are located on both sides of SH3 BD. Because of this structural feature of CAP1, it is believed that when resistin binds to CAP1, a structural change in CAP1 occurs, and AC3 and CAV1 can be separated from CAP1 (Figure 5G). However, in order to clarify the mechanism by which the CAP1-AC3-CAV1 complex is disrupted by resistin, detailed analysis of the structural change is necessary.

In vivo analysis, the increased transmigration of monocyte by the CAP1 knockdown in endothelial cells was also consistent with the transwell assay *in vitro*. In an animal model using carrageenan-induced edema formation, the inflammation of $CAP1^{+/-}$ mice themselves decreased overall. That is to say, the edema depth and the paw volume by carrageenan, an inflammatory substance, significantly decreased (Figure 8E, F, G). However, the infiltration of monocytes with an intact CAP1 was increased under the condition that only CAP1 of endothelial cells was depleted (Figure 8 H, I). Also, in the case of $CAP1^{+/-}$ mice, vascular leakage by mustard oil was increased and it was related to the change of junctional protein. That is, when CAP1 was depleted in endothelial cells, PECAM-1 was decreased, and VE-cadherin and ZO-2 were increased (Figure 8J, K, L).

However, there are still many conflicting reports of leukocyte transmigration related to junctional proteins[37, 38]. Therefore, it is considered that further investigations are needed to clarify the relationship between junctional integrity and CAP1 alteration in endothelial cells. Figure 9 illustrates the mechanism by which CAP1, as a functional receptor for resistin in endothelial cells, promotes the TEM of monocytes. In summary, AC3 is arrested by the CAP1-CAV1 complex in lipid raft, but the resistin-CAP1 binding dissociates this suppressive assembly and activates adenylyl cyclase. As a result, cAMP, hICAM-1 and hVCAM-1 are increased through PKA and NF-κB pathway, and consequently, it facilitates the TEM of monocytes.

I newly found that CAP1 of endothelial cells is an important regulator of the infiltration of mononuclear cells into inflamed tissues as a functional receptor for human resistin. In our previous study, downregulation of CAP1 in monocytes inhibited the inflammatory action of resistin[17]. However, unlike in the case of monocytes, the depletion of CAP1 in endothelial cells augmented adhesion molecules and increased leukocyte infiltration. This implies that CAP1 may function differently

according to the cell contexts. It seems that the main cause of different CAP1 mechanism each cell context is probably the difference of membrane complexes that bind to CAP1, such as CAV1 and AC isoforms. Therefore, the action mechanism of CAP1 may be different for each cell and tissue, so the development of therapeutic agents targeting CAP1 requires more research and careful approach. However, if we are well aware of the key mechanisms and can develop specific drugs targeting CAP1 of monocytes, it may be possible to inhibit the activation of inflammatory cells and block the tissue invasion of leukocytes.

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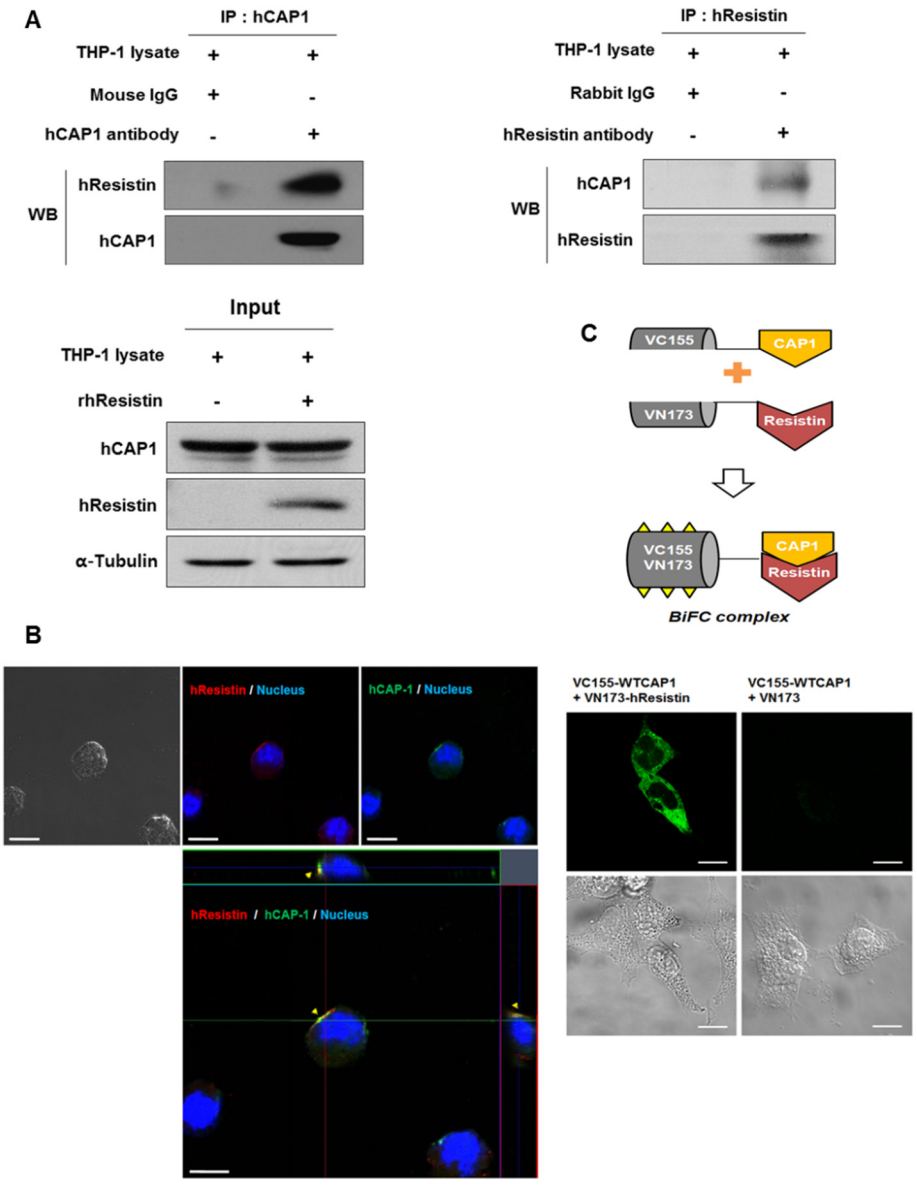
TABLE

Table 1. Real Time PCR Primer Sequences

Gene	Primer Sequence (5 ' −−→ 3 ')		Size (bp)
hCAP1	Forward	GGAGGGCAAGAAGTGGAGAG	184
	Reverse	CACGTCATCGAATACCAGGC	
mCAP1	Forward	GATTGCCATCTGGACCCTCT	148
	Reverse	GTG ATG CTT TCC CCC TGA TT	
hICAM−1	Forward	GGCACCCCAGCGGCTGACGT	184
	Reverse	CCCCATTCAGCGTCACCTTG	
hVCAM−1	Forward	AAGATGGTCGTGATCCTTGG	210
	Reverse	GGTGCTGCAAGTCAATGAGA	
AC1	Forward	CATGACCTGCGAGGACGAT	213
	Reverse	TCCCGTTCGACATGTTTGTACTT	
AC2	Forward	GGATCTCTCTCACGATCATCACCACA	234
	Reverse	CCCGCAGGAACACGGAACAGGAT	
AC3	Forward	CTCCAGTACTACACGGGACCCAG	274
	Reverse	GCTCTAAGGCCACCATAGGTAAGTCG	
AC4	Forward	CAGAGAGCACTAACAATTTCCACAGCC TCTAT	185
	Reverse	CCGCATGCATTCATGCTCCTTGGCA	
AC5	Forward	TTTGAAGACCCCAAGGACAAGAACG	150
	Reverse	AGGCTCCCTGAAGGTCAGGAGGAACT	

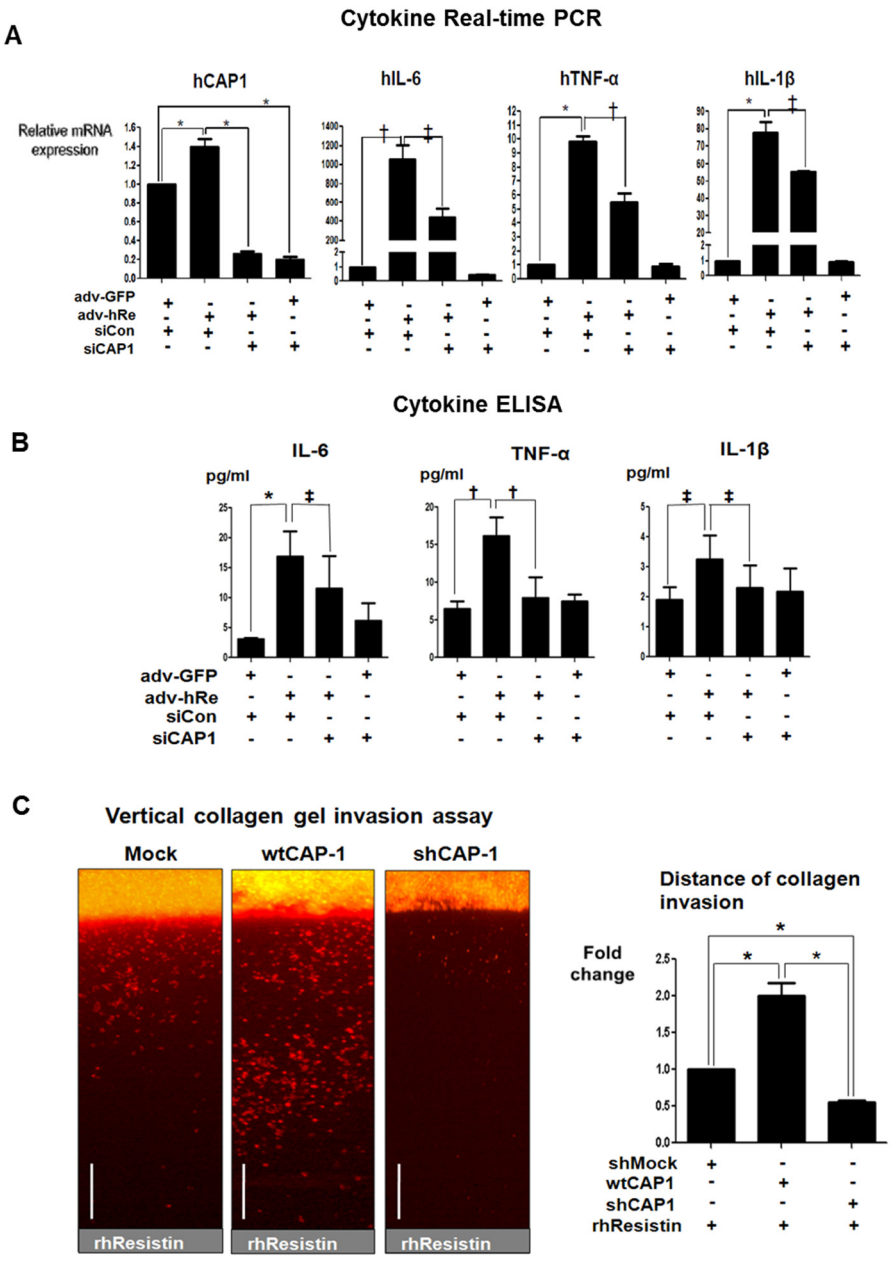
AC6	Forward	CCTGAGGATGAGGTGGATGAGTTC	251
	Reverse	CTGGCATAGATCCCAAGCATCAG	
AC7	Forward	ACGAGAAGTACCAGCTCACCA	192
	Reverse	CGACGTACATCAGCACAGAGA	
AC8	Forward	CAAACTCGAATCTGGAGGAATC	167
	Reverse	GACTGTCCTCAGGCTGCTTAAT	
AC9	Forward	CACCGCAAAATACTTAGATGACCGG	240
	Reverse	ACATTCCCTGATGACGCTGTCCCC	
AC10	Forward	CATTGATGAGGCCCAGTTTG	279
	Reverse	AATGGAATCCCACAGCTTCC	
GAPDH	Forward	GAGTCAACGGATTTGGTCGT	185
	Reverse	GACAAGCTTCCCGTTCTCAG	
Mouse 18SRNA	Forward	GTAACCCGTTGAACCCCATT	151
	Reverse	CCATCCAATCGGTAGTAGCG	



Supplemental Figure 1.

(A) Co-immunoprecipitation of human resistin and CAP1 in THP-1. (B) Co-localization of human resistin and CAP1 by immunofluorescence double staining in THP-1 cells. Scale,

10㎛. (C) Representative bimolecular fluorescence complementation (BiFC) assay visualizing the human resistin-CAP1 interaction in living cells. The highest fluorescence intensity was exhibited when both CAP1 and resistin fused to each fragment (pVC155, pVN173) were expressed (left panel). However, when human CAP1 was expressed alone, detectable fluorescence was not exhibited (right panel).



Supplemental Figure 2.

(A, B) CAP1 levels were reduced using specific small interfering RNAs (siRNAs) in THP-1 cells. siRNA targeting

CAP1 abolished resistin-induced increases in cytokine production (n = 3; *p < 0.001, †p < 0.01, ‡p < 0.05). (C) Vertical collagen gel invasion assay. Adenovirus-mediated CAP1 overexpression significantly enhanced the invasion of THP-1 cells toward resistin. In contrast, suppression of CAP1 expression by short hairpin RNAs (shRNAs) abrogated the resistin-mediated infiltration of monocytes. These data indicate that the chemotaxis of THP-1 cells to resistin is dependent upon CAP1 (n = 3; *p < 0.001). Scale, 500 ㎛.

국문 초록

배경

리지스틴은 비만 마우스 모델에서 처음 발견된 아디포카인(adipokine)으로 인슐린 저항성을 유발하며, 비만과 당뇨의 중요한 매개체로 알려져 있다. 마우스 리지스틴은 주로 지방세포에서 분비되지만 인간 리지스틴은 말초혈액 단핵구 세포와 대식세포가 주된 원천이다. 최근에 다양한 연구들에서 리지스틴이 당대사, 동맥경화, 심장질환, 비알콜성 지방간, 만성염증 등 다양한 인간질병의 매개체로서 역할을 하는 것으로 보고되고 있다. 그러나 리지스틴에 의해 유발되는 만성염증에 관한 많은 연구에도 불구하고, 리지스틴 수용체와 그 작용기전에 대해서는 아직까지 명확하게 밝혀진 바가 없다. 최근에 본 연구진은 단핵구 염증세포에서 adenylyl cyclase-associated protein 1 (CAP1)이 인간 리지스틴의 새로운 수용체이며, 단핵구 세포에서 수용체인 CAP1 을 통해 염증을 일으키는 세포내 신호전달기전을 밝혔다. 본 연구에서는 CAP1 이 인간 혈관내피세포에서도 리지스틴의 기능적인 수용체로 역할을 한다는 것을 새로이 밝혔다. 그 작용기전은 리지스틴이 혈관내피세포의 CAP1 과 결합하게 되면 adenylyl cyclase 가 활성화되고, cAMP-PKA-NFκB pathway 를 통해 내피세포의 면역세포 부착단백질의 발현을 증가시켜 단핵구 경내피세포 이동을 촉진시키는 것이다.

방법 및 결과

인간 혈관내피세포에서 CAP1 이 리지스틴의 기능적인 수용체인지를 실험하기 위해 CAP1 과발현 아데노 바이러스를 클로닝 하였고, 생체 내 연구를 위해 CAP1 유전자가 결핍된 유전자 조작 마우스를 제작하였다. 본 연구에서 CAP1 이 인간 혈관내피세포에서 리지스틴의 기능적인 수용체이며, CAP1-Adenylyl cyclase-Caveolin 복합체를 통해 혈관염증을 조절하는 새로운 기전을 확인하였다. 리지스틴이 CAP1 과 결합하면 CAP1-Adenylyl cyclase3-Caveolin1 복합체가 해체되고, 그 결과 cAMP-PKA-NFκB 신호전달체계가 활성화되어 면역세포 부착단백질의 발현이 증가하고, 이러한 결과들이 결국 단핵구 경내피세포 이동을 촉진시켰다.

결론

CAP1 은 인간 혈관내피세포에서도 리지스틴의 기능적인 수용체로서 단핵구 경내피세포 이동의 중요한 조절자 이다.

주요어 : Resistin, 염증, 경내피세포이동, CAP1, 세포간부착분자 1 (ICAM-1), 혈관세포접착단백질 1 (VCAM-1), Caveolin-1, 지질뗏목 (lipid raft), 인간 제대정맥 내피세포

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