Chorein Sensitivity of Actin Polymerization, Cell Shape and Mechanical Stiffness of Vascular Endothelial Cells

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Abstract
Background/Aims: Endothelial cell stiffness plays a key role in endothelium-dependent control of vascular tone and arterial blood pressure. Actin polymerization and distribution of microfilaments is essential for mechanical cell stiffness. Chorein, a protein encoded by the VPS13A gene, defective in chorea-acanthocytosis (ChAc), is involved in neuronal cell survival as well as cortical actin polymerization of erythrocytes and blood platelets. Chorein is expressed in a wide variety of further cells, yet nothing is known about the impact of chorein on cells other than neurons, erythrocytes and platelets. The present study explored whether chorein is expressed in human umbilical vein endothelial cells (HUVECs) and addressed the putative role of chorein in the regulation of cytoskeletal architecture, stiffness and survival of those cells.

Methods: In HUVECs with or without silencing of the VPS13A gene, VPS13A mRNA expression was determined utilizing quantitative RT-PCR, cytoskeletal organization visualized by confocal microscopy, G/F actin ratio and phosphorylation status of focal adhesion kinase quantified by western blotting, cell death determined by flow cytometry, mechanical properties studied by atomic force microscopy (AFM) and cell morphology analysed by scanning ion conductance microscopy (SICM).

Results: VPS13A mRNA expression was detectable in HUVECs. Silencing of the VPS13A gene attenuated the filamentous actin network, decreased the ratio of soluble G-actin over filamentous F-actin, reduced cell stiffness and changed cell morphology compared to HUVECs silenced with negative control siRNA. These effects were paralleled by a significant decrease in FAK phosphorylation following VPS13A silencing. Moreover, silencing of the VPS13A gene increased caspase 3 activity and induced necrosis in HUVECs.

Conclusions: Chorein is a novel regulator of cytoskeletal architecture, cell shape, mechanical stiffness and survival of vascular endothelial cells.
Introduction

Chorein, a protein encoded by the VPS13A (vacuolar protein sorting 13 homolog A) gene [1], is expressed in a wide variety of cells [2-5]. Loss of function mutations [5] of the VPS13A gene [6-9] leads to chorea-acanthocytosis (ChAc), an autosomal recessive genetic disease characterized by progressive hyperkinetic movement disorder with muscle dystrophy, cognitive dysfunction, behavioural abnormalities, chronic hyperkalemia and variable acanthocytosis of red blood cells [5, 10]. Clinically overt disease develops at an age between 30 and 70 years and progresses to disability and premature death [10]. The lack of chorein action in ChAc leads to disappearance of cortical actin filaments in erythrocytes and thus to the spectacular erythrocyte shape change of acanthocytosis [3, 11]. Chorein is further expressed and modifies actin polymerization in blood platelets [12]. Loss of chorein renders cells more vulnerable to apoptosis [3], possibly contributing to increased susceptibility of neurons to cell death and neurodegeneration.

Chorein interacts with phosphoinositide-3-kinase (PI3K) [13, 14] leading to stimulation of PI3K signaling with subsequent activation of p21 protein-activated kinase (PAK1) [3]. PI3K signaling further involves focal adhesion kinase (FAK) [15-17]. FAK may transmit extracellular signals by activating downstream molecules such as: PI3K [18-20], paxillin [21] α-actinin [22], PAK1 [23-25], Nhe1 [26, 27], or rho kinase [28-34] and by stimulating polymerization of actin [35-37]. FAK plays a critical role in the formation and maintenance of focal adhesion complexes, thereby preventing anoikis in vascular endothelial cells [38-41].

At least in theory, the effect of chorein on cortical actin polymerization could impact on cell stiffness, which may affect endothelial cell function [42]. Aldosterone and salt-sensitive insertion of ENaC into the cell membrane of endothelial cells is known to trigger early swelling and later stiffening of those cells [43-46]. Endothelial cell stiffness compromises endothelial nitric oxide (NO) formation and thus the ability of endothelial cells to trigger vasodilation [43]. Accordingly, endothelial cell stiffening is thought to increase blood pressure [42]. ENaC insertion into the cell membrane and thus cell stiffness is further enhanced by C-reactive protein [47], an effect, which may well contribute to C-reactive protein-induced vascular lesions [48] including atherosclerosis [49]. Stiff endothelial cell syndrome (SECS) [42] may thus bear considerable pathophysiological relevance.

The present study explored whether chorein influences FAK signaling, actin polymerization, cell morphology, mechanical stiffness and cell survival of human umbilical vein endothelial cells.

Materials and Methods

Cell culture and silencing of HUVECs

Human umbilical vein endothelial cells (HUVECs) [50, 51] from Promocell (Heidelberg, Germany) were routinely cultured in VascuLife basal medium (Lifeline Cell Systems, Kirkland, USA) supplemented with 2% fetal bovine serum (FBS), 10 mM L-Glutamine, 5 ng/ml rh VEGF, 5 ng/ml rh EGF, 5 ng/ml rh FGF basic, 15 ng/ml rh IGF-1, 50 µg/ml ascorbic acid, 1 µg/ml hydrocortisone hemisuccinate and 0.75 units/ml heparin sulfate (Lifeline Cell Systems, Kirkland, USA). For silencing, HUVECs were cultured on six-well plates. After 24 hours, the cells were subsequently transfected with 5 nM validated VPS13A siRNA (ID no. S23342, Ambion, Life Technologies GmbH, Germany) or with 5 nM negative control siRNA (ID no. 4390843, Ambion, Life Technologies GmbH, Germany) using siPORT amine transfection agent (Ambion, Life Technologies GmbH, Germany) according to the manufacturer’s protocol. The cells were used 48 hours after transfection.

Quantitative RT-PCR

To determine VPS13A mRNA levels and verify the silencing efficiency of the VPS13A gene in HUVECs, quantitative real-time PCR was performed [52-55]. Total RNA was isolated from HUVECs using Trifast
Reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. Reverse transcription of 2µg RNA was performed using oligo(dT)_{12-18} primers (Invitrogen, Life Technologies GmbH, Germany) and SuperScriptIII Reverse Transcriptase (Invitrogen, Life Technologies GmbH, Germany). cDNA samples were treated with RNaseH (Invitrogen, Life Technologies GmbH, Germany). Quantitative real-time PCR was performed with the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using Universal TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. TaqMan primers and probes for VPS13A (ID no. Hs00362901_m1; Unigene ID: Hs.459790) and HPRT1 (ID no. Hs02800695_m1; Unigene ID: Hs.412707) were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). All PCRs were performed in duplicate, and mRNA fold changes were calculated by the 2^ΔΔCt method using HPRT1 as internal reference.

**Measurement of the G/F actin ratio by Triton X-100 fractionation**

To quantify actin polymerization in HUVECs [3, 56], cells were incubated in 500 µl of Triton X-extraction buffer containing 0.3% Triton X-100, 5 mM Tris, pH 7.4, 2 mM EGTA, 300 mM sucrose, 2 µM phalloidin, 1 mM PMSF, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 1 mM sodium orthovanadate, and 50 mM NaF for 5 min on ice. The supernatant containing the soluble proteins was removed by aspiration. The Triton X-insoluble pellet was scraped from the plate directly into 500 µl of RIPA buffer (Cell Signaling, Danvers, MA). Any remaining insoluble material was removed by centrifugation. Equal volumes of each fraction were boiled in Roti Load 1 protein loading buffer (Carl Roth, Karlsruhe, Germany) at 100°C for 10 min. Proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with monoclonal rabbit anti-β-Actin (13E5)- HRP conjugated antibody (1:1000, Cell Signaling, Danvers,MA). Antibody binding was detected with the ECL Western Blotting Substrate (Pierce, Rockford, IL) and bands were quantified using Quantity One Software (Bio-Rad, München, Germany).

**Western Blotting**

HUVECs were lysed with ice-cold RIPA lysis buffer (Cell Signaling, Danvers, MA) supplemented with complete protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL). After centrifugation at 10000 rpm for 5 min, 30 µg of proteins were boiled in Roti Load 1 protein loading buffer (Carl Roth, Karlsruhe, Germany) at 100°C for 10 min. Proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes [55, 57]. The membranes were incubated overnight at 4°C with rabbit phospho-FAK (Tyr397) antibody (1:1000, Cell Signaling, Danvers, MA), rabbit polyclonal FAK antibody (A-17) (1:1000; Santa Cruz, Dallas, Texas, USA) or rabbit anti-GAPDH antibody (1:1000; Cell Signaling, Danvers, MA) and then with secondary anti-rabbit HRP-conjugated antibody (1:1000; Cell Signaling, Danvers, MA) for 1 hour at room temperature. For loading controls, the membranes were stripped in stripping buffer (Thermo Scientific,USA) at RT for 10 min. Antibody binding was detected with the ECL Western Blotting Substrate (Pierce, Rockford, IL) and bands were quantified using Quantity One Software (Bio-Rad, München, Germany).

**Immunocytochemistry and confocal microscopy**

HUVECs grown on chamber slides were fixed for 15 min with 4% paraformaldehyde, washed with PBS and permeabilized for 10 min in 0.1% Triton X-100/PBS. The slides were blocked with 5% goat serum in 0.1% Triton X-100/PBS for 1 hour at RT. Actin was stained with rhodamine-phalloidin (1:100, Invitrogen, Karlsruhe, Germany) for 1 hour at RT, in the dark. The slides were mounted with ProLong Gold antifade reagent (Invitrogen, Karlsruhe, Germany). Confocal microscopy was performed as described previously [3, 58] with a confocal laser-scanning microscope (LSM 510, Carl Zeiss MicroImaging GmbH) using a water immersion A-Plan 40x/1.2W DICIII. At least 30 different fields were counted (about 70 cells), 8 independent experiments were performed and analysed.

**AFM force mapping**

AFM force mapping [59] was performed on live HUVECs plated onto fibronectin-coated Petri dishes and silenced with negative control siRNA or with VPS13A siRNA. The force mapping experiments were performed with a commercial AFM setup (MFP3D Bio, Asylum Research, Santa Barbara, CA) at 37°C about 48 hours after transfection. Right before the AFM experiments the cell culture medium was replaced with CO_{2}-independent Leibovitz’s L-15 medium (Invitrogen, Life Technologies GmbH, Germany).
Two-dimensional arrays of force-distance-curves were recorded on the cells (with a scan area of 80 x 80 µm², a force curve rate of 3 Hz corresponding to a vertical tip velocity of 20 µm/s, and a maximum force of 1 nN) using a single sphere-tip cantilever (Nanosensors SD-ST-CONT-M, NanoWorld, Neuchâtel, Switzerland) with a radius of \( R = 1 \) µm. The cantilever’s spring constant was calibrated by the thermal noise method \([60]\) to 0.38 N/m. The local mechanical stiffness in terms of Young’s modulus \( E \) was obtained by fitting the force-indentation-curves with the spherical Hertz model \([61]\):

\[
F = \frac{4}{3} \frac{E R \delta^{3/2}}{1 - \nu^2},
\]

where \( \delta \) is the sample indentation and \( \nu \) is the Poisson’s ratio of the sample (assumed as \( \nu = 0.5 \)). Two representative force curves and fits of the spherical Hertz model are shown in Fig. 2D and H.

A single value for the mechanical stiffness of a single cell was derived by averaging \( E \) over the cell area (exemplarily outlined by the dashed curves in Fig. 2C and G), where regions with a cell thickness below 1 µm were excluded to avoid an influence of the underlying substrate on the derived stiffness value. The average mechanical stiffness \( \langle E \rangle \) was then obtained by averaging the stiffness values of 24 negative control siRNA silenced HUVECs and of 24 VPS13A siRNA silenced HUVECs, respectively. Since the mechanical stiffness of live cells is log-normally distributed \([62]\) averaging and statistical tests were carried out with the logarithmic stiffness.

**SICM topography imaging**

SICM topography imaging \([63, 64]\) was performed on HUVECs plated onto fibronectin-coated Petri dishes and silenced with negative control siRNA or with VPS13A siRNA. SICM allows non-contact and compression-free topography imaging of soft samples such as membranes \([65]\) or cells \([66, 67]\). Recently, SICM has been used for stiffness mapping of living cells \([68]\). The images were recorded in backstep/hopping mode \([66, 69]\) using a home-built SICM setup. The nanopipettes were fabricated from borosilicate glass capillaries (1B100F-4, World Precision Instruments Inc., Sarasota, FL) using a CO₂-laser-based micropipette puller (P-2000, Sutter Instruments, Novato, CA) and had typical opening radii of 100-150 nm, providing a high signal-to-noise ratio while still allowing sub-micrometer lateral image resolution \([70]\). The SICM topography images were recorded on live cells at 37°C about 48 hours after transfection. Right before the SICM experiments the cell culture medium was replaced with CO₂-independent Leibovitz’s L-15 medium (Invitrogen, Life Technologies GmbH, Germany). Height profiles of the cells (from one edge of a cell through its center to its other edge) were obtained from the topography images (20 negative control siRNA silenced HUVECs and 20 VPS13A siRNA silenced HUVECs, 3 sections per cell). For each profile the cell height, \( h_{\text{max}} \), was obtained as the maximum value of the profile; the lamellipodium height, \( h_{\text{lam}} \), was obtained by averaging the outer 17% on each side of the profile (s. Fig. 3C and F). The relative lamellipodium height was defined as the ratio of lamellipodium height to cell height, \( h_{\text{lam}}/h_{\text{max}} \).

**Caspase 3 activity**

The cultured HUVECs were detached from the 6-well plate by treatment with 1x trypsin-EDTA (PAA, Austria) for 5 minutes. The cells were washed with Vasculife complete VEGF medium (Lifesience Inc. Germany) with 10% FBS by centrifugation at 1600 rpm for 3 minutes. Active caspase-3 was measured by CaspGlow Fluorescein Active Caspase-3 Staining kit (BioVision, CA, USA) according to the manufacturer’s instructions. Briefly, \( 10^5 \) cells were suspended in 100 µl Vasculife complete VEGF medium, stained for 1 hour with 0.1µl active caspase-3-FITC antibody under cell culture conditions. Then the cells were washed once in 200 µl wash buffer supplied in the kit, resuspended in 200 µl wash buffer and fluorescence was determined by flow cytometry using a BD FACS Calibur (BD Biosciences, CA, USA).

**Analysis of necrosis**

To determine necrosis, \( 10^5 \) cells/100µl in complete Vasculife VEGF medium were incubated in 70% ethanol (Sigma, USA) on ice for 30 minutes, centrifuged at 1600 rpm for 3 minutes at 4°C and added to 200 µl of hypotonic buffer (0.1% sodium citrate, 0.1% triton X-100, 2mM CaCl₂, 20U/ml RNase A in deionized water) together with 24 µl/ml Annexin V-FITC (Mabtag, Germany) and 50 µg/ml propidium iodide (Mabtag, Germany) and incubated on ice in the dark for 60 minutes. The cells were washed once at 1600 rpm for 3
minutes, resuspended in PBS/1% BSA and Annexin V-FITC fluorescence as well as PI fluorescence intensity were determined by flow cytometry using a BD FACS Calibur (BD Biosciences, CA, USA).

Statistics

Data are provided as means ± SEM, n represents the number of experiments. All data were tested for significance using Student’s unpaired t-test or Mann-Whitney test, as indicated in the figure legends. Only results with p values <0.05 were considered statistically significant.

Results

The present study explored whether chorein is expressed in vascular endothelial cells and whether it participates in the regulation of actin polymerization, cell shape and mechanical stiffness of those cells. Quantitative RT-PCR was employed to determine chorein expression. As illustrated in Fig. 1A, VPS13A transcript levels are detectable in human
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umbilical vein endothelial cells (HUVECs). Further experiments explored whether chorein influences the actin cytoskeleton organization in HUVECs. To this end, RNA interference was used to suppress endogenous VPS13A mRNA levels. To confirm the effects of silencing, VPS13A mRNA expression was examined by quantitative RT-PCR. As shown in Fig. 1A and B, VPS13A mRNA levels were significantly reduced in HUVECs silenced with VPS13A siRNA but not in HUVECs transfected with negative control siRNA. As illustrated in Fig. 1C and D, the ratio of soluble G-actin over filamentous F-actin was significantly higher in HUVECs silenced with VPS13A siRNA than in HUVECs silenced with negative control siRNA, an observation pointing to increased actin depolymerization. This was confirmed by confocal microscopy showing that HUVECs silenced with negative control siRNA had a clear filamentous actin network, which was markedly decreased in HUVECs silenced with VPS13A siRNA (Fig. 1E). Images are representative of 8 independent experiments. Filamentous actin network was reduced in approximately ¾ of investigated HUVECs silenced with VPS13A siRNA. Thus, in human umbilical vein endothelial cells, chorein influences the polymerization of actin filaments.

Fig. 2. AFM stiffness analysis with or without VPS13A silencing in HUVECs. Representative optical image (A), AFM topography image (B), AFM stiffness map (C), and representative force curve (D) of HUVECs after 48 hours silencing with 5 nM negative control siRNA. Representative optical image (E), AFM topography image (F), AFM stiffness map (G), and representative force curve (H) of HUVECs after 48 hours silencing with 5 nM VPS13A siRNA. AFM scan area 80 × 80 µm² (indicated by the dashed box in A and E). To avoid an influence of the underlying substrate on the stiffness, only regions with a cell thickness above 1 µm (exemplarily outlined by dashed curve in C and G) were considered. I. Geometric means ± SEM (n = 24/group) of the averaged stiffness of HUVECs after 48 hours silencing with 5 nM negative control siRNA or with 5 nM VPS13A siRNA, showing that the VPS13A silenced HUVECs are softer by about 53% (p = 1.5·10⁻⁵). ***(p<0.001) indicates statistically significant difference from HUVECs silenced with negative control siRNA; t-test.
Actin depolymerization indicates impaired filament stability [56] and is expected to modify the mechanical properties of HUVECs. In view of the impact of chorein silencing on actin polymerization we explored the possibility that chorein influences the stiffness and morphology of the human umbilical vein endothelial cells. Atomic force microscopy was thus employed to determine the stiffness of HUVECs following transfection with either negative control siRNA or with VPS13A siRNA (Fig. 2). Individual endothelial cells were localized using optical phase contrast microscopy and subsequently imaged with AFM force mapping (Fig. 2A-H). Chorein silencing results in a softer cell body (Fig. 2G and H) compared to the negative control silenced HUVECs (Fig. 2C and D). An analysis of the AFM force mapped cells revealed that chorein silenced HUVECs are, on average, significantly softer as compared to HUVECs transfected with negative control siRNA ($\bar{E} = 0.8\pm0.1$ kPa and $1.7\pm0.2$ kPa, respectively, Fig. 2I).

To analyse the cell morphology SICM topography imaging was performed on HUVECs in a confluent monolayer following transfection with either negative control siRNA or with VPS13A siRNA (Fig. 3). In comparison with the negative control siRNA treated HUVECs (Fig. 3A), the VPS13A siRNA silenced HUVECs (Fig. 3D) exhibited a distinct change in cell morphology, as their lamellipodium became more prominent and extended. Cross sections through single representative cells (Fig. 3B and E) revealed a smaller relative lamellipodium height for the VPS13A siRNA silenced HUVECs ($h_{\text{lam}}/h_{\text{max}}=0.1$, Fig. 3F) as compared to the negative control siRNA silenced HUVECs ($h_{\text{lam}}/h_{\text{max}} \approx 0.3$, Fig. 3C). On average, the relative
Fig. 4. Phosphorylation of focal adhesion kinase (FAK) with or without VPS13A silencing in HUVECs. A. Representative original western blots showing phosphorylated-FAK (Tyr397), total FAK and GAPDH protein abundance in HUVECs after 48 hours silencing with 5 nM negative control siRNA (Neg. siRNA) or with 5 nM VPS13A siRNA (VPS13A siRNA). B. Arithmetic means ± SEM (n = 8, arbitrary units) of phosphorylated-FAK/total FAK protein ratio normalized to GAPDH in HUVECs after 48 hours silencing with 5 nM negative control siRNA (Neg. siRNA) or with 5 nM VPS13A siRNA (VPS13A siRNA). *(p<0.05) indicates statistically significant difference from HUVECs silenced with negative control siRNA; t-test.

Fig. 5. Effect of VPS13A silencing on apoptosis and necrosis of HUVECs. A. Representative dot plots and arithmetic means ± SEM (n = 10, %) of the percentage of HUVECs with active caspase 3 quantified by active caspase 3-FITC fluorescence in flow cytometry analysis after 48 hours silencing with 5 nM negative control siRNA (Neg. siRNA) or with 5 nM VPS13A siRNA (VPS13A siRNA); cleaved caspase3-FITC: FL1-Height. B. Representative dot plots and arithmetic means ± SEM (n = 10, %) of the percentage of necrotic HUVECs quantified by Annexin V/PI flow cytometry analysis after 48 hours silencing with 5 nM negative control siRNA (Neg. siRNA) or with 5 nM VPS13A siRNA (VPS13A siRNA); Annexin V: FL1-Height, PI: FL2-Height. *(p<0.05) indicates statistically significant difference from HUVECs silenced with negative control siRNA; Mann-Whitney test.

The lamellipodium height of the VPS13A siRNA silenced HUVECs was significantly smaller than that of the negative control siRNA silenced HUVECs (Fig. 3G).
To gain some insight into the signaling involved in chorein-sensitive actin filament organization, cell shape and stiffness of HUVECs and in view of previous findings that focal adhesion kinase (FAK) is involved in cytoskeletal dynamics and organization as well as maintenance of cell stiffness [29], further experiments explored the role of chorein in the regulation of focal adhesion kinase. As illustrated in Fig. 4, VPS13A gene silencing was followed by a significant decrease of FAK phosphorylation in HUVECs pointing to a decrease of FAK activity.

In view of previous reports showing that loss of chorein leads to apoptosis of K562-erythrocytic cells [3] and that FAK signaling plays an important role in prevention of apoptosis [38, 39, 41], we explored the possibility that chorein influences HUVECs survival. As a result, VPS13A gene silencing was followed by a significant increase of caspase 3 activity in HUVECs (Fig. 5A). Furthermore, as shown in Fig. 5B, the percentage necrotic cells was significantly increased in HUVECs silenced with VPS13A siRNA as compared to negative control siRNA silenced HUVECs.

Discussion

The present observations disclose a novel function of chorein, i.e. regulation of cytoskeletal reorganization, cell morphology and mechanical stiffness of vascular endothelial cells. In human umbilical vein endothelial cells, decreased chorein expression markedly reduced the filamentous actin network which led to a distinct change in cell morphology with more prominent lamellipodium and softening of those cells. Furthermore, loss of chorein leads to caspase 3 activation and decreases survival of the human umbilical vein endothelial cells. The present observations provide compelling evidence that chorein is not only important for erythrocyte shape and neuronal function [3, 6-9] but may be effective in a wide variety of further functions.

At least in theory, endothelial cell stiffening may decrease the sensitivity of endothelial nitric oxide (NO) formation to shear stress and thus interfere with endothelial-dependent vasodilation [43]. For instance, aldosterone has been suggested to enhance endothelial cell stiffness and thus to decrease NO release and counteract vasodilation by up-regulating expression of the epithelial Na⁺ channel ENaC [44]. Along those lines, the mineralocorticoid receptor antagonist spironolactone reversed aldosterone induced endothelial cell stiffness and enhanced NO release [57]. Accordingly, endothelial cell stiffening was considered to predispose for decrease of NO release and increase of blood pressure [42]. In view of the present observations, not only decreased but as well increased chorein expression or function may be pathophysiologically relevant. According to the present observations increased chorein expression in endothelial cells could theoretically result in a "stiff endothelial cell syndrome" (SECS) [42] with potential impact on NO release and blood pressure regulation [43, 46]. Thus, our results suggest that endothelial chorein expression is involved in the regulation of endothelial function and may affect blood pressure regulation. Future studies are required, however, to test whether indeed excessive chorein influences endothelial NO release and systemic blood pressure.

The effects of chorein on actin cytoskeleton organization and cell stiffness are paralleled by the respective alterations of focal adhesion kinase (FAK) phosphorylation in HUVECs. FAK is a component of focal adhesions (FAs) considered as a point of convergence of integrin and growth factor mechanical signaling [58-62]. FAK is important for maintaining cell rigidity (stiffness) through promoting a static and highly aligned contractile cytoskeleton [29, 30, 34]. It is a powerful regulator of actin polymerization and distribution of microfilaments [21, 35-37] by activating downstream molecules such as: PI3K [18-20], paxillin [21, 35], PAK1 [23-25], Nhe1 [26, 27], Src [28, 32, 34], rho kinase [28-34] or α-actinin [22]. In FAK-deficient cells, reduced cytoskeletal stability is attributed to a compensatory increase of rhoA-kinase and ROCK activity [28, 29, 32, 33]. The FAK-sensitive regulation of cortical actin filament
architecture and dynamics impacts on exocytosis, membrane blebbing and receptor function [63-70].

Furthermore, focal adhesion kinase directly interacts with multiple cell surface receptors and signaling proteins [34, 60, 71-76] thus mediating a variety of physiological responses. As a regulator of focal adhesion dynamics, FAK plays an important role in vascular endothelial mechano-transduction induced by various stimuli [77-81] and contributes to the regulation of vascular cell proliferation and apoptosis [39, 41, 82-84], tissue remodelling [85, 86], angiogenesis and cell migration [82, 87, 88], leukocyte adhesion and vascular inflammation [89] as well as endothelial barrier function [82, 90]. Therefore, by regulating the activity of FAK, chorein may contribute to regulation of other physiological processes beyond regulating actin polymerization and cell stiffness. The present observations indeed reveal that chorein silencing leads to caspase 3 activation and increased necrosis in HUVECs. Thus, our results indicate that chorein is important for cell survival and that disruption of chorein-dependent signaling results in reduced viability of vascular endothelial cells.

In ChAc patients, the neuronal symptoms are most striking but possibly only the tip of the iceberg [5, 10]. However, additional studies are needed to define the physiological and pathophysiological role of chorein-dependent regulation of vascular endothelial cell stiffness and survival.

In conclusion, chorein is expressed in vascular endothelial cells and regulates endothelial cytoskeletal architecture and endothelial cell survival. Decreased chorein expression depolymerizes the actin filaments and thus leads to a distinct change in cell morphology and to softening of the vascular endothelial cells.

Abbreviations

AFM (atomic force microscopy); ChAc (chorea-acanthocytosis); EGF (epidermal growth factor); ENaC (amiloride-sensitive epithelial sodium channel); FAK (focal adhesion kinase); FGF (fibroblast growth factor); HPRT1 (hypoxanthine phosphoribosyltransferase 1); HUVECs (human umbilical vein endothelial cells); IGF (insulin-like growth factor); NHE1 (Na+/H+ exchanger 1); NO (nitric oxide); PAK1 (p21 protein-activated kinase); PI3K (Phosphoinositide 3-kinase); SECS (stiff endothelial cell syndrome); SICM (scanning ion conductance microscopy); siRNA (short interference RNA); Src (proto-oncogene tyrosine-protein kinase); ROCK (Rho-associated kinase); VEGF (vascular endothelial growth factor); VPS13A (vacuolar protein sorting 13 homolog A).

Conflict of Interests

The authors state that they have not any conflict of interests.

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