

Role of vasodilator-stimulated phosphoprotein in human cytomegalovirus-induced hyperpermeability of human endothelial cells

YIHAO TIAN¹, YANQI HE², LING ZHANG², JIE ZHANG², LIU XU²,
YANBIN MA², XIAOLONG XU² and LEI WEI²

¹Department of Human Anatomy, Histology and Embryology; ²Hubei Provincial Key Laboratory of Developmentally Originated Disease, Department of Pathology and Pathophysiology, School of Basic Medical Sciences, Wuhan University, Wuhan, Hubei 430071, P.R. China

Received September 6, 2017; Accepted May 11, 2018

DOI: 10.3892/etm.2018.6332

Abstract. Atherosclerosis (AS) is a common chronic vascular disease and epidemiological evidence demonstrates that infection is closely associated with the occurrence of AS, including infection by human cytomegalovirus (HCMV) and *Chlamydomphila pneumoniae*. The aim of the present study was to investigate the effect of HCMV AD169 infection on the barrier function of human umbilical vein endothelial cells (HUVECs) and to understand the role of vasodilator-stimulated phosphoprotein (VASP) during this process. In cultured HUVEC-CRL-1730 cells, knockdown of VASP expression with small interfering (si)RNA-VASP resulted in impaired cellular barrier function. Furthermore, knockdown of Ras-related C3 botulinum toxin substrate 1 (Rac1) using siRNA-Rac1 could induce downregulation of VASP expression in HUVEC-CRL-1730 cells. Additionally, following the infection of the cells by HCMV, cellular morphological alterations could be observed under an inverted microscope, the mRNA and protein levels of Rac1 and VASP were transiently reduced, and what appeared to be a time-dependent impairment of the barrier function was observed. Finally, transfection of siRNA-VASP or siRNA-Rac1 into HCMV-infected HUVEC-CRL-1730 cells resulted in increased impairment of the cellular barrier function. Taken together, these data demonstrated that HCMV infection could induce impairment

of the barrier function in monolayer HUVEC-CRL-1730 cells via interference with Rac1/VASP expression.

Introduction

Atherosclerosis (AS) is a common chronic vascular disease that may lead to cardiovascular and cerebrovascular disease, affecting the health of the individual (1). A number of epidemiological studies have revealed that AS occurrence is closely associated with infection, including infection with human cytomegalovirus (HCMV) or *Chlamydomphila pneumonia* (2,3). Endothelial cells (ECs) expressing the 36 kDa annexin II receptor on their surface are potential hosts of HCMV (4). Bentz *et al* (5) revealed that HCMV infection of human microvascular ECs could result in a decrease in the cellular barrier function and an increase in permeability. This effect was associated with cytoskeletal rearrangements, including actin stress fiber depolymerization, and decreased expression of tight junction proteins, containing occludin and vascular endothelial-cadherin (6). Vascular endothelial impairment and barrier function dysfunction are important factors in the initiation of AS lesions; they facilitate the movement of monocytes accompanied by peroxidized lipids across the vascular endothelium, which are then deposited in the intima where monocytes absorb lipids, resulting in the formation of foam cells, which accumulate into atherosclerotic plaques (7). Therefore, elucidating the mechanism by which HCMV infection leads to the reduction of EC barrier function and promotes increased permeability through the rearrangement of the cytoskeleton may improve understanding of the process of AS formation.

Ena/vasodilator-stimulated phosphoprotein (VASP) homology (EVH) proteins are actin-associated proteins involved in a range of dynamic processes that are dependent on cytoskeletal remodeling and cellular polarity, including axon guidance and formation, lamellipodial and filopodial dynamics, platelet activation and cell migration (8). Additionally, as a main member of the EVH family, VASP was also revealed to serve a crucial role in establishing and maintaining the barrier functions of endothelial and epithelial

Correspondence to: Professor Lei Wei, Hubei Provincial Key Laboratory of Developmentally Originated Disease, Department of Pathology and Pathophysiology, School of Basic Medical Sciences, Wuhan University, 185 Donghu Road, Wuhan, Hubei 430071, P.R. China
E-mail: leiwei@whu.edu.cn

Key words: atherosclerosis, human cytomegalovirus, endothelial cells, hyperpermeability, vasodilator-stimulated phosphoprotein, Ras-related C3 botulinum toxin substrate 1

cells, which are closely associated with tight junction protein ZO-1 (ZO-1) at tight junctions (9,10). ZO-1, which is located near the tightly connected EC envelope, has a molecular weight of 225 kDa (11,12) and contains an SH3 domain (10). VASP consists of three functional regions: EVH1, EVH2 and proline-rich regions (PRR), of which PRR can bind to an SH3 domain. In a previous study utilizing human umbilical vein endothelial cells (HUVECs), VASP was phosphorylated by protein kinase A and distributed to the cell-cell junction, while the binding between phosphorylated VASP and ZO-1 was significantly enhanced; also, the polymerization of tight junctions was increased and EC permeability was significantly reduced (9). These data demonstrated that VASP and ZO-1 could jointly regulate EC barrier function. However, further studies are required.

The Rho family of GTPases contains >20 members, of which transforming protein RhoA (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (Cdc42) are the main players involved in the regulation of cell-cell connections and potential actomyosin networks (13,14). RhoA- and Rac1-mediated signaling pathways can respectively disrupt or maintain cell barrier function by coordinating actomyosin contractions and barrier alterations in various cell types (15,16). Furthermore, Rac1 and Cdc42 activities are required to maintain barrier integrity (17,18) by mediating the formation of actin filaments that associate with proteins from junctional complexes, including ZO-1 and α -catenin at the cell periphery (19). In addition, Rac1 regulates the alterations of endothelial permeability by mediating skeletal protein remodeling (20). It has been demonstrated that VASP is a downstream effector of Rac1 in osteosarcoma cells (21). Therefore, Rac1-mediated VASP activation may be involved in maintaining the barrier function of ECs.

In the present study, HCMV-induced EC barrier dysfunction was used to investigate the role of Rac1-mediated VASP activation in regulating vascular permeability, which may contribute to elucidating the molecular mechanism underlying the development of AS following HCMV infection.

Materials and methods

Plasmids, small interfering (si)RNAs and antibodies. To generate green fluorescent protein (GFP)-VASP overexpression plasmids, the VASP cDNA sequence was cloned into the pEGFP-C1 (304 mg/ml; Clontech Laboratories, Inc., Mountainview, CA, USA) multicloning site between the *EcoRI* and *BamHI* restriction sites. To generate flag tag protein (Flag)-Rac1 overexpression plasmids, the Rac1 DNA sequence was cloned into the pflag-CMV (285 mg/ml; Clontech Laboratories, Inc.) multicloning site between the *EcoRI* and *BamHI* restriction sites. The 21-nucleotide RNA oligos corresponding to human Rac1 (Genbank accession number, 006908) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) as follows: siRNA1 targeted nucleotides 439-459 (sense, 5'-GGAGATTGGTGCTGTA AAA-3' and anti-sense, 5'-UUUUACAGCACCAUCCUCC-3'), siRNA2 targeted nucleotides 199-217 (sense, 5'-CUACUGUCUUUG ACAUUA-3' and anti-sense, 5'-UAAUUGUCAAGACA GUAG-3') and siRNA3 targeted nucleotides 328-346 (sense, 5'-CAUCCUAGUGGGAACUAAA-3' and anti-sense, 5'-UUU

AGUUCCCACUAGGAUG3'), with dTdT overhangs at each 3' terminus. The following RNA oligos containing 21 nucleotides corresponding to human VASP (Genbank accession number, 003370) were also synthesized by Shanghai GenePharma Co., Ltd.: siRNA1 targeted nucleotides 942-960 (sense, 5'-CAA CCUUGCCAAGGAUGAATT-3' and anti-sense, 5'-UUCAUC CUUGGCAAGGUUGTG-3'), siRNA2 targeted nucleotides 1,002-1,020 (sense, 5'-CGCCCAGCUCCAGUGAUUATT-3' and anti-sense, 5'-UAAUCACUGGAGCUGGGCGTG-3') and siRNA3 targeted nucleotides 1,024-1,042 (sense, 5'-GGA CCUACAGAGGGUGAAATT-3' and anti-sense, 5'-UUU CACCCUCUGUAGGUCCGA-3'). A negative control siRNA (5'-AGUUCAACGACCAGUAGUCdTdT-3') was also used in the experiment (Shanghai GenePharma Co., Ltd). The anti-Rac1 (cat. no. 610651; dilution 1:1,000; BD Biosciences, Franklin Lakes, NJ, USA), anti-VASP (cat. no. 0010-10; dilution, 1:1,000; ImmunoGlobe GmbH, Himmelstadt, Germany), anti-GAPDH (cat. no. SC-69778; dilution, 1:5,000) and anti-tubulin (cat. no. SC-73242; dilution, 1:5,000) (both Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies were used in the study.

Cell culture and transfection. Two endothelial cell lines, the primary HUVECs and HUVEC-CRL1730, were obtained from the Key State Laboratory of Virology of Wuhan University (Wuhan, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone; GE Healthcare, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin G and 100 U/ml streptomycin sulfate at 37°C in a humidified incubator supplemented with 5% CO₂. Prior to transfection, the HUVEC-CRL1530 cells were seeded at a density of 2x10⁵ cells/well in 6-well plates and grown overnight in DMEM (without FBS or antibiotics) until they reached 60-70% confluence. Transfection of siRNAs or plasmids was carried out using Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Lipofectamine and siRNAs or plasmids were each diluted in DMEM. Diluted Lipofectamine was then mixed with diluted siRNAs or plasmids, and this mixture was incubated for 20 min at room temperature for complex formation. Following the addition of DMEM (2 ml/well) to the cells, the Lipofectamine/siRNA or Lipofectamine/plasmid mixture was added to each well, resulting in a final concentration of 100 pmol siRNAs or 4 μ g plasmids. At 48 h after transfection, the cells were collected for western blotting or incubated with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) for reverse transcription-quantitative (RT-q) PCR analysis.

HCMV titration assay and observation of cell morphology. The HCMV AD169 He was obtained from the Perinatal Laboratory of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The HUVEC-CRL1730 cells were seeded at 5x10³ cells/well in 96-well plates and cultured in DMEM (supplemented with 2% FBS and antibiotics) at 37°C in 5% CO₂. To determine the CCID₅₀/ml, serial 10-fold dilutions followed by serial 8-fold dilutions of the HCMV viral sample were made in DMEM. The following day the cells were inoculated a 96-well plate with the same dilution of virus, 6 replicate wells were set up for each dilution each well was inoculated with 0.1 ml diluted virus solution in a 37°C, 5%

CO₂ incubator. Following HCMV infection, the cells become larger and rounder and specific 'hawk-eye' changes appeared, which were observed under a light microscope (Olympus IX 73 DP80, Olympus Corporation, Tokyo, Japan). Following inoculation, depending on the speed of the HUVEC-CRL1530 cell lesions, observations were continued for several days until the cytopathies did not progress. Cytopathic effect (CPE) is a specific lesion that occurs when cells are infected with a virus. The virus dilution corresponding to 50% CPE is the titer of the virus. The dilution of half of the lesions was taken as the 50% infection unit; CCID50 (where CCID50 represents the 50% cell culture infectious dose in HUVEC-CRL1530 cells).

Endothelial monolayer cell permeability assay. *In vitro* cell permeability analysis was performed with fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) as follows. Following treatment with a time gradient HCMV (10⁻⁵ dosage) infection at 0, 4, 8, 12 and 24 h or plasmid and siRNA transfection, the HUVEC-CRL-1730 cells were seeded into the upper chambers of a Costar Transwell 24-well plate at a density of 1x10³ cells/cm² and initially plated with 1% gelatin (membrane diameter, 6.5 μm; pore size, 0.4 μm). Following adherence, the cells were cultured in serum-free DMEM for 24 h. The medium in the upper layer was subsequently replaced with medium containing 100 μg/ml FITC-dextran (100 μl) and the lower chamber was filled with normal medium. After incubation for 45 min, the fluorescence intensity of the sample was measured in a black 96-well plate with 100 μl sample from the upper and lower chambers. An excitation wavelength of 490 nm and an emission wavelength of 520 nm were used to measure the fluorescence in each well using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the volume of liquid in the lower chamber was measured. The permeability of the EC monolayer to FITC-labeled dextran was expressed as 'Pa' and calculated as follows: $Pa = [A]/t \times 1/A + V/[L]$. In the formula, 't' was the time in seconds; '[A]' was the FITC-labeled dextran concentration in the upper layer (expressed in terms of fluorescence intensity); 'V' was the volume of liquid in the lower chamber in ml; '[L]' was the FITC-labeled dextran concentration in the lower chamber (expressed in terms of fluorescence intensity); and 'A' was the filter area in cm².

Isolation of RNA and RT-qPCR analysis. The HUVEC-CRL-1730 cells were seeded into the 6-well plate with HCMV infection and/or plasmid and siRNA transfection and the mRNA expression was measured at 0, 4, 8, 12, 16, 24 and 48 h. Total RNA was extracted from cells using TRIzol reagent, and 2 mg total RNA was used for first-strand cDNA synthesis with a RevertAid™ First-Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The semi-quantitative PCR was performed using the CFX96 Real Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in the presence of SYBR Green (Thermo Fisher Scientific, Inc.). All qPCRs were run in triplicate and repeated at least three times. Relative mRNA expression was calculated using the 2^{-ΔΔCq} method (22) using GAPDH as the internal reference. The primers used were as follows: VASP, sense 5'-GGAAAGTCA

GCAAGCAGG-3' and antisense 5'-TGTGCGGAAAGGAGA AGC-3'; Rac1, sense 5'-AGACGGAGCTGTAGGTA AAA-3' and antisense 5'-GCAGGACTACAAGGA-3'; GAPDH, sense 5'-AGGTCCACCACTGACACGTT-3' and antisense 5'-GCCTCAAGATCATCAGCAAT-3'. Rac1 reaction parameters were 94°C for 40 sec, 54°C for 30 sec and 72°C for 30 sec for 40 cycles. VASP reaction parameters were 94°C for 40 sec, 60°C for 30 sec and 72°C for 30 sec for 40 cycles.

Western blotting. The HUVEC-CRL-1730 cells were seeded into the 6-well plate treatment with HCMV infection and/or plasmid and siRNA transfection at the protein expression was measured at 0, 4, 8, 12, 16, 24 and 48 h. Following that the cells were washed with PBS and lysed in 200 μl modified RIPA buffer (Thermo Fisher Scientific, Inc.). Protein concentration was measured with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of total protein (10 μg/lane) were loaded, electrophoresed on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with Tris-buffered saline containing 5% nonfat milk for 2 h at room temperature, then probed with antibodies against VASP (1:1,000 dilution), Rac1 (1:1,000 dilution) and GAPDH at 4°C overnight. The membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit IgG (cat. no. 16473-1-AP; 1:1,000 dilution) and anti-mouse IgG (cat. no. 10285-1-AP; 1:1,000 dilution) (both ProteinTech Group, Inc., Chicago, IL, USA) secondary antibodies for 2 h at room temperature. The bound antibodies were detected by Pierce™ ECL Western Blotting substrate (Thermo Fisher Scientific, Inc.). Quantification of band densities was performed using ImageJ software (version 1.5.0.26; National Institutes of Health, Bethesda, MD, USA). The experiments were repeated three times.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical significance between groups was tested using one-way analysis of variance followed by a Bonferroni's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

HCMV infection induces increased EC permeability. The normal primary HUVECs appeared closely linked and flat with typical spindle shapes (Fig. 1A). After 6 passages, the morphology of primary HUVECs had changed when viewed under an inverted microscope, with an increased number of irregular protrusions decreased density and more cellular debris in the cytoplasm (Fig. 1B). After 7 days of HCMV infection, the morphology of the primary HUVEC cells was altered, with evident nuclear enlargement and cell swelling, and gradual rounding of the cells (Fig. 1C). Typical dense basophilic inclusion bodies surrounded by a halo in the nucleus and 'owl eye'-like alterations of the nuclei were observed. The normal HUVEC-CRL-1730 cells appeared closely linked and flat with typical spindle shapes (Fig. 1D). After 10 days of HCMV infection, the HUVEC-CRL-1730 cell boundaries became blurred and numerous cells merged to form giant cells, and a number of the cells died (Fig. 1E).

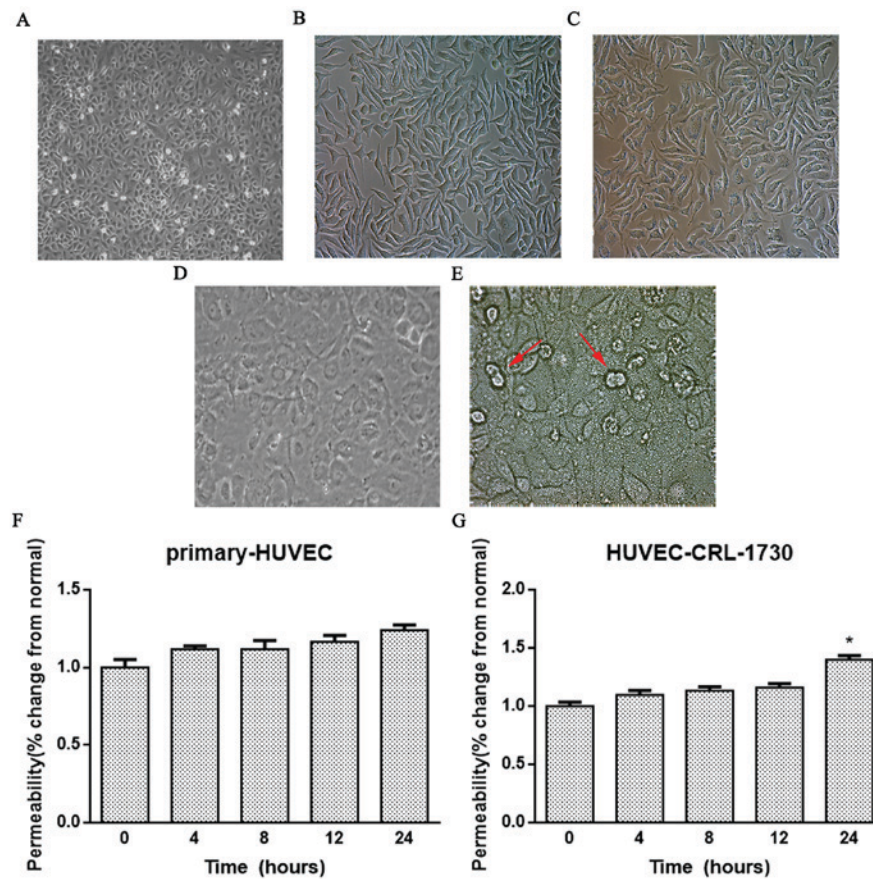


Figure 1. HCMV infection induces increased EC permeability. Cell morphology was observed in cultures of (A) normal primary HUVECs (magnification, x100), (B) primary HUVECs following 6 passages (magnification, x400) and (C) primary HUVECs 7 days after HCMV infection (magnification, x400). Cell morphology was observed in cultures of (D) normal HUVEC-CRL-1730 cells (magnification, x400). (E) HUVEC-CRL-1730 10 days after HCMV infection (magnification, x400), the red arrows indicate the 'owl eye'. (F) Primary HUVECs and (G) HUVEC-CRL-1730 monolayer cells were infected with *tissue culture infective dose*₅₀ (10^{-5}) HCMV AD169 for 0, 4, 8, 12 and 24 h. A Transwell assay was then performed to detect endothelial permeability using fluorescein isothiocyanate-labeled dextran. Data are expressed as the mean \pm standard error of the mean ($n=3$ /group). * $P<0.05$ vs. the 0 h group. HUVEC, human umbilical vein endothelial cell; HCMV, human cytomegalovirus; EC, endothelial cell.

Next, the effect of HCMV infection on the permeability of the two EC cell lines was studied. First, the 50% *cell culture infective dose* ($CCID_{50}$) of HCMV was determined to be 10^{-5} by a virus titration assay. The monolayer cells were infected with $CCID_{50}$ HCMV AD169 for 4, 8, 12 and 24 h. A Transwell assay was subsequently used to analyze the intercellular permeability of FITC-labeled dextran. As revealed in Fig. 1F and G, the permeability of HUVEC-CRL-1730 cells was significantly increased after HCMV infection compared with the negative control group ($P<0.05$), while no significant difference was identified in primary HUVECs. These results indicated that HCMV infection increased the permeability of monolayer HUVEC-CRL-1730 cells, leading to a decline in cell barrier function.

VASP is involved in maintaining EC permeability. VASP is involved in maintaining EC permeability in HUVEC-CRL-1730 cells via binding to ZO-1 at the cell junction (9). In order to verify whether the expression level of VASP affects the permeability of ECs, a loss-of-function assay was utilized by transfecting HUVEC-CRL-1730 cells with siRNA-VASP or the overexpression plasmid pEGFP-VASP. siRNA-VASP significantly inhibited VASP gene transcription in the cells, which resulted in the protein expression level being decreased

to 85% ($P<0.05$; Fig. 2A and B) and the mRNA expression level of VASP being reduced to 79% ($P<0.05$; Fig. 2C) compared with the siRNA-NC group. On the contrary, compared with the pEGFP-C1 group, transfection with pEGFP-VASP upregulated VASP protein and mRNA expression by 72 and 68%, respectively (both $P<0.05$; Fig. 2B and C).

In addition, a Transwell assay was used to detect permeability 24 h after transfection. VASP knockdown revealed a $40\pm 2\%$ increase in endothelial permeability (both $P<0.05$; Fig. 2D) compared with the control or siRNA-NC groups, whereas the overexpression plasmid resulted in no significant difference in endothelial permeability.

HCMV infection reduces the expression of Rac1 and VASP in HUVEC-CRL-1730 cells. The Rac1/VASP signaling pathway is involved in the regulation of endothelial permeability (23) and HCMV infection increased the permeability of the monolayer HUVEC-CRL-1730 cells. To investigate the potential signaling pathway involved in HCMV infection-induced hyperpermeability in ECs, the expression of Rac1 and VASP at the mRNA and protein levels were examined in HCMV-pretreated cells over a range of times. The results demonstrated that HCMV infection significantly inhibited Rac1 and VASP mRNA expression starting at 8 and

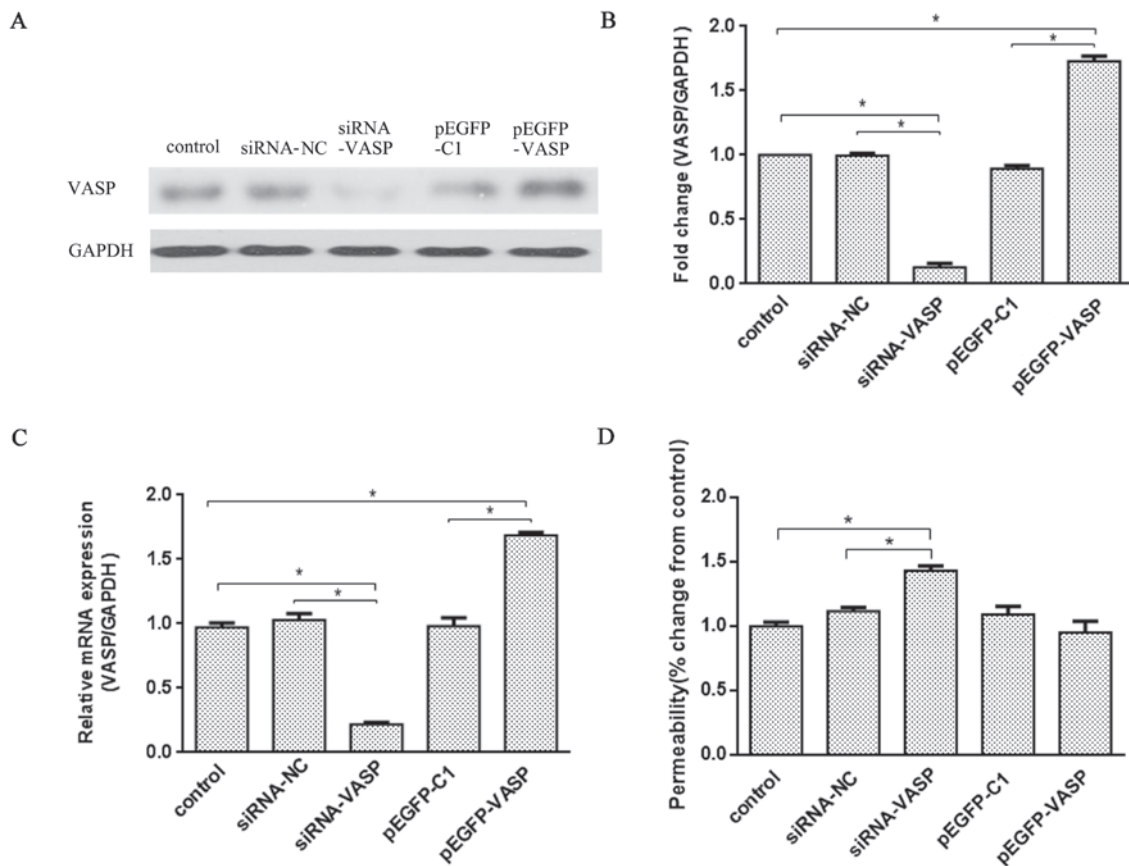


Figure 2. VASP is involved in maintaining endothelial permeability. HUVEC-CRL-1730 cells were treated with siRNA-VASP, siRNA-NC, pEGFP-C1 or over-expression plasmid pEGFP-VASP for 48 h (mRNA and protein expression) or 24 h (permeability). VASP expression was (A) assessed (B) quantified by western blot analysis. (C) VASP mRNA expression was quantified by reverse transcription-polymerase chain reaction. (D) The permeability of HUVEC-CRL-1730 cells. Data are expressed as the mean \pm standard error of the mean (n=3/group). *P<0.05. siRNA, small interfering RNA; HUVEC, human umbilical vein endothelial cells; VASP, vasodilator-stimulated phosphoprotein; GFP, green fluorescent protein; NC, negative control.

12 h, respectively, compared to the 0 h group (all P<0.05; Fig. 3A and B); maximum inhibition was achieved at 12 h. HCMV infection also significantly inhibited Rac1 and VASP protein expression starting at 8 and 12 h, respectively, compared with the 0 h group (all P<0.05; Fig. 3A). Maximum Rac1 and VASP protein inhibition was achieved at 24 and 48 h, respectively. Therefore, the data demonstrated that Rac1 and VASP expression levels can be affected by HCMV infection, which also suggested that VASP expression was positively associated with Rac1. These data suggest that HCMV infection-induced increases in EC permeability may be achieved by altering the expression levels of VASP and/or Rac1.

HCMV infection induces hyperpermeability through the Rac1/VASP signaling pathway in HUVEC-CRL-1730 cells. HCMV infection inhibited the expression of Rac1 and VASP, and, therefore, to investigate the association between Rac1 and VASP, silencing or over-expression of Rac1 was carried out using siRNA-Rac1 or a pflag-Rac1 plasmid, respectively. At the mRNA and protein level, siRNA-Rac1 oligonucleotides demonstrated a significant inhibitory effect on Rac1 and VASP expression, and the pflag-Rac1 plasmid significantly increased Rac1 and VASP expression compared with the control group and their respective negative controls (all P<0.05; Fig. 4A-C).

These data indicated that VASP may act as a downstream effector of Rac1 in HUVEC-CRL-1730 cells.

Following transfected with siRNA-Rac1 or the pflag-Rac1 plasmid in HUVEC-CRL-1730 cells, a Transwell assay was used to detect endothelial permeability at 24 h (Fig. 4D). Rac1 knockdown revealed significant increase in endothelial permeability compared with the control and siRNA-NC groups (P<0.05), which is a 45 \pm 2% increase compared with the control group, whereas the Rac1 overexpression plasmid resulted in no significant difference in endothelial permeability. Furthermore, the potential role of the Rac1/VASP signaling pathway in the regulation of HCMV infection-induced hyperpermeability in the cells was analyzed.

After HCMV infection for 24 h, the cells were transfected with siRNA-Rac1, the pflag-Rac1 overexpression plasmid, siRNA-VASP, the pEGFP-VASP overexpression plasmid or their respective controls using Lipofectamine 2000. After 48 h, HUVEC-CRL-1730 cell permeability was assessed using a Transwell assay. The endothelial permeability of Rac1 knockdown cells significantly increased compared with the control and siRNA-NC groups (both P<0.05), which was an increase of 58 \pm 3% compared to the control cells (Fig. 4E). The endothelial permeability of the Rac1 overexpressing cells revealed no significant alteration. As demonstrated in Fig. 4F, the endothelial permeability of VASP knockdown cells was

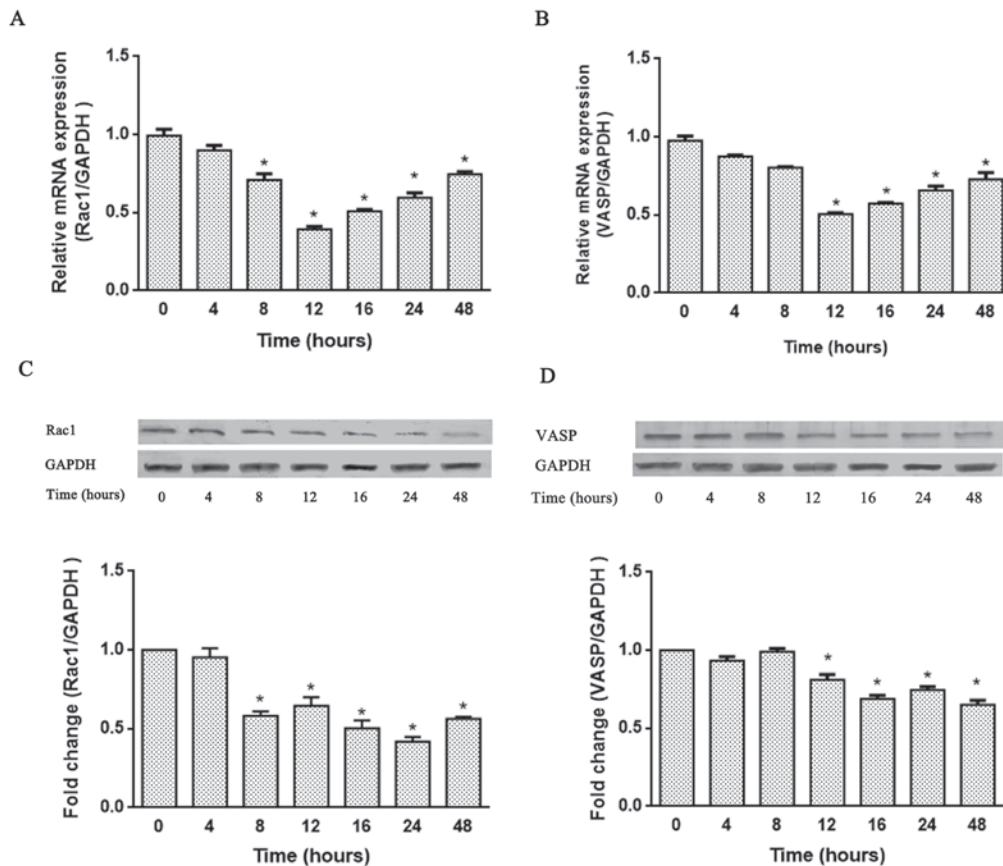


Figure 3. HCMV infection reduces the expression of Rac1 and VASP. HUVEC-CRL-1730 cells were infected with HCMV for 0, 4, 8, 12, 16 and 24 h. mRNA expression of (A) Rac1 and (B) VASP was quantified by reverse transcription-polymerase chain reaction. Protein expression of (C) Rac1 and (D) VASP was assessed and quantified by western blotting. Data are expressed as the mean \pm standard error of the mean ($n=3$ /group). * $P<0.05$ vs. the control group. HUVEC, human umbilical vein endothelial cell; HCMV, human cytomegalovirus; VASP, vasodilator-stimulated phosphoprotein; Rac1, Ras-related C3 botulinum toxin substrate 1.

also significantly increased compared with the control and siRNA-NC groups (both $P<0.05$), an increase of $50\pm 3\%$ compared to the control cells. The endothelial permeability of the VASP overexpressing cells revealed no significant alteration. These data suggested that HCMV infection induces hyperpermeability via the Rac1/VASP signaling pathway.

Discussion

AS is a common chronic vascular pathological process and the most common cause of cardiovascular and cerebrovascular diseases (24). The pathogenesis of AS is complex; the high etiological risk factors associated with the development of AS include genetic polymorphisms and susceptibility, long-term exposure to various chemicals, smoking, hypertension and hyperlipidemia (25). Furthermore, numerous epidemiological studies and clinical retrospective analyses have demonstrated that viral or bacterial infections, including HCMV (26) and *Chlamydomphila pneumonia* (27), may affect the formation of AS.

EC injury is the first step in AS development and is frequently caused by specific chemicals, high fat diets, genetic factors, trauma and pathogen infection (3). Yi *et al* (28) found HCMV DNA, antigens, and immediate-early (IE) genes and proteins in the internal carotid arteries of patients

suffering ischemic stroke. Blum *et al* (29) found a high-titer of immunoglobulin G anti-CMV antibodies in the sera of patients with high-risk atherosclerotic cardiovascular disease. Additionally, a clinical cohort study revealed that CMV infection is an independent risk factor for AS and coronary heart disease (30). These studies suggest that the viral infection is closely associated with the development and progression of AS.

ECs are potential hosts of HCMV, and the entry of HCMV into cells is mediated by cellular annexin II (4), a 36 kDa HCMV-binding protein that exists on the cellular membrane. In the present study, in order to demonstrate that endothelial damage can be caused by viral invasion, the effect of HCMV infection on the morphology of ECs was observed. Under normal physiological conditions, HUVECs were closely connected, flat, long and spindle-shaped, and exhibited the typical 'paving stone' morphology. After the cells were infected with HCMV for 7-10 days, the nuclei had increased in size and some of the cells had become rounded. In addition, the boundaries between cells became blurred and numerous cells merged to form giant cells, while a number of cells died. The data from the current study are consistent with that of a previous report, which demonstrated that, following HCMV infection, the cells from the two HUVEC cell lines became rounded and intercellular fissure formation occurred (27). This

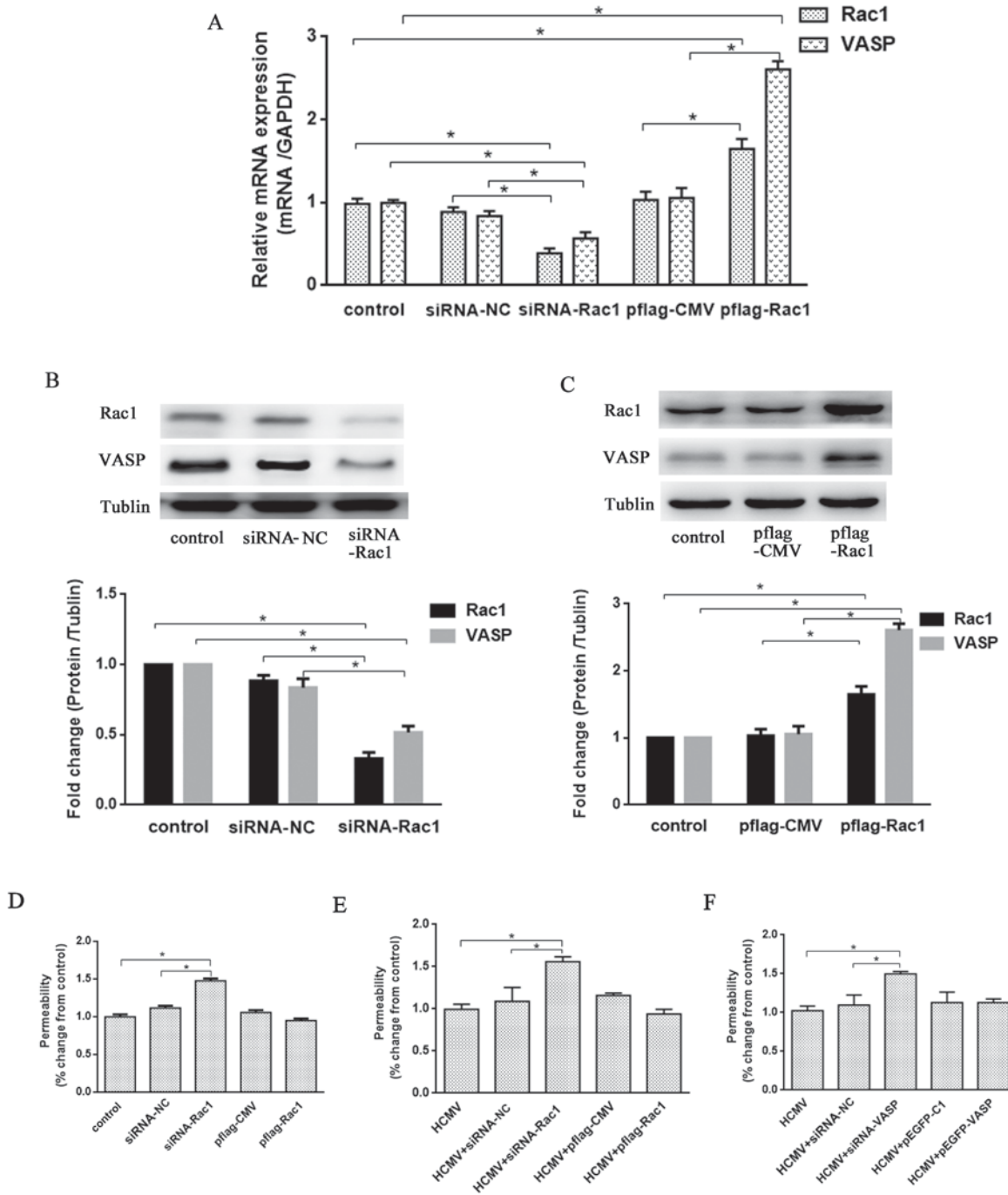


Figure 4. HCMV infection induces hyperpermeability through the Rac1/VASP signaling pathway in HUVEC-CRL-1730 cells. (A) Rac1 and VASP mRNA quantification, relative to GAPDH mRNA, was assessed by reverse transcription-polymerase chain reaction, in cells treated with siRNA-NC, siRNA-Rac1 or pflag-Rac1 for 48 h. Rac1 and VASP protein expression was determined and quantified by western blot analysis in cells treated with (B) siRNA-NC or siRNA-Rac1 and (C) pflag-CMV or pflag-Rac1 for 48 h. (D) The permeability of HUVEC-CRL-1730 cells, which were treated with siRNA-NC, siRNA-Rac1, pflag-CMV or the overexpression plasmid pflag-Rac1 for 24 h, was assessed using FITC-labeled dextran in a Transwell assay. After 24 h of HCMV infection, the permeability of HUVEC-CRL-1730 cells, which were treated with siRNA-NC and (E) siRNA-Rac1, pflag-CMV or the overexpression plasmid pflag-Rac1, or (F) siRNA-VASP, pEGFP-C1 or the overexpression plasmid pEGFP-VASP for 24 h, was assessed using FITC-labeled dextran in a Transwell assay. Data are expressed as the mean \pm standard error of the mean (n=3 per group). *P<0.05 vs. the control group. si, small interfering; HUVEC, human umbilical vein endothelial cell; VASP, vasodilator-stimulated phosphoprotein; flag, tag protein; GFP, green fluorescent protein; NC, negative control; Rac1, Ras-related C3 botulinum toxin substrate 1; FITC, fluorescein isothiocyanate.

alteration promoted the attraction of a variety of inflammatory cells and factors through ECs, which began to accumulate peroxidized lipids from the blood vessels, and gradually evolved into foam cells, which are involved in atherosclerotic plaque formation (31). These phenomena suggest that the

reduction of barrier function, which is closely associated with the morphological alterations of ECs, is crucial to the development of AS.

In the present study, an increase in Rac1 expression was demonstrated to induce hyperpermeability, which disrupted

EC barrier function. Baumer *et al* (32) demonstrated that Rac1 activation likely contributed to the barrier-stabilizing effects of cyclic adenosine monophosphate in the microvascular endothelium. These effects may in part, be mediated by the Epac/Rap1 signaling pathway (33). Cytoskeletal rearrangements are the structural basis for the modification of EC morphology and associated barrier function (34). VASP, an actin-associated protein, belongs to a family of Ena/VASP proteins, which localize to the intercellular junction, focal adhesion terminals of stress fibers and highly dynamic areas of change at the cellular membrane (35). VASP has been revealed to serve an important role in the maintenance of endothelial barrier function (36,37), and paracellular permeability (38). Luyer *et al* (39) demonstrated that the expression of the tight junction protein, ZO-1, was abolished and levels of phosphorylated VASP increased under low intestinal blood flow conditions in a rat model hemorrhagic shock and, at the same time, the permeability of the intestinal mucosa and bacteria translocation also increased. Bogatcheva *et al* (40) reported that the bacterial endotoxin lipopolysaccharide secreted by human pulmonary microvascular ECs can cause the redistribution of VASP- and ZO-1-associated proteins. In VASP-depleted pulmonary microvascular ECs, the cell barrier function was reduced, and the mechanism involved may be associated with the cell-cell tight junction damage caused by the disorder of the ZO-1-F-actin arrangement (41).

In the current study, after HCMV infection, endothelial permeability and the expression of Rac1 and VASP were analyzed at the mRNA and protein levels in two HUVEC cell lines. The mRNA level of Rac1 and VASP reached the minimum level at 12 h. The reason for the increase after 12 h may be associated with HCMV infection-induced activation of cytokine-associated signaling pathways. For example, following HCMV infection, the cellular factor, nuclear factor- κ B p105 subunit, was activated and its expression increased, which was important for the efficient transcription of IE genes (42); also, hypoxia-inducible factor (HIF)-1 α transcription was clearly stimulated (43). The aforementioned results also demonstrated that HCMV-induced hyperpermeability was accomplished by decreasing Rac1 and VASP expression in HUVEC-CRL-1730 cells.

In addition, a further decrease of VASP expression using siRNA-VASP in HCMV-infected cells aggravated the permeability increase. These results indicated that HCMV-induced endothelium hyperpermeability may be mediated by Rac1 and VASP-associated cell-cell junction remodeling. In addition, as VASP is a downstream effector of Rac1 in osteosarcoma cells, interference of Rac1 in the cells led to a significant reduction in VASP expression (21). Additionally, consistent with previous reports, the EC data from the current study also revealed that interfering with the expression of Rac1 using siRNA-Rac1 could inhibit the expression of VASP. Therefore, HCMV-induced endothelial cytoskeleton remodeling may be controlled by the Rac1/VASP signaling pathway. In conclusion, the current study demonstrated that HCMV infection could induce impairment of the barrier function in monolayer HUVEC-CRL-1730 cells via downregulation of Rac1-mediated VASP expression. The present study identified that the presence of pathogens, which cause a breakdown in the barrier function of endothelial cells had a high correlation

with the occurrence and development of atherosclerosis. These results indicate that an effective strategy for the prevention of atherosclerosis would be to strengthen the protection of endothelial cells.

Acknowledgements

Not applicable.

Funding

The present study was supported by a grant from the National Natural Science Foundation of China (grant no. 81572943).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YT, LW and YH designed the study and wrote the manuscript. XX and YM analyzed the data. LZ, JZ and LX performed the cell experiments.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Tarp JB, Jensen AS, Engstrom T, Holstein-Rathlou NH and Sondergaard L: Cyanotic congenital heart disease and atherosclerosis. *Heart* 103: 897-900, 2017.
2. Zhou YF, Leon MB, Waclawiw MA, Popma JJ, Yu ZX, Finkel T and Epstein SE: Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. *N Engl J Med* 335: 624-630, 1996.
3. Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, Gallimore JR and Pepys MB: Low grade inflammation and coronary heart disease: Prospective study and updated meta-analyses. *BMJ* 321: 199-204, 2000.
4. Pietropaolo RL and Compton T: Direct interaction between human cytomegalovirus glycoprotein B and cellular annexin II. *J Virol* 71: 9803-9807, 1997.
5. Bentz GL, Jarquin-Pardo M, Chan G, Smith MS, Sinzger C and Yurochko AD: Human cytomegalovirus (HCMV) infection of endothelial cells promotes naive monocyte extravasation and transfer of productive virus to enhance hematogenous dissemination of HCMV. *J Virol* 80: 11539-11555, 2006.
6. Conforti G, Zanetti A, Colella S, Abbadini M, Marchisio PC, Pytela R, Giancotti F, Tarone G, Languino LR and Dejana E: Interaction of fibronectin with cultured human endothelial cells: Characterization of the specific receptor. *Blood* 73: 1576-1585, 1989.
7. Etingin OR, Silverstein RL and Hajjar DP: von Willebrand factor mediates platelet adhesion to virally infected endothelial cells. *Proc Natl Acad Sci USA* 90: 5153-5156, 1993.

8. Loureiro JJ, Rubinson DA, Bear JE, Baltus GA, Kwiatkowski AV and Gertler FB: Critical roles of phosphorylation and actin binding motifs, but not the central proline-rich region, for Ena/vasodilator-stimulated phosphoprotein (VASP) function during cell migration. *Mol Biol Cell* 13: 2533-2546, 2002.
9. Comerford KM, Lawrence DW, Synnestvedt K, Levi BP and Colgan SP: Role of vasodilator-stimulated phosphoprotein in PKA-induced changes in endothelial junctional permeability. *FASEB J* 16: 583-585, 2002.
10. Lawrence DW, Comerford KM and Colgan SP: Role of VASP in reestablishment of epithelial tight junction assembly after Ca²⁺ switch. *Am J Physiol Cell Physiol* 282: C1235-C1245, 2002.
11. Ross R: Atherosclerosis-an inflammatory disease. *N Engl J Med* 340: 115-126, 1999.
12. Lum H and Malik AB: Mechanisms of increased endothelial permeability. *Can J Physiol Pharmacol* 74: 787-800, 1996.
13. Hall A: Rho family GTPases. *Biochem Soc Trans* 40: 1378-1382, 2012.
14. Rojas AM, Fuentes G, Rausell A and Valencia A: The Ras protein superfamily: Evolutionary tree and role of conserved amino acids. *J Cell Biol* 196: 189-201, 2012.
15. Abbasi T and Garcia JG: Sphingolipids in lung endothelial biology and regulation of vascular integrity. *Handb Exp Pharmacol*: 201-226, 2013.
16. van Nieuw Amerongen GP, Beckers CM, Achekar ID, Zeeman S, Musters RJ and van Hinsbergh VW: Involvement of Rho kinase in endothelial barrier maintenance. *Arterioscler Thromb Vasc Biol* 27: 2332-2339, 2007.
17. Samarín S and Nusrat A: Regulation of epithelial apical junctional complex by Rho family GTPases. *Front Biosci (Landmark Ed)* 14: 1129-1142, 2009.
18. Kouklis P, Konstantoulaki M, Vogel S, Broman M and Malik AB: Cdc42 regulates the restoration of endothelial barrier function. *Circ Res* 94: 159-166, 2004.
19. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT and Horwitz AR: Cell migration: Integrating signals from front to back. *Science* 302: 1704-1709, 2003.
20. Winge MCG and Marinkovich MP: Epidermal activation of the small GTPase Rac1 in psoriasis pathogenesis. *Small GTPases* 5: 1-6, 2017.
21. Wu G, Wei L, Yu A, Zhang M, Qi B, Su K, Hu X and Wang J: Vasodilator-stimulated phosphoprotein regulates osteosarcoma cell migration. *Oncol Rep* 26: 1609-1615, 2011.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
23. Schlegel N and Waschke J: VASP is involved in cAMP-mediated Rac 1 activation in microvascular endothelial cells. *Am J Physiol Cell Physiol* 296: C453-C462, 2009.
24. Writing Group M, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, *et al*: Heart disease and stroke statistics-2016 update: A report from the American heart association. *Circulation* 133: e38-e360, 2016.
25. Ross R: The pathogenesis of atherosclerosis-an update. *N Engl J Med* 314: 488-500, 1986.
26. Wang Z, Cai J, Zhang M, Wang X, Chi H, Feng H and Yang X: Positive expression of human cytomegalovirus phosphoprotein 65 in atherosclerosis. *Biomed Res Int* 2016: 4067685, 2016.
27. Kreutmayer S, Csordas A, Kern J, Maass V, Almanzar G, Offterdinger M, Ollinger R, Maass M and Wick G: Chlamydia pneumoniae infection acts as an endothelial stressor with the potential to initiate the earliest heat shock protein 60-dependent inflammatory stage of atherosclerosis. *Cell Stress Chaperones* 18: 259-268, 2013.
28. Yi L, Lin JY, Gao Y, Feng ZJ and Wang DX: Detection of human cytomegalovirus in the atherosclerotic cerebral arteries in han population in china. *Acta Virol* 52: 99-106, 2008.
29. Blum A, Peleg A and Weinberg M: Anti-cytomegalovirus (CMV) IgG antibody titer in patients with risk factors to atherosclerosis. *Clin Exp Med* 3: 157-160, 2003.
30. Adam E, Melnick JL, Probstfield JL, Petrie BL, Burek J, Bailey KR, McCollum CH and DeBakey ME: High levels of cytomegalovirus antibody in patients requiring vascular surgery for atherosclerosis. *Lancet* 2: 291-293, 1987.
31. Badimon L and Vilahur G: Thrombosis formation on atherosclerotic lesions and plaque rupture. *J Intern Med* 276: 618-632, 2014.
32. Baumer Y, Drenckhahn D and Waschke J: cAMP induced Rac 1-mediated cytoskeletal reorganization in microvascular endothelium. *Histochem Cell Biol* 129: 765-778, 2008.
33. Birukova AA, Zagranichnaya T, Fu P, Alekseeva E, Chen W, Jacobson JR and Birukov KG: Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation. *Exp Cell Res* 313: 2504-2520, 2007.
34. Li YS, Haga JH and Chien S: Molecular basis of the effects of shear stress on vascular endothelial cells. *J Biomech* 38: 1949-1971, 2005.
35. Schmit MA, Mirakaj V, Stangassinger M, König K, Köhler D and Rosenberger P: Vasodilator phosphostimulated protein (VASP) protects endothelial barrier function during hypoxia. *Inflammation* 35: 566-573, 2012.
36. Rentsendorj O, Mirzapozazova T, Adyshev D, Servinsky LE, Renne T, Verin AD and Pearse DB: Role of vasodilator-stimulated phosphoprotein in cGMP-mediated protection of human pulmonary artery endothelial barrier function. *Am J Physiol Lung Cell Mol Physiol* 294: L686-L697, 2008.
37. Schlegel N, Burger S, Golenhofen N, Walter U, Drenckhahn D and Waschke J: The role of VASP in regulation of cAMP- and Rac 1-mediated endothelial barrier stabilization. *Am J Physiol Cell Physiol* 294: C178-C188, 2008.
38. Profirovic J, Han J, Andreeva AV, Neamu RF, Pavlovic S, Vogel SM, Walter U and Voyno-Yasenetskaya TA: Vasodilator-stimulated phosphoprotein deficiency potentiates PAR-1-induced increase in endothelial permeability in mouse lungs. *J Cell Physiol* 226: 1255-1264, 2011.
39. Luyer MD, Buurman WA, Hadfoune M, Jacobs JA, Konstantinov SR, Dejong CH and Greve JW: Pretreatment with high-fat enteral nutrition reduces endotoxin and tumor necrosis factor-alpha and preserves gut barrier function early after hemorrhagic shock. *Shock* 21: 65-71, 2004.
40. Bogatcheva NV, Zemskova MA, Kovalenkov Y, Poirier C and Verin AD: Molecular mechanisms mediating protective effect of cAMP on lipopolysaccharide (LPS)-induced human lung microvascular endothelial cells (HLMVEC) hyperpermeability. *J Cell Physiol* 221: 750-759, 2009.
41. Ikari A, Ito M, Okude C, Sawada H, Harada H, Degawa M, Sakai H, Takahashi T, Sugatani J and Miwa M: Claudin-16 is directly phosphorylated by protein kinase A independently of a vasodilator-stimulated phosphoprotein-mediated pathway. *J Cell Physiol* 214: 221-229, 2008.
42. Caposio P, Dreano M, Garotta G, Gribaudo G and Landolfo S: Human cytomegalovirus stimulates cellular IKK2 activity and requires the enzyme for productive replication. *J Virol* 78: 3190-3195, 2004.
43. Frede S, Stockmann C, Freitag P and Fandrey J: Bacterial lipopolysaccharide induces HIF-1 activation in human monocytes via p44/42 MAPK and NF-kappaB. *Biochem J* 396: 517-527, 2006.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.