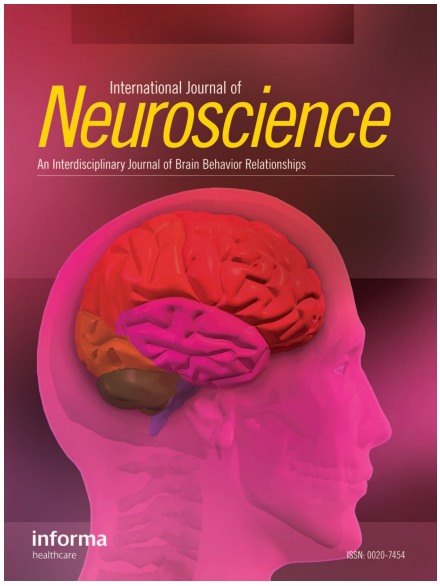

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Hypertension alters phosphorylation of VASP in brain endothelial cells

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ABSTRACT

Hypertension impairs cerebral vascular function. Vasodilator-stimulated phosphoprotein (VASP) mediates active reorganization of the cytoskeleton via membrane ruffling, aggregation, and tethering of actin filaments. VASP regulation of endothelial barrier function has been demonstrated by studies using VASP$^{-/-}$ animals under conditions associated with tissue hypoxia. We hypothesize that hypertension regulates VASP expression and/or phosphorylation in endothelial cells, thereby contributing to dysfunction in the cerebral vasculature. Because exercise has direct and indirect salutary effects on vascular systems that have been damaged by hypertension, we also investigated the effect of exercise on maintenance of VASP expression and/or phosphorylation. We used immunohistochemistry, Western blotting, and immunocytochemistry to examine the effect of hypertension on VASP expression and phosphorylation in brain endothelial cells in normotensive [Wistar–Kyoto (WKY)] and spontaneously hypertensive (SH) rats under normal and exercise conditions. We also analyzed VASP regulation in normoxia- and hypoxia-induced endothelial cells. Brain endothelial cells exhibited significantly lower VASP immunoreactivity and phosphorylation at the Ser157 residue in SHR versus WKY rats. Exercise reversed hypertension-induced alterations in VASP phosphorylation. Western blotting and immunocytochemistry indicated reduction in VASP phosphorylation in hypoxic versus normoxic endothelial cells. These results suggest that diminished VASP expression and/or Ser157 phosphorylation mediates endothelial changes associated with hypertension and exercise may normalize these changes at least in part by restoring VASP phosphorylation.

Hypertension alters phosphorylation of VASP in brain endothelial cells

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ABSTRACT

Hypertension impairs cerebral vascular function. Vasodilator-stimulated phosphoprotein (VASP) mediates active reorganization of the cytoskeleton via membrane ruffling, aggregation, and tethering of actin filaments. VASP regulation of endothelial barrier function has been demonstrated by studies using $VASP^{-/-}$ animals under conditions associated with tissue hypoxia. We hypothesize that hypertension regulates VASP expression and/or phosphorylation in endothelial cells, thereby contributing to dysfunction in the cerebral vasculature. Because exercise has direct and indirect salutary effects on vascular systems that have been damaged by hypertension, we also investigated the effect of exercise on maintenance of VASP expression and/or phosphorylation. We used immunohistochemistry, Western blotting, and immunocytochemistry to examine the effect of hypertension on VASP expression and phosphorylation in brain endothelial cells in normotensive [Wistar–Kyoto (WKY)] and spontaneously hypertensive (SH) rats under normal and exercise conditions. We also analyzed VASP regulation in normoxia- and hypoxia-induced endothelial cells. Brain endothelial cells exhibited significantly lower VASP immunoreactivity and phosphorylation at the Ser157 residue in SHR versus WKY rats. Exercise reversed hypertension-induced alterations in VASP phosphorylation. Western blotting and immunocytochemistry indicated reduction in VASP phosphorylation in hypoxic versus normoxic endothelial cells. These results suggest that diminished VASP expression and/or Ser157 phosphorylation mediates endothelial changes associated with hypertension and exercise may normalize these changes at least in part by restoring VASP phosphorylation.

INTRODUCTION

Hypertension, a condition associated with endothelial dysfunction, is a critical risk factor for cerebrovascular and cardiovascular diseases. It also has a role in the development of vascular cognitive impairment and vascular dementia [1, 2]. Cerebral blood vessels have many unique structural and functional characteristics that differentiate them from vessels in other organs. These include absence of fenestrations and presence of tight junctions, which are the foundation of the blood-brain barrier (BBB). Endothelial cells also play an important role in the regulation of vascular tone by releasing potent vasoactive factors such as nitric oxide (NO), free radicals, prostacyclin, endothelium-derived hyperpolarizing factor, and endothelin [3]. Neurovascular structure is disrupted early in pathological conditions such as hypertension and ischemic stroke, leading to metabolic deficiency in neuronal tissue. The deleterious action of reactive oxygen species on cerebral blood vessels also mediates cerebrovascular dysregulation [6]. Furthermore, acute and chronic increases in blood flow and/or shear stress in endothelial cells are known to enhance the expression and activity of endothelial nitric oxide synthase (eNOS), thereby resulting in the release of nitric oxide (NO). This mechanism has been shown to be associated with Ca2+-independent hypertension [7, 8].

VASP (vasodilator-stimulated phosphoprotein), first described in human platelets, is a 46 kDa membrane-associated protein that is part of the Ena/VASP family [9]. It is widely accepted that VASP is concentrated at focal adhesions, and cell-cell contacts where it regulates actin microfilaments; this suggests an intracellular modulatory role in the adhesion functions of many cells including platelets, vascular smooth muscle cells, endothelial cells, and fibroblasts. VASP is functionally activated by phosphorylation at

serine 157, serine 239, and threonine 278. The serine 157 and serine 239 sites are primarily phosphorylated by the cAMP and cGMP signaling pathways, respectively. Previous studies have shown that VASP mediates regulation of vascular endothelial cell functions such as endothelial barrier integrity, vascular permeability, and platelet aggregation under *in vivo* and *in vitro* conditions [10-12].

Hypertension reduces expression and/or activity of eNOS in endothelial cells, causing endothelium-dependent relaxation of cerebral blood vessels to deteriorate [14]. Previous studies have shown that VASP phosphorylation is NO/cGMP-dependent in brain capillary endothelial cells [15]. VASP modulates cytoskeletal dynamics in vascular endothelial cells, a crucial structural and functional unit of the blood-brain barrier. Other studies have shown that VASP regulates Rac1 Rho-GTPase, resulting in stabilization of endothelial barrier function [6]. Maintaining the endothelial barrier requires intercellular adhesion and cell-matrix connections for which VASP is thought to be essential. It has also been shown that VASP phosphorylation is decreased in hypertensive rats [16]. We hypothesize that hypertension-related hypoxia decreases the expression and/or phosphorylation of VASP in brain endothelial cells, thereby contributing to the development of dysfunction in cerebral vasculature.

METHODS

Animal Experiments

Spontaneously hypertensive rats (SHR) (11–12 weeks of age) and age-matched normotensive WKY rats (Harlan Laboratories, Indianapolis, IN, USA) served as the source for brain tissues. The animals were housed at 23 ± 2°C under a 12:12 h light:dark cycle and had free access to standard rat chow and drinking water. Systolic blood

pressure (SBP) was monitored daily via a tail-cuff method during the week before the experiments and every 2 weeks during the 8-week study period. All animal protocols were performed in accordance with the standards established and approved by the Animal Care Use and Animal Ethics Committee at Akdeniz University Faculty of Medicine, Turkey.

Rats were assigned randomly to WKY sedentary (WKY; n = 20), WKY-exercising (WKY-E; n = 20), SHR-sedentary (SHR; n = 20), and SHR-exercising (SHR-E; n = 20) groups. The exercise training groups were subjected to swimming exercise (60 min/day, 5 days/wk for 8 wks) in a glass tank (100 × 50 × 50 cm) filled with tap water (32–34°C). The duration of the first swimming experience was limited to 10 min and increased by 10 min daily until 60 min was reached. The experimental protocol was approved by the Animal Care and Usage Committee of Akdeniz University and in accordance with the guidelines for using animals in experimental research. Animals were sacrificed under thiopental sodium anesthesia (80 mg/kg/body weight) [17]. After anesthesia, extracted brain samples were fixed in 10% formalin for 8 h, dehydrated in an ascending ethanol series, and embedded in paraffin for immunohistochemical analysis.

Blood Pressure Monitoring

Systolic blood pressure was measured using a non-invasive tail-cuff method at baseline and every 2 weeks during the 8-week study period. Data were obtained with a MAY-BPHR 9610-PC unit and MP 150 data acquisition system (BIOPAC Systems; Santa Barbara, CA, USA). Final measurements were performed on exercised animals one day after the last swimming session.

Immunohistochemistry

Total VASP, Ser157-phosphoVASP, and Ser238-phosphoVASP immunohistochemistry were performed with antibodies developed specifically for total and phosphorylated forms of

VASP, as described previously [18]. Serial sections were incubated with mouse monoclonal total VASP antibody (Alexis Biochemicals), rabbit polyclonal Ser157-phosphoVASP (Cell Signaling Technology, Beverly, MA; both at 1:50 dilution), and rabbit polyclonal Ser238-phosphoVASP (Novus Biologicals, Littleton, CO; at 1:75 dilution) in TBS overnight in a humidified chamber at 4°C.

Non-specific mouse IgG1 and normal rabbit IgG antibodies were used at the same concentrations as the primary antibodies for negative controls. 100-M access peptide (Novus Biologicals, catalogue # NB100-82254PEP Littleton, CO) blocking was performed on the negative control VASP-Ser238 slides for 30 min before primary antibody application to determine the specificity of anti-Ser238 VASP antibody. After washing, the slides were incubated with biotinylated horse anti-mouse secondary antibody and biotinylated goat anti-rabbit secondary antibody (1:400; Vector Labs, Burlingame, CA) for 30 min at room temperature. After washing twice in TBS, the antigen-antibody complex was detected with streptavidin-biotin peroxidase complex (Vector Labs). DAB (3, 3-diaminobenzidine tetrahydrochloride dihydrate) (Vector Labs) was used as the chromogen to detect total VASP, phospho-Ser157 VASP, and phospho-Ser238 VASP. Slides were lightly counterstained with hematoxylin and mounted with a cover slip.

The distribution and intensity of total, phosphoSer157, and phosphoSer238 VASP immunostaining were semi-quantitatively evaluated using HSCORE analysis [19]. The intensity of endothelial immunoreactivity was scored as follows: 0, no staining; 1, weak, but detectable staining; 2, moderate staining; and 3+, intense staining. An HSCORE value was derived for each specimen by calculating the sum of the percentage of stained cells in each category multiplied by its respective score, using the formula HSCORE = $\sum_i$ i*Pi, where i represents the intensity score and Pi is the corresponding percentage of cells [20]. For each slide, five different fields were evaluated microscopically at 250× magnification. HSCORE evaluation was performed

independently by two investigators blinded to the source of the samples. Investigator scores were averaged.

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA), and mouse brain microvascular endothelial cells (bEND.3s) were kindly provided by Michael Ostrowski (Department of Molecular and Cellular Biochemistry, The Ohio State University College of Medicine, Columbus, OH). Both HUVECs and bEND.3s were grown in endothelial cell basal medium-2 (Clonetics, BioWhittaker, San Diego, CA) supplemented with human recombinant epidermal growth factor (10 pg/mL), human recombinant basic fibroblast growth factor (4 pg/mL), vascular endothelial growth factor, human recombinant IGF, ascorbic acid, heparin, hydrocortisone (0.4 µg/mL), gentamicin (50 µg/mL), amphotericin B (50 ng/mL), and 5% fetal bovine serum (Clonetics, BioWhittaker). HUVECs and bEND.3s were plated on 6-well plates and 4-well chamber slides and incubated in hypoxia (0.5% O_2 and 5% CO_2 with a balance of N_2 in a specialized environmental chamber (C-Chamber and ProOx Model C21, BioSpherix) and normoxia (5% CO2/95% air) for 12 and 24 h. The plates and chamber slides were used for Western blotting and immunocytochemistry. Experiments were conducted with third subcultures at 70–80% confluence. Before each experiment, cells were treated with phenol red-free media prepared with 5% charcoal-stripped calf serum for 24 h. Each experiment was repeated at least three times.

Western Blotting

Total protein from hypoxia- and normoxia-incubated HUVECs were extracted using T-PER protein extraction reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail (1 mM Na3VO4, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and

1 mM phenylmethylsulfonyl fluoride; Calbiochem, San Diego, CA). Western blotting was performed as described previously [20]. Briefly, 20 µg protein was loaded into each lane, separated by SDS-PAGE using 10% Tris-HCl Ready Gels (Bio-Rad Laboratories), and electroblotted onto nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked with 5% nonfat dry milk in TBS-T buffer (0.1% Tween 20 in Tris-buffered saline) for 1 h to reduce nonspecific binding. The membrane was incubated with mouse anti-human VASP or mouse anti-human phospho-Ser157 VASP or mouse anti-human phospho-Ser238 VASP monoclonal antibodies (1:1000 dilution; Cell Signaling Technology, Beverly, MA) overnight at 4°C and washed three times with TBS-T for 30 min. The membrane was incubated for 1 h with peroxidase-labeled anti-mouse IgG (1:10,000; Vector Laboratories) and washed with TBS-T three times for 30 min. Total and phospho-VASP were detected with chemiluminescent detection reagents (PerkinElmer Life Sciences, Boston, MA) and exposure to BioMax film (Kodak, Rochester, NY).

Membranes were stripped with stripping solution (Pierce) and reprobed with mouse monoclonal anti-β-actin (Cell Signaling Technology, Beverly, MA). Immunoblot bands for total VASP (T-VASP), phospho-Ser157 VASP, phospho-Ser238 VASP, and β-actin were quantified with ImageJ software (National Institutes of Health, Bethesda, MD). Each band for T-VASP was normalized to the value obtained from the corresponding β-actin band, whereas each band for phospho-Ser157 or phosphor-Ser238 VASP was normalized to the value obtained from the corresponding T-VASP band.

Immunocytochemistry

Chamber slides were fixed in 4% paraformaldehyde for 30 min at 4°C, then washed three times in TBS-T followed by the immunohistochemistry protocol, starting from endogenous peroxidase quenching as described above (see immunohistochemistry protocol).

Statistical Analyses

Normal SHR with or without exercise were normally distributed as determined by ANOVA for repeated measures followed by Bonferroni correction. These results were used to compare blood pressure levels. Statistical significance was defined as $p < 0.001$. HSCORE results from immunostaining were also normally distributed and analyzed by one-way ANOVA with *post hoc* Tukey testing. Statistical significance was defined as $p < 0.05$.

RESULTS

Blood Pressure Outcomes

Systolic blood pressure was significantly higher in SHR than in WKY rats (Mean ± SEM; SBP SHR = 190.7 ± 1.2 mmHg *vs.* SBP WKY = 130.3 ± 1.1 mmHg; $p < 0.001$). At the end of the study (after 8 weeks), exercise training had produced a significant decrease in systolic blood pressure in SHR-E animals compared to the SHR group (Mean ± SEM; SHR-E = 182.5 ± 2.2 *vs.* SHR = 204.7 ± 1.4; $p < 0.001$). In contrast, blood pressure levels were similar in the WKY-E and WKY rats (Mean ± SEM; WKY-E = 134.2 ± 1.6 *vs.* WKY = 134.0 ± 1.0; ***Table 1***).

VASP Expression in Brain Endothelial Cells

Arteriolar and capillary endothelial cells have different functions; the former is responsible for blood flow and the latter mediates nutrient and gas exchange and blood-brain-barrier function. Brain endothelial cells were evaluated in arteriolar and capillary

endothelia. VASP immunoreactivity was observed in brain endothelial cells of arteriolar and capillary vessels in both SHR and WKY rats (Fig. 1). HSCORE analysis revealed that SHR brain arteriolar and capillary endothelial cells expressed significantly less VASP than normotensive (WKY) brain endothelial cells ($p < 0.05$, Figs. 1, 2A and 4A). However, exercise did not change VASP expression in arteriolar endothelial cells in WKY *vs*. WKY+E ($p < 0.08$) and SHR *vs*. SHR+E ($p < 0.78$) (Fig. 2A). Similarly, exercise had no significant effect on capillary endothelial VASP expression in WKY *vs*. WKY+E ($p < 0.31$) and HT *vs*. HT+E ($p < 0.42$; Fig. 4A).

Exercise Reverses the Decreased VASP Phosphorylation in HT Brain Endothelial Cells

HSCORE analyses revealed that VASP phosphorylation at the Ser157 residue in SHR brain endothelial cells is significantly reduced in comparison to WKY brain arteriolar and capillary endothelial cells ($p < 0.05$ and $p < 0.02$, respectively; Fig. 3A *vs*. B; Figs. 2B and 4B). Moreover, exercised WKY+E rats showed an increase in phospho-Ser157 VASP immunoreactivity in arteriolar and capillary endothelium in comparison to unexercised WKY rats ($p < 0.006$*;* Fig. 3A *vs*. C; 2 and 4B). Similarly, arteriolar and capillary endothelial cells of exercised SHR+E rats revealed a significant increase in Ser157 phosphorylation when compared with unexercised SHR rats (*p<0.001*; Fig. 3B *vs*. D; Figs 2B and 4B).

There was no significant difference in VASP ser238 phosphorylation in brain arteriolar and capillary endothelial cells between WKY and SHR rats (*p<0.06*; Fig. 3E *vs*. F; Figs. 2C, and 4C). Furthermore, there was no significant difference in VASP Ser238 phosphorylation in brain endothelial cells between WKY rats vs. WKY+E rats (*p<0.49*

Fig. 3E *vs.* G; Figs.2C and 4C) and SHR vs. SHR+E rats ($p < 0.1$; Fig. 3F *vs.* H*;* Figs. 2C and 4C).

Hypoxia Inhibits VASP Phosphorylation in Cultured Endothelial Cells

To characterize the association between the hypertension-related decrease in VASP phosphorylation and hypoxia, endothelial cells were incubated in normoxia and hypoxia conditions. Western blotting showed that VASP phosphorylation at Ser157 was significantly reduced following 12 and 24 h incubations under hypoxic *versus* normoxic conditions (Fig. 5). In contrast, VASP phosphorylation at Ser238 did not differ between normoxia and hypoxia after 12 and 24 h incubation (Fig. 5).

Immunocytochemistry verified that hypoxia-induced HUVECs exhibited lower phospho-Ser157, but not phospho-Ser238 levels in endothelial cells (Fig. 6A-D). Furthermore, compared to normoxic conditions (Fig. 6 E), phospho-VASP Ser157 levels in bEND.3s cells incubated in hypoxic conditions (Fig. 6F) were also reduced. This confirms that hypoxia also induces changes in VASP phosphorylation in brain endothelial cells, similar to HUVECs.

DISCUSSION

VASP is important for F-actin assembly in endothelial cells [21] and is required to maintain heart and lung microvascular endothelial barrier functions *in vivo* and *in vitro* [22-24]. We analyzed the *in vivo* expression and phosphorylation levels of VASP in the brain vascular endothelium of normal rats and determined the effects of hypertension and exercise on these levels. This study was performed in an *in vivo* animal model and verified by *in vitro* immunocytochemistry and western blots.

Genetic studies in mice suggest Ena/VASP mediates neuritogenesis, endothelial barrier formation, and neural tube closure [23, 25]. Mutations in all three Ena/VASP family members result in severe endothelial barrier dysfunction, which is consistent with earlier studies suggesting a link between VASP and barrier function [15, 26]. Although our results do not provide a direct link between VASP expression and hypertension, the lower level of VASP expression in hypertensive rats suggests that VASP in the brain endothelium is sensitive to and down-regulated by hypertension. This observation is consistent with the results of another study that demonstrated reduced VASP phosphorylation associated with hypertension in platelets [16]. We found similar changes in VASP expression and/or its phosphorylation in microvascular and arteriolar endothelium, suggesting these transformations occur independent of vessel type and/or specific function in regulating blood flow and tissue-blood exchanges and/or blood-brain barrier functions, respectively [27].

Hypoxia is associated with hypertension in the vascular system [28]. Our in vitro finding which demonstrates reduced VASP phosphorylation by hypoxia supports the hypothesis that hypertension-related hypoxia decreases the phosphorylation of VASP in brain endothelial cells. Furthermore, this interaction occurs without changing VASP levels. A recent study showed that VASP is downregulated in response to hypoxic conditions, illustrating another mechanism by which Ena/VASP family members regulate barrier function [29]. Targeting VASP by siRNA reduces barrier function in human microvascular endothelial cells, whereas overexpression of VASP increases basal barrier function and protects cells from hypoxia-induced barrier dysfunction [23].

Phosphorylation of VASP at serine 157, serine 239, and threonine 278 results in functional activation of the protein [11, 16, 23]. The serine 157 and serine 239 sites are primarily phosphorylated by cAMP and cGMP, respectively. Our results show that hypertension affects VASP phosphorylation at serine 157 but not at serine 239, suggesting that decreased VASP Ser-157 phosphorylation in the endothelial cells of SH rats most likely affects the cAMP signaling cascade. cAMP- and cGMP-dependent phosphorylation of VASP is also responsible for the negative regulation of collagen-induced fibrinogen binding and platelet aggregation [30]. VASP also regulates vascular smooth muscle cell proliferation [31] and studies suggest it is involved in the endothelium-driven development of arteriosclerotic diseases [32, 33]. We speculate that alterations in VASP expression and phosphorylation may explain why endothelial cytoskeletal disruption results in hypertension-related endothelial changes. In a similar manner, hypertension alters endothelium-dependent relaxation of cerebral blood vessels [14] by reducing the expression and/or activity of eNOS in endothelial cells. This supports our hypothesis, as phosphorylation of VASP is NO/cAMP and or NO/cGMP-dependent in capillary endothelial cells [15, 34].

In this study, we demonstrated that exercise increases VASP phosphorylation at the Ser157 residue without changing its total expression level in normal and hypertensive rats; thus, the reduction in VASP phosphorylation in the brain endothelium of SH rats is reversible. This, in turn, may result in a functional improvement of vasculature in exercised rats. Previous studies have shown that regular exercise may represent a non-pharmacological therapeutic option to delay the degradation of endothelial function associated with aging [35] and may reverse endothelial impairment in individuals with

atherosclerosis [36] or heart failure [37]. Studies of a variety of vessels and animal models have shown that the repetitive increases in blood flow and shear stress that accompany regular exercise elicit an adaptive response that alters the intrinsic responsiveness of the endothelium by increasing mRNA expression of NOS [38]. This in turn increases the synthesis and release of NO and improves endothelial function. Because exercise is associated with increased NOS synthesis, our results showing increased VASP phosphorylation in exercised rats could be due to increased NO production; this will be addressed in future studies. In addition, it has been shown that expression of eNOS in arterial segments was significantly higher in exercising groups than in the controls, and this upregulation expression was expected because the increased shear stress during exercise is known to induce eNOS expression [39]; this may improve endothelial function. Indeed, increased bioavailability of endothelium-derived NO in the bloodstream induces PKG-dependent Ser157 and Ser238 phosphorylation of VASP in endothelial cells and platelets [15, 40].

This study demonstrates for the first time that hypertension-related hypoxia alters expression and phosphorylation of VASP in brain endothelial cells. This may be a contributing factor in hypertension-related endothelial changes. Our findings also show that exercise can reverse the loss of VASP phosphorylation in hypertensive rats and thus may improve brain endothelium related vascular changes. Pharmacologic agents targeting VASP expression and/or phosphorylation should be tested in this rat model as a potential treatment for hypertensive patients.

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TABLE AND FIGURE LEGENDS

Table 1. Systemic blood pressure before and after swimming exercise

Rats were divided into normotensive control (WKY), normotensive training (WKY-E), hypertensive control (SHR), and hypertensive training (SHR-E) groups (n = 20 per group). Changes in the mean arterial pressure (MAP, mmHg) were measured by the tail-cuff method. Data are means ± SD. *P < 0.001 vs. Wistar-Kyoto (WKY); #P < 0.001 relative to basal levels.

Figure 1

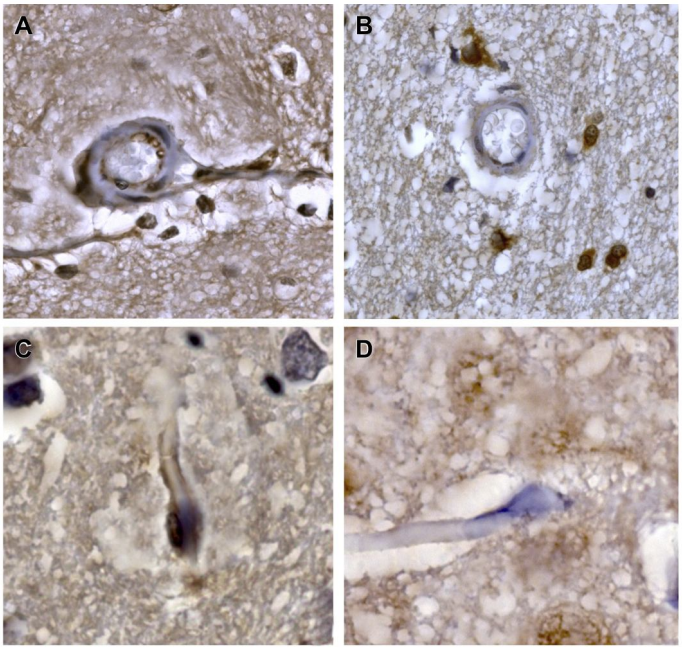

Figure 1. Immunostaining of total VASP in brain vascular endothelial cells

Immunohistochemical staining of total-VASP in brain arteriolar (A) and capillary (C) endothelial cells of WKY rats is and that of total-VASP in arteriolar (B) and capillary (D) endothelial cells of SHR. Original magnification A–D, 250X

Figure 2

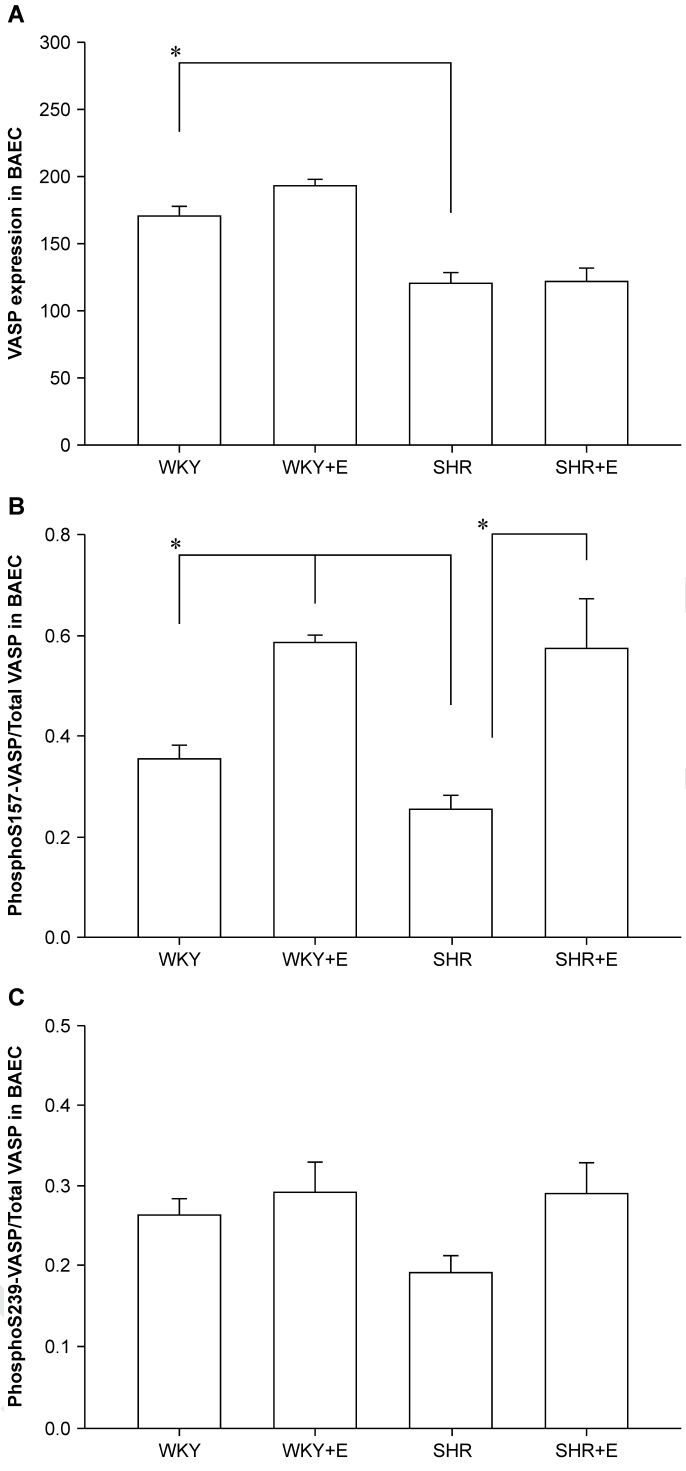

Figure 2. HSCORE analysis of VASP, Ser157-Phospho VASP, and Ser238-PhosphoVASP in brain arteriolar endothelium of WKY sedentary (WKY), WKY-exercised (WKY-E), SHR-sedentary (SHR), and SHR-exercised (SHR-E) rats

Total VASP expression (A); Ser157-PhophoVASP (B); and Ser238 Phospo-VASP (C) in brain arteriolar endothelial cells. *$P < 0.05$

Figure 3

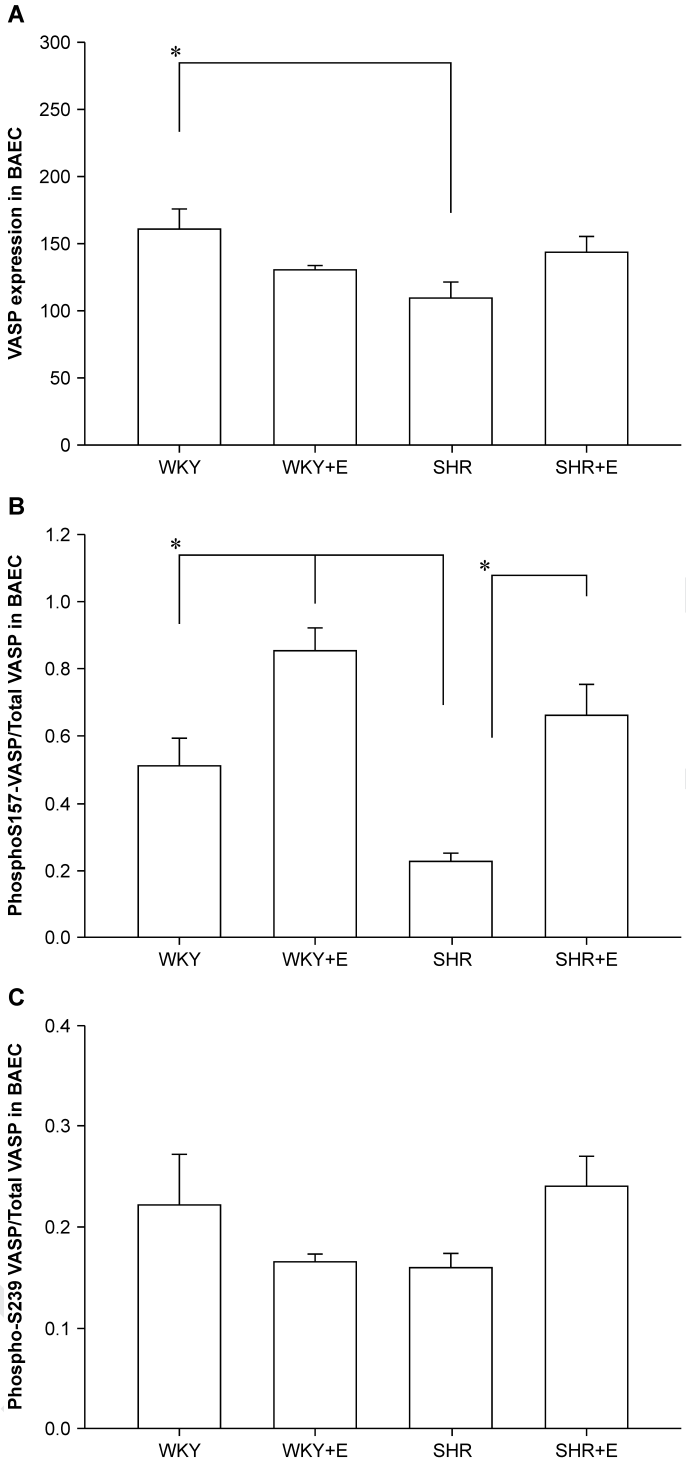

Figure 3. Immunostaining of Ser157 phospho-VASP and Ser238 phospho-VASP in brain endothelial cells

Ser157-VASP immunoreactivity in rat brain capillary endothelium of WKY rats (A), SHR (B), WKY-E (C), and SHR-E (D) rats. Ser238-VASP immunoreactivity in rat brain endothelium of WKY (E), SHR (F), WKY-E (G) and SHR-E (H) rats. Original magnification A–D, 250×

Figure 4

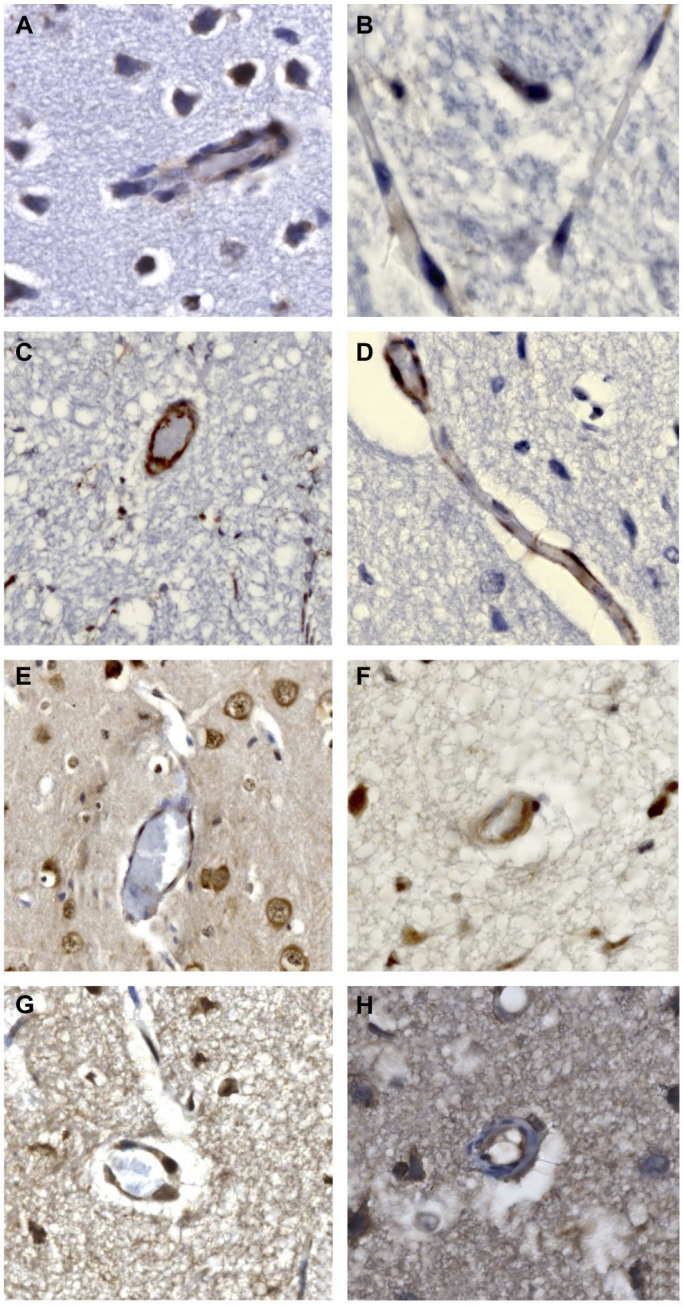

Figure 4. HSCORE analysis of VASP, Ser157-Phospho VASP, and Ser238-PhosphoVASP in brain capillary endothelium of WKY sedentary (WKY), WKY-exercised (WKY-E), SHR-sedentary (SHR), and SHR-exercised (SHR-E) rats. Total VASP expression (A); Ser157-PhophoVASP (B), and Ser238 Phospho-VASP (C) in brain capillary endothelial cells. *$P < 0.05$

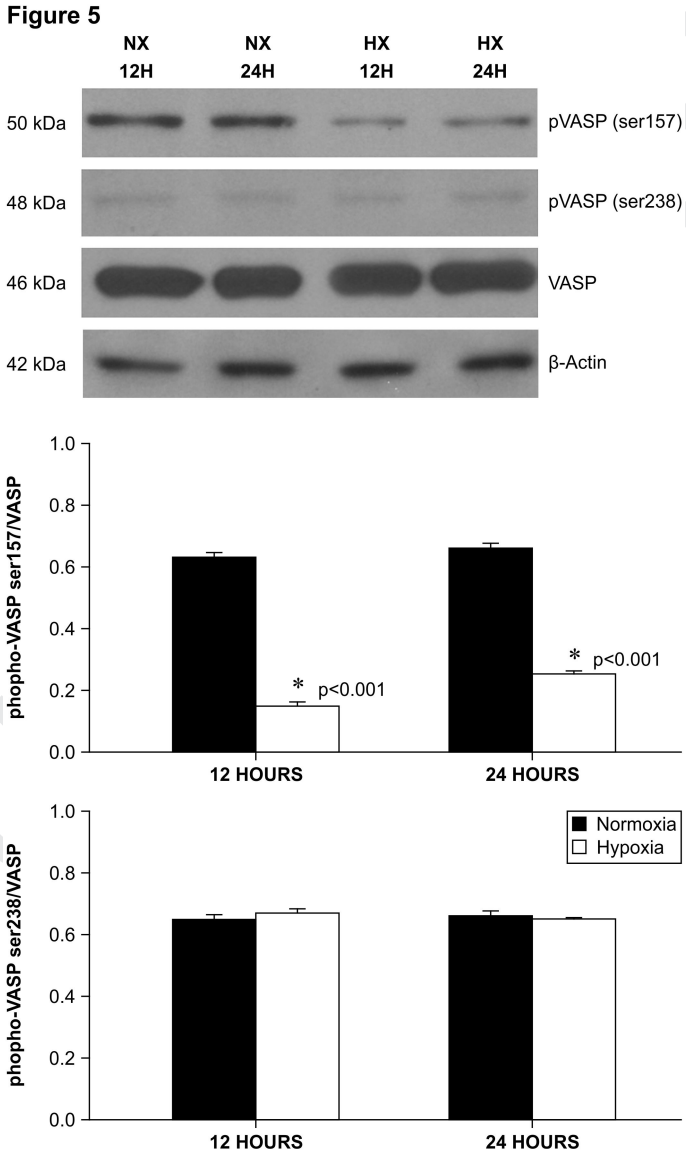

Figure 5. VASP expression and phosphorylation in hypoxic cultured endothelial cells Confluent endothelial cells were incubated under normoxic (Nx) and hypoxic (Hx) conditions for 12 and 24 h in 6-well plates. Western blots of VASP, Ser157-Phospho VASP, and Ser238-PhosphoVASP. * *versus* Normoxia. Experiments were repeated three times. Representative blots from one experiment are shown. Bars represent mean ± SEM.

Figure 6

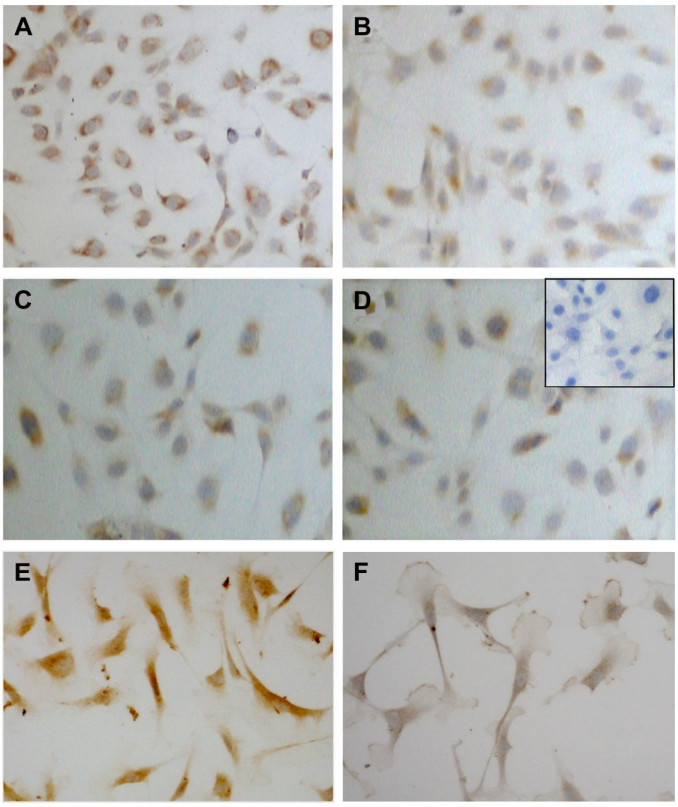

Figure 6. Immunocytochemistry of VASP phosphorylation in hypoxic cultured endothelial cells

Cells on tissue chamber slides were immunostained for phospho-VASP Ser157 and phospho-VASP Ser238 after incubation under normoxic and hypoxic conditions.

Immunostaining of normoxic (A) and hypoxic (B) endothelial cells for phospho-VASP Ser157 and normoxic (C) and hypoxic (D) endothelial cells for phospho-VASP Ser238. Staining with a rabbit isotype was used as a negative control (D, inset). Expression of phospho-VASP Ser157 in cultured mouse brain microvascular endothelial cells under normoxic (E) and hypoxic conditions (F). Original magnification: 200× (A–F and D inset).

Table 1. Systemic blood pressure before and after swimming exercise

*p < 0.001 WKY; #p < 0.001 difference from basal

	Normotensive rats		**Hypertensive rats**	
	Control	**Exercised**	**Control**	**Exercised (SHR-**
Basal	130.3 ±1.1	132.3 ± 1.4	190.7 ±	191.6 ± 1.6*
After 8	134.0 ±	134.2 ± 1.6	204.7 ±	182.5 ± 2.2*#