Role of dual-specificity protein phosphatase-5 in modulating the myogenic response in rat cerebral arteries

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Wickramasekera NT, Gebremedhin D, Carver KA, Vakeel P, Ramchandran R, Schuett A, Harder DR. Role of dual-specificity protein phosphatase-5 (DUSP-5) in modulating the myogenic response in rat cerebral arteries. J Appl Physiol 114: 252–261, 2013. First published November 21, 2012; doi:10.1152/japplphysiol.01026.2011.—The present study examined the role of the dual-specificity protein phosphatase-5 (DUSP-5) in the pressure-induced myogenic responses of organ-cultured cerebral arterial segments. In these studies, we initially compared freshly isolated and organ-cultured cerebral arterial segments with respect to responses to step increases in intravascular pressure, vasodilator and vasoconstrictor stimuli, activities of the large-conductance arterial Ca2+-activated K+ (KCa) single-channel current, and stable protein expression of DUSP-5 enzyme. The results demonstrate maintained pressure-dependent myogenic vasoconstriction, DUSP-5 protein expression, endothelium-dependent and -independent dilations, agonist-induced constriction, and unitary KCa channel conductance in organ-cultured cerebral arterial segments similar to that in freshly isolated cerebral arteries. Furthermore, using a permeabilization transfection technique in organ-cultured cerebral arterial segments, gene-specific small interfering RNA (siRNA) induced knocking down of DUSP-5 mRNA and protein, which were associated with enhanced pressure-dependent cerebral arterial myogenic constriction and increased phosphorylation of PKCβII. In addition, siRNA knockdown of DUSP-5 reduced levels of phosphorylated ROCK and ERK1 with no change in the level of phosphorylated ERK2. Pharmacological inhibition of ERK1/2 phosphorylation significantly attenuated pressure-induced myogenic constriction in cerebral arteries. The findings within the present studies illustrate that DUSP-5, native in cerebral arterial muscle cells, appears to regulate signaling of pressure-dependent myogenic cerebral arterial constriction, which is crucial for the maintenance of constant cerebral blood flow to the brain.

myogenic response; DUSP-5; KCa channel; vasoreactivity; endothelium; vascular smooth muscle; organ culture; cerebral arteries

MATERIALS AND METHODS

Myogenic response studies of cannulated cerebral arteries. All procedures were approved by the Animal Care and Use Committee (IACUC) of the Medical College of Wisconsin. Age-matched 10- to 12 wk-old male Sprague-Dawley (SD) rats were purchased from Harlan (Indianapolis, IN). The rats were euthanized with Beuthanasia-D (200 mg/kg), administered intraperitoneally (Schering-Plough Animal Health, Union, NJ). The brain was removed and placed in ice-cold phosphate-buffered saline containing (in mM) 138 NaCl, 3 KCl, 10 Na2HPO4, 2 NaH2PO4, 5 glucose, 0.1 CaCl2, and 0.1 MgSO4 (pH = 7.4). Middle cerebral arteries were carefully dissected free of the surrounding connective tissue. Immediately after dissection and following the reversible permeabilization procedure described below, arterial segments were cultured for 72 h by adopting methods previously developed by several investigators (2, 4, 9, 44). This technique allows us to manipulate the vessel ex vivo and modify a variety of signaling molecules including DUSP-5. It is possible that organ culture of isolated arteries, which are composed of mature, differentiated, and contracting cells, could offer an alternative approach without phenotypic drift observed in dissociated cells grown in culture. The potential advantage of a vascular organ culture model is that, in addition to its suitability for transfection of cells in the arterial wall, it could also permit examination of vascular reactivity, ion channel function, and screening of different signaling mechanisms known to regulate vascular function. The present studies were initiated to first determine pressure-dependent myogenic constriction, vascular reactivity, K+ channel function, and expression of genes of interest that are known to regulate cerebral arterial function, with special emphasis placed on actions of the protein DUSP-5.

We used rat cerebral arteries, which were cultured for 72 h, to examine the level of expression and role of DUSP-5, also known as mitogen-activated protein kinase (MAPK) phosphatase (MKP) encoded by the DUSP-5 gene, in the regulation of pressure-induced myogenic cerebral arterial constriction by decreasing its expression via gene-specific small interfering RNA (siRNA) transfection of cultured cerebral arterial segments. Since the extracellular signal-regulated kinase (ERK1/2), a member of the MAPK pathway, is known to be involved in pressure-dependent myogenic constriction (40) and is subject to negative regulation by protein phosphatases (5, 22, 23, 33, 47), its fate following siRNA knockdown of DUSP-5 was also examined.
ously described by other investigators (2, 4, 9, 44). The cultured arteries and the freshly isolated segments (8–10 mm in length and 0.15–0.2 mm in diameter) were placed in a perfusion chamber containing physiological salt solution (PSS) with the following ionic composition (in mM): 140 NaCl, 4.7 KCl, 0.72 MgCl₂, 1.18 NaH₂PO₄, 2.5 CaCl₂, 25 NaHCO₃, 10 glucose, and 5 HEPES (pH = 7.4), cannulated at both ends with glass micropipettes and secured in place with 8-0 polyethylene suture (Ethicon, Somerville, NJ) using a stereo microscope. The cannulated cerebral arterial segment was gently extended to its approximate in situ length, and the side branches were tied off to stop leakage. The cannulated arterial segment was continuously perfused and superfused with PSS of the above-mentioned composition at 37°C and pH 7.4, aerated with 21% O₂, 5% CO₂, 74% N₂ gas mixture, and the intraluminal pressure was maintained at 80 mmHg. In some experiments, the endothelium lining the inner wall of the cerebral arterial segments was removed or damaged by brief passage of air through the lumen. The internal diameter of the arteries was measured using a video-microscopy system composed of a television camera and a video micrometer (Nikon Instruments, Melville, NY). Fluorescent images were all taken with the same exposure time and intensity to compare the relative vessel size.

Single-channel Kₐᵥ currents. Single-channel Kₐᵥ currents were recorded at room temperature from excised inside-out membrane patches of dissociated smooth muscle cells of either freshly isolated or cultured cerebral arterial muscle cells, as previously described (10, 13, 14, 17). Briefly, recording pipettes were fabricated from borosilicate glass pulled on a two-stage micropipette puller (PC-84) and heat polished under a microscope (Narishige MF-83 heat polisher). The recording pipettes were mounted on a three-way hydraulic micromanipulator (Narishige, Tokyo, Japan) for placement of the tips on the cell membrane. High-resistance seals (>1 Gig Ω) were established by applying a slight suction between fire-polished pipette tips (3–10 MΩ) and cell membranes. The junction potentials between pipette and bath solution were corrected with an offset circuit before each experiment. Pipette potential was clamped, and single-channel Kₐᵥ currents were recorded at different patch potentials from excised inside-out patches, as described below through a List EPC-7 patch-clamp amplifier (List Biological Laboratories, Campbell, CA). The amplifier output was low-pass filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). Current signals were digitized at a sampling rate of 2.5 kHz. Single-channel Kₐᵥ currents were analyzed using a pClamp software package (pClamp versions 10.2, Axon Instruments, Foster City, CA) to determine mean current amplitudes, unitary conductance, and open-state probability.

Patch-clamp solutions. Recording pipette solution contained (in mM) 145 KCl, 1.8 CaCl₂, 1.1 MgCl₂, 5 HEPES, 5 EGTA, with pH 6.8 adjusted to 7.2 with KOH. This resulted in a calculated final bath [Ca²⁺] of 10⁻⁷ M (14). To study the sensitivity to changes in [Ca²⁺], the single-channel Kₐᵥ currents recorded from inside-out membrane patches of freshly isolated or cultured cerebral arterial muscle cells, the different [Ca²⁺], in the bath solution were calculated using a computer program (15). The unitary conductances, sensitivity to changes in voltage, or Ca²⁺ concentrations of the single-channel Kₐᵥ current native in freshly isolated or cultured cerebral arterial muscle cells was characterized as previously described (10, 13).

Responses to vasodilator and vasoconstrictor stimuli. To examine and compare the integrity of vascular function, the effects of the endothelium-dependent vasodilator acetylcholine (ACh), the endothelium-independent relaxant sodium nitroprusside (SNP), and the vasoconstrictor agonist serotonin (5-HT) were examined in cannulated and pressurized cultured or freshly isolated cerebral arteries. The cannulated arterial segments were pressurized to 20 mmHg and equilibrated in PSS bubbled with a 21% O₂, 5% CO₂, 74% N₂ gas mixture at 37°C and pH 7.4 for 45 min. At the end of the equilibration period, measurement of the internal diameter was made, and intraluminal pressure was increased to 80 mmHg and maintained for 60 min to develop myogenic tone. The internal diameter was measured using a video-micrometer system (Living Systems, St. Albans, VT). In separate experiments, the vasodilator response of the cannulated and pressurized (80 mmHg)-constricted cerebral arterial segments to cumulative addition of ACh or SNP to the solution bathing the arterial segments was determined. The cannulated and pressurized arterial segments treated with ACh were extensively washed, and, following pressurization to 80 mmHg and constriction with 50 μM 5-HT, the endothelium-dependent relaxation response of the arterial segments was assessed by cumulative addition of ACh (10⁻¹⁰ to 10⁻⁶ M) to the vessel chamber. At the end of each experiment, the cannulated arterial segments were repeatedly washed with PSS, and maximum vasodilator response of the arterial segments was determined following superfusion with Ca²⁺⁻free PSS having the following composition (in mM): 140 NaCl, 4.7 KCl, 1.18 NaH₂PO₄, 2.9 MgCl₂, 25 NaHCO₃, 10 glucose, and 5 HEPES (pH = 7.4). Diameter responses were expressed as [(Diametermaximum − Diametercontrol)/Diametermaximum] × 100, where Diametermaximum is the maximum diameter in Ca²⁺⁻free solution and Diametercontrol is the control diameter measured at 80 mmHg in normal PSS solution.

Transfection with siRNA. siRNA against DUSP-5 was examined. The DUSP-5 siRNA (on-target plus smart pool Rat DUSP-5, NM_135378) purchased from Thermo Fisher Scientific (Lafayette, CO) was dissolved according to the manufacturer’s instructions in a siRNA suspension buffer (Thermo Fisher Scientific). SignalSilence Control siRNA (Fluorescein Conjugate) was obtained from Cell Signaling Technology (Danvers, MA). This nonsense siRNA sequence that would not lead to specific degradation of any cellular message was used as a control siRNA to verify the successful entry of siRNA into the cerebral vascular smooth muscle cells (7). Control siRNA or gene-specific siRNA molecules were introduced into cultured intact cerebral arterial segments from two rat brains using a reversible permeabilization procedure previously described by other investigators (6, 30, 36, 44). For permeabilization, the arterial segments in culture dishes were incubated for 20 min at 4°C in the following solution (in mM): 10 EGTA, 120 KCl, 5 ATP, 2 MgCl₂, and 20 TES and 100 nM siRNA (pH = 6.8) for 30 min. Subsequently, permeabilization was reversed by placing the arteries in a fourth solution containing (in mM) 140 NaCl, 5 KCl, 10 MgCl₂, 5 glucose, and 2 3-(N-Morpholino)propanesulfonic acid (MOPS) (pH = 7.1, 22°C, 30 min) in which [Ca²⁺] was gradually increased from 0.01 to 1.8 mM every 15 min. Following the reversible permeabilization procedure, the arterial segments were cultured in DMEM/F-12 culture medium (supplemented with 1 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin) and maintained in an incubator (37°C, 21% O₂, 5% CO₂, 74% N₂) for 72 h (6, 30, 36, 44). In experiments where the control FITC-conjugated siRNA was used, arterial segments were placed on a glass slide, and the incorporation of FITC-tagged siRNA was visualized using a Nikon Eclipse E600 fluorescence microscope (Nikon Instruments, Melville, NY). Fluorescent images were all taken with the same exposure time and intensity to compare the relative
fluorescent intensity. The images were obtained using a Spot RT slider color camera (Diagnostic Instruments, Sterling Heights, MI). For mRNA analysis, Real-time PCRs were used to quantify DUSP-5 and RNA polymerase II (Polr2) mRNA in cerebral arterial segments from two rat brains, treated either with nonselective or DUSP-5-targeted siRNA and cultured for 72 h. For RNA extraction, arterial tissue was first homogenized on ice in lysis buffer RI (Ambion Biotechnologies, Union City, CA) using a glass mortar and pestle and then further homogenized by sonication and passage through a QIAshredder column (Qiagen, Hilden, Germany). Total RNA was isolated using the AxyPrep Multisource Total RNA Miniprep Kit (Axyon Biosciences). To prevent contamination of RNA with genomic DNA, samples were treated with DNase I (Thermo Fisher Scientific, Waltham, MA) during the isolation process. RNA integrity of samples was checked by electrophoresis on a formaldehyde agarose gel. RNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific), and 250 ng of RNA per 20-µl reaction was used to make cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). For RT-PCR, 1 µl of the 20-µl cDNA reaction product containing 12.5 ng of cDNA was used per 25-µl RT reaction containing iQ SYBR Green Supermix (Bio-Rad Laboratories) with the following protocol: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Primers were obtained from Qiagen (RT² qPCR Primer Assay for rat DUSP-5, product ID PPR45099A) and Operon Biotechnologies (Huntsville, AL) for RNA polymerase II (Polr2 forward: 5'-cctgatgcgggtgctgagtcagaagg-3'; reverse: 5'-gctgtgaccccatgacgagtg-3'). Linearity of amplification for both primer sets was verified by analysis of serially diluted cDNA samples. Product singularity and specificity were confirmed by melt curve analysis and agarose gel electrophoresis, respectively. Relative quantification of expression was determined by measuring the threshold (Ct) values of each sample using the 2-ΔΔCt method (32). Relative abundance of DUSP-5 was normalized to the levels of Polr2. The highest control value was set to 100, and the other values were normalized accordingly.

**Effect of inhibition of extracellular signal-regulated kinase phosphorylation by DUSP-5 on the pressure-induced myogenic tone.** UO126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminoethyl]thiobutadiene) was used as a selective inhibitor of extracellular signal-regulated kinase (ERK) isoform 1 and 2 (10) and was dissolved in DMSO. The freshly isolated and cannulated cerebral arteries were treated for 3 h with the vehicle (DMSO) or UO126 (10 µM) (48) by adding to the bath to determine its effect on pressure-induced myogenic tone. Following the 3-h incubation with vehicle or UO126, the cannulated arteries were pressurized from 20 to 120 mmHg in 20-mmHg increments. Changes in diameter in response to step increases in intraluminal pressure under control condition or following treatment with UO126 were determined and normalized to the diameter measured at 20 mmHg (control diameter). Arteries from two rat brains were treated with vehicle or UO126 and homogenized in cold homogenization buffer (25 mM Tris, pH = 7.0, 10% Triton-X100) containing protease inhibitors and phosphatase inhibitors (protease and phosphatase inhibitor cocktail, Calbiochem), and homogenized using a tissue glass homogenizer. Between 15 and 20 vessels were homogenized to prepare protein for Western blot analysis. The amount of protein was determined by the Bio-Rad protein assay method. Laemmli buffer was added to the samples and boiled for 5 min. Protein (25–50 µg) was loaded per well for analysis of the level of expression of CYP 4A and CYP 4F proteins. Protein (50 µg) was loaded per well for analysis of DUSP-5 protein. Protein samples were run on 10% acrylamide gels and subjected to SDS-PAGE using the Bio-Rad minigel system. Proteins were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) and probed with primary antibodies [cytochrome P450 (CYP) 4A, 4F, DUSP-5 (ab54939; AbCam, Cambridge, MA), ERK1/2, P-ERK1/2 (Thr202/Tyr204), and P-PKC (B (II Ser600) (Cell Signaling Technology, Danvers, MA) overnight (4°C) at a dilution of 1:500 or 1:1,000. Membranes were washed three times and then incubated with the appropriate secondary antibody (Bio-Rad). Membranes were exposed to films, and the bands were visualized using an enhanced chemiluminescence reagent (ECL; Amersham Biosciences). In all Western blot experiments, β-actin was used as an internal control for equal protein loading. Western blots were repeated three times for each protein. For some experiments, proteins separated using SDS-PAGE on Bio-Rad Minigel gradient gels (4–20%) stained with Coomassie brilliant blue were used for determination of total protein profile in fresh and organ-cultured arteries.

**Data analysis.** Western blot films were scanned on an Epson expression scanner, and protein bands were quantified by using Image J software (NIH). The data were plotted by using GraphPad Prism 4 software (La Jolla, CA), and final figures were assembled and labeled with Canvas software (Deneba Systems, Miami, FL). Calculations and statistics were performed using the Graph Pad Prism 4 software. The pEC50 values for ACh and SNP were expressed as −log of the molar concentration that produces 50% of the maximal response. P values of <0.05 were considered statistically significant. Data are expressed as means ± SE, and n values indicate the number of vessels or the number of times an experiment was performed.

**Drugs and chemicals.** All chemicals were analytical grade and obtained from Sigma (St. Louis, MO). U0126 [mitogen-activated extracellular signal-regulated kinase (MEK) 1/2 inhibitor] was purchased from Cell Signaling Technology (Danvers, MA).

**RESULTS**

**Voltage sensitivity.** Representative tracings of single-channel K+ currents recorded from excised inside-out membrane patches of vascular smooth muscle cells (VSM) obtained from fresh and cultured cerebral arterial muscle cells during recording at different patch potentials using symmetrical KCl (145 mM) solution are presented in Fig. 1A. The amplitudes and opening frequencies of single-channel K+ currents in inside-out membrane patches of fresh and cultured cerebral arterial muscle cells increased in response to changes in patch potential between −60 and +60 mV in steps of 20 mV, and were similar in the two groups of cells types. The mean single-channel slope conductance averaged 238 ± 16 pS (n = 3–6 cells) in freshly isolated arterial muscle cells and 241 ± 11 pS (n = 2–7 cells) in cultured arterial muscle cells (Fig. 1B). The voltage sensitivity determined by measuring the open-state probability (NPO) during step depolarization of the excised inside-out membrane patches of VSM of fresh or cultured cerebral arteries from a patch potential of −40 to +60 mV in steps of 20 mV revealed similar voltage-dependent levels of NPO as depicted in Fig. 1C between the two groups of excised membrane patches at all patch potentials studied.

**Sensitivity to changes in [Ca2+]i.** The Ca2+ dependence of the openings of the single-channel K+ currents was determined by varying the concentration of [Ca2+]i on the cytosolic surface of the excised inside-out membrane patches of arterial muscle cells obtained from freshly isolated and cultured cere-

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bral arteries during recording at a patch potential of +40 mV is depicted in Fig. 1D. Elevation of bath [Ca²⁺]i from 0.1 µM to 0.3 µM and then to 1 µM induced a concentration-dependent increase in the NPo of the 238 pS single-channel K⁺ currents recorded in inside-out patches of freshly isolated arterial muscle cells from 0.0029 ± 0.0003 to 0.011 ± 0.0014 and then to 0.0177 ± 0.0014, and that of the 241 pS single-channel K⁺ currents recorded in excised inside-out membrane patches of the cultured arterial muscle cells from 0.0033 ± 0.0002 to 0.0114 ± 0.001 and then to 0.0186 ± 0.003, respectively (n = 3–7 for each group). These data indicate that the 238 pS and 241 pS single-channel cerebral arterial muscle cell membranes, respectively, were similarly activated by about two- to threefold in response to increases in [Ca²⁺]i in the sub-micromolar range on the cytosolic surface of the excised inside-out membrane patches. These findings indicate that the single-channel KCa currents recorded from freshly isolated and cultured arterial muscle cells when recorded at a patch potential of +40 mV.

Endothelium-dependent and -independent relaxation of organ-cultured arterial segments. Although vascular smooth muscle cells are solely responsible for the generation of myogenic reactivity in vascular tissue (27), such responses can be modulated by factors released from the endothelium (9). ACh is a classical endothelium-dependent relaxant, whereas the NO donor SNP is commonly used to study vascular relaxations that are independent of the endothelium. We determined and compared the effects of ACh- and SNP-induced dilation of freshly isolated and cultured arteries in an attempt to verify functional integrity. Summarized in Fig. 2A is the response of pressure...
removal of the organ cultured cerebral arterial segments (maxi-
mal was also significantly reduced following endothelium
removal). As depicted in Fig. 2A, cumulative application of
ACh to the bath induced a concentration-dependent
vasodilation of the cannulated and pressurized (80 mmHg) cerebral
arterial segment with the endothelium-independent vasorelax-
ation of ACh to the bath induced a concentration-dependent
vasodilation (maximal response: 2.56 ± 0.2 M; n = 10) in freshly
isolated and cultured cerebral arteries (data not shown). The other endoge-


nous enzymes that are known to be involved in the pressure-
induced myogenic response and myogenic autoregulation of
cerebral blood flow, namely, the CYP 4A CYP 4F enzymes,
which catalyze the formation of 20-HETE that mediates con-
striction of cerebral arteries (12, 13), have identical expression
between freshly isolated and cultured cerebral arteries in the
present study (data not shown). A reverse permeabilization
protocol similar to that previously described by other investi-
gators (6, 37, 42, 43, 47) was employed to permit transient
transfection of siRNA into cultured cerebral arterial smooth
muscle. Isolated cerebral arterial segments were first reversibly
permeabilized in the presence of FITC-tagged siRNA (100
nM). As clearly shown in Fig. 3A, right, we obtained strong
fluorescence from FITC-labeled siRNA (100 nM) of perme-
abilized arteries, whereas in contrast there was low level of
detectable fluorescence from the arteries that were not perme-
abilized but exposed to FITC-labeled siRNA in PBS alone
(Fig. 3A, left). Thus the reversible permeabilization method we
utilized was able to efficiently transfer FITC-tagged siRNA
into the cultured cerebral arterial muscle cells.

This method was then employed to suppress DUSP-5 mRNA
and protein expression in cultured cerebral arterial muscle. Cere-
breal arterial segments were reversibly permeabilized in the pres-
ence of 100 nM DUSP-5 targeted siRNA, designed to block the
translational step in the biogenesis of DUPS-5. Another set of
arterial segments were reversibly permeabilized in the presence of	nonsense siRNA to serve as a control and were subsequently
cultured for 72 h to provide adequate time for the induction of
knockdown of DUSP-5. Real-time PCR was used to monitor
mRNA levels after the culture period. Message for DUSP-5 was
identified in intact cerebral arteries treated with control siRNA as
well as DUSP-5 targeted siRNA. Results of RT-PCR studies
revealed amplicons (159 bp) of appropriate size for rat DUSP-5
(Fig. 3B, i and ii). When compared with the control siRNA,siRNA targeted against DUSP-5 gene significantly reduced
DUSP-5 mRNA expression by 53 ± 4% (n = 3) in cultured
erteries (Fig. 3B, ii). As depicted in Fig. 3C, i and ii, immunoblot
analysis revealed a reduced immunoreactive band corresponding to
DUSP-5 protein in cerebral arteries treated with DUSP-5-
specific siRNA compared with the cerebral arteries treated with
control [nonspecific (NS)] siRNA. Densitometric analysis of the
Western blots from three separate experiments normalized to
β-actin indicated that treatment of arteries with 100 nM DUSP-
5-specific siRNA for 72 h induced a 35 ± 2% (n = 3; P < 0.05)
reduction in the density of the immunoreactive band correponding
to DUSP-5 protein compared with cerebral arteries treated with
control or nonspecific siRNA (Fig. 3C, ii). For a proof of
principle, we also examined whether treatment with siRNA specific
to DUSP-5 affects the expression of another dual-specificity
protein phosphatase, namely, DUSP-3 in cerebral arterial muscle cells. As depicted in Fig. 3D, i and ii, of data obtained from three independent experiments, treatment of cultured arterial segments with 100 nM siRNA specific to DUSP-5 did not influence the expression level of DUSP-3 protein, revealing the absence of cross inactivation by the specific siRNA to DUSP-5. Furthermore, in all studies performed, expression of the internal standard β-actin was comparable between treatment and control arteries, demonstrating that siRNA specific for DUSP-5 or the nonspecific control siRNA did not cause nonspecific silencing (Fig. 3C, i). These findings convincingly verify that the culture procedure is suitable for studies involving silencing of functional genes in cerebral arteries.

**Functional role for DUSP-5 in rat cerebral arteries.** To attain convincing evidence for the functional role of DUSP-5 in cerebral arterial smooth muscle, we examined the effects of permeabilization-facilitated transfection of cerebral arterial segments with control siRNA or with siRNA targeted against DUSP-5 on pressure-induced myogenic response in cerebral arteries. The effect of transfection with control siRNA and siRNA specific for DUSP-5 on myogenic reactivity of rat middle cerebral arteries is shown in Fig. 4A. We observed that
the pressure-induced constriction of cerebral arterial segments was significantly higher in DUSP-5-targeted siRNA-treated arteries than in arteries treated with control siRNA (Fig. 4A). Thus arterial segments treated with siRNA targeting DUSP-5 showed markedly enhanced constriction and, on average, a significantly greater reduction in arterial diameter compared with control siRNA-treated vessels at all pressures >80 mmHg (Fig. 4A). siRNA-induced suppression of DUSP-5 expression significantly increased the constrictor responses to intraluminal pressure over a pressure range of 100–160 mmHg. Compared with control siRNA-treated cerebral arteries, pressure-induced constriction of DUSP-5 siRNA-treated cerebral arteries was increased by 10% in response to 100-mmHg intraluminal pressure and by 18% in response to 120-mmHg pressure, whereas increasing the intravascular pressure to 140 mmHg and then to 160 mmHg enhanced the pressure-induced constriction by 31 and 32%, respectively, compared with the corresponding increase in intravascular-pressure induced constriction of control siRNA-treated cerebral arterial segments. 

Additional experiments examined the mechanisms underlying the increased contractile gain of DUSP-5-depleted arteries. PKC has been shown to modulate myogenic tone (23, 26, 29, 30, 36, 38). During the past years, accumulating evidence suggested that components of the mitogen-activated protein kinase (MAPK) cascade may be involved in vascular contraction (1, 2, 4, 25, 40). Crucial components of this MAPK cascade include the ERK1/2 subfamily, the activation of which is dependent on dual phosphorylation of a tyrosine (Try185) and a threonine (Thr187) residue (27). Several studies have demonstrated that ERK1/2 phosphorylation and/or its upstream MAPK kinase (MEK) play a role in the contraction of numerous vessels (1, 26, 28). DUSP-5 is an inducible ERK-specific MKP and functions as both a nuclear anchor and inactivator of ERK MAPK in mammalian cells (5, 24, 25, 34). We speculated, therefore, that DUSP-5 depletion was perhaps activating one of the signaling pathways that is negatively regulated by itself and is responsible for enhancing myogenic tone development following DUSP-5 inactivation. Thus, in subsequent studies, we examined whether the reduced expression of DUSP-5 in cerebral arteries was associated with a change in the expression levels of ERK1/2 and PKC βII. Figure 4B, i and ii, depicts Western immunoblots and summary of P-ERK1/2 and P-ROCK1 expression levels (i; n = 3; * P < 0.05 for each group).

The expression levels of ERK1/2 and PKC βII and preserved phosphorylation of ERK2 but reduced the phosphorylation of ERK1 and ROCK1 (Fig. 4B, i and ii). These findings suggest that endogenous depletion of DUSP-5 protein in cerebral arteries potentiates pressure-induced myogenic constriction. These data support the supposition that manipulation of DUSP-5-regulated signaling cascades affects arterial reactivity by modulating the extent of development of myogenic depolarization evoked by increased intraluminal pressure.

Fig. 4. Suppression of DUSP-5 protein with siRNA enhances pressure-dependent myogenic constriction of cerebral arteries. A: line graphs depicting pressure-induced changes in diameter expressed as percent of control diameter measured at 20 mmHg of nonsense siRNA (100 nM)-treated arteries (n = 10) and arteries treated with siRNA targeting DUSP-5 (n = 12). Cerebral arterial segments were treated with nonsense siRNA or siRNA targeted against DUSP-5 and following 72 h culture were subjected to 20 mmHg step elevations in intraluminal pressure from 20 to 160 mmHg. The treatment with 100 nM siRNA targeted against DUSP-5 significantly enhanced pressure-induced constriction at pressures >80 mmHg compared with the nonsense siRNA-treated group (n = 5–7 vessels; *P < 0.05). Inset: linear correlation of pressure-diameter relation curves between 20 and 160 mmHg in 20 mmHg steps in cultured arteries treated with nonsense siRNA or DUSP-5 targeted siRNA yielded the regression equations y = −0.23x + 95.0 with a correlation coefficient of R² = 0.58, and y = −0.47x + 104.3 with a correlation coefficient of R² = 0.93, respectively. Treatment with the DUSP-5 targeted siRNA significantly enhanced the pressure-diameter relationships compared with the effect of treatment with the control nonsense siRNA (***P < 0.001). B: DUSP-5 depletion by treatment with siRNA targeted to DUSP-5 induced phosphorylation of PKC βII and preserved phosphorylation of ERK2 but reduced the phosphorylation of ERK1 and ROCK1 (i; n = 3 for each group) in cultured arteries compared with treatment with nonsense siRNA following 72 h of culture. Summary of the effects of siRNA knock down of DUSP-5 on P-PKC βII and P-ERK1 expression levels (ii; n = 3; * P < 0.05 for each group).
DUSP-5 siRNA, depletion of DUSP-5 led to the detection of unchanged phosphorylation of ERK2, with total ERK2 protein level unaffected. DUSP-5 suppression was, however, associated with an apparent elimination of ERK1 phosphorylation (Fig. 4B, i). In addition, depletion of DUSP-5 was also associated with increased levels of P-PKC βII phosphorylation, whereas it resulted in marked reduction of the level of P-ROCK. However, the expression of the internal standard, β-actin, was similar between lanes.

Inhibition of ERK1/2 phosphorylation inhibits pressure-dependent myogenic response of isolated rat cerebral arteries. To further understand the DUSP-5-ERK interactions in mediating pressure-induced myogenic constriction, we additionally examined the mechanism of DUSP-5 depletion-elicited contraction by studying the effects of pharmacological inhibition of ERK1/2 phosphorylation on the pressure-dependent myogenic constriction of cannulated rat isolated cerebral arterial segments. In these studies, pretreatment with the selective inhibitor of ERK1/2 phosphorylation U0126 (10 μM) for 3 h significantly inhibited the pressure-dependent myogenic constriction of cerebral arterial segments (Fig. 5A; n = 10). As depicted in Fig. 5B, results of immunoblot analysis studies revealed that treatment of the pressurized cerebral arterial segments for 3 h with 10 μM U0126 markedly reduced the level of phosphorylated ERK1/2 (n = 3 independent experiments), whereas treatment for 3 h with the vehicle control had no effect. As also shown in Fig. 5B, treatment with U0126 did not alter the total expression of ERK1/2 protein (n = 3 independent experiments). These findings clearly demonstrate that the ERK1/2 inhibitor markedly attenuates pressure-induced myogenic constrictions as well as phosphorylation of ERK1 and 2.

DISCUSSION

In the present study, we examined the effect of reduced expression of the DUSP-5 on pressure-induced myogenic tone development in cannulated intact cerebral arteries. We report that siRNA targeted against DUSP-5 selectively reduced mRNA and protein expression levels in cultured intact arteries, and these targeted reductions significantly enhanced pressure-induced myogenic tone development. For the first time, the findings of our present studies indicate that DUSP-5 is critically important in regulating and maintaining pressure-induced myogenic tone development in rat cerebral arteries.

Similar to our present study, Bolz et al. (4) reported a 48-h skeletal muscle resistance artery culture technique in which cultured and freshly isolated cannulated arteries were found to maintain similar levels of spontaneous tone and myogenic responsiveness (4). Furthermore, prior studies of pressure-induced myogenic tone of rat cerebral arteries have been demonstrated in 4- to 5-day cultured cerebral arteries following siRNA knockdown of Rho kinase and TRPC3/6 channels (6, 47). Although these previous studies did demonstrate a reduction in pressure-induced myogenic tone following siRNA-induced knock down, there was no comparison of pressure-dependent myogenic constriction responses with freshly isolated cerebral arterial segments. In the present study, the cultured arterial segments studied for siRNA knockdown of DUSP-5 were found to elicit similar responses to an increase in intravascular pressure and concentration-dependent reactivity to vasodilator and vasoconstrictor agonists, and expressed KCa channel currents with similar unitary conductance and sensitivity to changes in voltage or Ca2+ concentration to those

Fig. 5. Inhibition of ERK1/2 phosphorylation by U0126 attenuates pressure-dependent myogenic response of rat cerebral arteries. A: freshly isolated cerebral arteries exposed to either vehicle (DMSO) (n = 5) or 10 μM of U0126 (n = 5) for 3 h were subjected to stepwise increases (20-mmHg increments) of intraluminal pressure from 20 to 120 mmHg. The diameter responses were normalized and are expressed as a percentage of control diameter measured at 20 mmHg. Values are means ± SE. B: Western immunoblot of inhibition of P-ERK1 and P-ERK2 in cerebral arteries following treatment of pressurized (120 mmHg) cerebral arterial segments with vehicle or with the ERK1/2 inhibitor U0126 (10 μM for 3 h). Scanned images of representative anti-ERK1/2 (n = 3) and anti-Phosho-ERK1/2 (n = 3) immunoblots from vehicle-treated (DMSO) and U0126-treated arteries are shown. Total ERK1/2 expression was measured using an anti-ERK1/2 antibody. ERK1/2 phosphorylation is demonstrated by using an anti-phosphorylated ERK1/2 antibody. The two bands detected correspond to the 44-kDa isoform (ERK1) and 42-kDa isoform (ERK2). Treatment with the ERK1/2 inhibitor markedly reduced phosphorylation of ERK1/2 compared with the vehicle control-treated group. β-Actin was used as a reference for equal protein loading.
measured in excised inside-out membrane patches of freshly isolated cerebral arterial muscle cells. These findings revealed existence of a comparable level of vascular reactivity in the cultured and freshly isolated cerebral arterial segments that could justify the use of the organ-cultured cerebral arterial segments as surrogates to study gene-specific siRNA knockdown of DUSP-5 protein expression on pressure-induced myogenic cerebral arterial constriction.

Previous studies by other investigators have documented that the MEK inhibitor U0126 eliminates the PKC activator, PBDu-induced increased formation of phosphorylated ERK1 and ERK2 with no influence on total ERK1/2 protein levels (48). In the present study, we found that siRNA-induced knockdown of DUSP-5 protein resulted in enhanced pressure-dependent cerebral arterial myogenic constriction and increased phosphorylation of PKC-βI associated with reduced phosphorylation of ERK1 without changing the level of phosphorylated ERK2. This new finding appears to indicate that an increase in the level of phosphorylated PKC-βI may account for the enhanced pressure-induced myogenic constriction of cerebral arterial segments observed following siRNA knockdown of endogenous DUSP-5. This finding may also further suggest prevalence of a negative feedback-type effect between endogenous DUSP-5 activity and PKC-βI that could operate to enhance pressure-induced myogenic contractile response of cerebral arteries. The influence of siRNA knockdown of DUSP-5-induced reduced phosphorylated level of ERK1, without changing the phosphorylated level of ERK2, on the pressure-induced cerebral arterial myogenic constriction of the present study was not previously known and may require further investigation. However, it has been previously reported that the MEK (ERK1 and ERK2) inhibitor U0126 caused increased activation of PKC- or pressure-induced contractile force (45). Given that the role of DUSP-5 has not been examined in this previous study, any probable discrepancy observable between this previous report and our present finding could likely be attributed to the influence of activity of endogenous DUSP-5.

On the other hand, in studies of pressurized cerebral arterial segments in which native DUSP-5 protein activity was not manipulated, we found that pretreatment with the MEK/MAPK inhibitor U0126 significantly blocked ERK1 and ERK2 phosphorylation and markedly attenuated pressure-induced cerebral arterial myogenic constriction (Fig. 5A). These data indicate that phosphorylation of ERK1 and ERK2 is requisite for the development of pressure-induced myogenic tone. This later finding is in part in agreement with a previous report that documented the involvement of activation of PKC-induced phosphorylation of ERK1 and ERK2 in pressure-induced myogenic constriction in ovine cerebral arteries (48). Our present finding that the MEK/MAPK (ERK1/2) inhibitor U0126 blunted the pressure-induced myogenic tone, however, contrasts with a previous report that proposed the existence of a negative feedback effect of phosphorylated ERK1 and ERK2 on activation of PKC, which implies that inhibition of phosphorylation of ERK1 and ERK2 could be expected to elicit enhanced pressure-induced myogenic constriction (48). In these studies, however, the influence of possible interaction between the ERK1 and ERK2 pathway and other signaling cascades capable of modulating the development of pressure-induced myogenic cerebral arterial constriction is not known and could be a potential factor to account for possible variation between our present findings and those reported by other investigators (17, 48).

The other intriguing finding of the present study is that the siRNA-induced knockdown of DUSP-5 is associated with a reduction in the level of phosphorylated ROCK, the functional implication of which in the present study is not known. However, these findings could indicate that, in addition to regulating the activities of PKC-βI and ERK1/2, DUSP-5 also appears to modulate the RhoA/ROCK signaling pathway, which is known to play a role in pressure-induced cerebral arterial myogenic constriction (46). In the present studies, although the functional roles of the reduced level of phosphorylated ERK1 or ROCK is not clear, the increased phosphorylation of PKC-βI might be regarded to play a dominant role in mediating pressure-induced myogenic constriction in a condition where endogenous DUSP-5 is inactivated. In summary, the findings of the present studies suggest that DUSP-5, native in cerebral arterial muscle cells, may possess the capacity to regulate the activities of different signaling pathways capable of modulating pressure-induced myogenic constriction of cerebral arteries that could have a significant bearing on the control of the distribution of nutritive blood flow to different regions of the brain. Although the exact signaling pathway through which modulation of elevated transmural pressure-induced activation of cerebral arterial tone by DUSP-5 remains to be elucidated, our present findings may raise the possibility that this dual-specificity phosphatase could operate to contribute to the maintenance of pressure-induced myogenic cerebral arterial constriction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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