In Vivo and Ex Vivo Dysfunction of Neurovascular Coupling in a Mouse Model of Subarachnoid Hemorrhage

Masayo Koide, Kathryn M. Dunn, Evelyn A. Bulkeley, Mark T. Nelson, George C. Wellman
Department of Pharmacology, University of Vermont College of Medicine, Burlington, VT

Neurovascular coupling (NVC) represents activity-dependent focal increases in cerebral blood flow (CBF) crucial for the enhanced delivery of nutrients to maintain brain function in regions of high metabolic demand. We have previously reported inversion of NVC, with neuronal activation causing vasoconstriction rather than vasodilation, in brain slices from subarachnoid hemorrhage (SAH) model rats (Koide et al, 2012). Here, we examined ex vivo NVC, in vivo functional hyperemia and sensory motor function using a mouse endovascular perforation SAH model. In brain slices, astrocytic endfoot Ca2+ and adjoining parenchymal arteriolar diameter were measured using two-photon and infrared-differential interference contrast microscopy. Neuronal activation caused increased endfoot Ca2+ that was followed by an inverted neurovascular response (i.e. vasoconstriction rather than vasodilation) in ~80% of brain slices from day 1 and day 2 SAH mice and ~30% of slices from SAH day 4 mice. In vivo functional hyperemia (whisker stimulation-induced CBF increases) measured by laser Doppler flowmetry in SAH day 1 mice was also significantly attenuated. Consistent with impaired NVC, SAH mice showed a decreased ability to perform sensory motor tasks. These data demonstrate dysfunction of neurovascular coupling occurs both ex vivo and in vivo following SAH, which may contribute to the development of neuronal deficits. Supported by Totman Research Trust, P. Martin Endowment, NIH P01HL095488, P30RR032135 & P30GM103498.

Subarachnoid Hemorrhage Suppresses KV1 And KV2 Currents Via Different Mechanisms In Rat Parenchymal Arteriolar Myocytes

Koide M, O’Connor KP, Pappas AP, Syed AU and Wellman GC
Department of Pharmacology, University of Vermont College of Medicine, Burlington, VT

Subarachnoid hemorrhage (SAH) leads to membrane potential depolarization in arteriolar myocytes and enhanced arteriolar tone in brain parenchymal arterioles (Nystoriak et al, 2011). However the mechanism underlying this augmented constriction is currently unknown. Here, we studied the impact of SAH on voltage-gated potassium (KV) currents. In rat parenchymal arterioles from control animals, KV1.2, 1.5, 2.1 and 2.2 mRNA was expressed, and KV1 (correolide-sensitive) and KV2 (stromatoxin-sensitive) currents were detected by whole cell K+ current measurement. Both KV1 and KV2 currents as well as total KV (4-AP-sensitive) currents were significantly suppressed in arteriolar myocytes after SAH, however KV channel subtype expression was not changed. Our previous work demonstrated that a blood component oxyhemoglobin causes KV current suppression through heparin binding EGF-like growth factor (HB-EGF). HB-EGF caused significant K+ current suppression in myocytes from control animals, but not after SAH. Further, HB-EGF suppressed K+ currents in the absence and presence of the KV2 channel blocker stromatoxin, but failed to alter currents in the presence of 4-AP or correolide. These data suggest SAH causes KV1 current suppression through HB-EGF shedding, while KV2 current suppression is independent of the HB-EGF pathway. Supported by NIH P01 HL095488, Totman Medical Research Trust and the Peter Martin Fund.

Inversion of neurovascular coupling after subarachnoid hemorrhage

Masayo Koide, Adrian D. Bonev, Mark T. Nelson, and George C. Wellman
Department of Pharmacology, University of Vermont College of Medicine, Burlington, VT

Aneurysmal subarachnoid hemorrhage (SAH) is associated with high rates of morbidity and mortality. The cellular events contributing to SAH-induced ischemic neuronal damage, a major cause of poor outcome, are still elusive. Here we examined the impact of SAH on neurovascular coupling (NVC) in brain slices from control and SAH model rats. Brain slices
were loaded with the fluorescent Ca2+ indicator dye, fluo-4, and astrocytic endfoot Ca2+ concentration and adjoining parenchymal arteriolar diameter were simultaneously measured using two-photon and infrared-differential interference contrast (IR-DIC) microscopy. As anticipated, neuronal activation by electrical field stimulation (EFS) caused an increase in endfoot Ca2+ followed by arteriolar dilation in brain slices from control rats. Remarkably, EFS caused a similar increase in astrocyte endfeet Ca2+ but induced vasoconstriction rather than vasodilation in brain slices from day 4 SAH animals. Similarly, Ca2+ uncaging in astrocyte endfeet caused vasodilation in control brain slices and vasoconstriction in brain slices from SAH rats. Paxilline, a blocker of large-conductance Ca2+-activated K+ (BK) channels, greatly diminished both EFS-induced vasodilation and vasoconstriction in brain slices from control and SAH rats, respectively. Interestingly, we also observed an increase in the magnitude of spontaneous astrocytic Ca2+ oscillations in brain slices from SAH animals. The peak amplitude of spontaneous astrocytic Ca2+ oscillations in brain slices after SAH was ~490 nM compared to ~320 nM in brain slices from control animals. Our data are consistent with a model in which SAH increases the amplitude of spontaneous astrocytic Ca2+ oscillations leading to increased activity of endfoot BK channels and elevation of basal extracellular K+ in the restricted perivascular space between astrocytic endfeet and parenchymal arteriole myocytes. This SAH-induced elevation in basal perivascular K+ combined with further K+ efflux stimulated by neuronal activity elevates K+ above the dilation/constriction threshold, switching the polarity of arteriolar responses from vasodilation to vasoconstriction. This inversion of NVC may contribute to decreased cerebral blood flow and the development of delayed ischemic neuronal deficits following SAH.

**Glucocorticoid signaling mediates stress-induced impairment of neurovascular coupling**

Thomas Longden, Fabrice Dabertrand, Sayamwong Hammack and Mark Nelson

*University of Vermont*

Stress influences the progression and severity of many diseases. Here we examined the effects of stress on neurovascular coupling (NVC), which matches cerebral blood flow increases to local neuronal activity. NVC was studied in brain slices from the amygdala, where dilation of parenchymal arterioles (PAs) was measured in response to neuronal stimulation. We administered a 7-day stress paradigm to male Sprague-Dawley rats, resulting in anxiety-like behavior and attenuated weight gain. After stress, PA vasodilation evoked by neuronal stimulation was reduced by 66%. This reduction was similar to that previously reported for the effect of blocking inward rectifier K+ (Kir) channels during NVC(1). Indeed, blocking Kir channels with barium inhibited NVC in control but not stressed slices. We thus hypothesized that the impairment of NVC by stress reflects a loss of PA Kir channels. Consistent with this, we found that Kir channel current density in smooth muscle cells from PAs was reduced by 90%. Moreover, elevation of external K+ from 3 to 8 mM, which normally causes profound dilation of PAs through Kir activation, was almost without effect on arterioles from stressed rats. In vivo delivery of corticosterone (without stressors) or the glucocorticoid receptor blocker RU486 (prior to stressors) mimicked or prevented NVC impairment by stress, respectively. We conclude that stress causes a glucocorticoid-mediated decrease in functional Kir channels in PA myocytes in the amygdala. This renders arterioles less responsive to K+ released from endfeet during NVC, thus impairing vasorelaxation. These studies may pave the way for the development of novel treatment options for brain disorders with a stress component. Reference: 1. Filosa et al. (2006) Nat Neurosci 9(11): 1397-1403

**Contribution of voltage-gated potassium channels in cerebrovascular dysfunction associated with a genetic model of ischemic small vessel disease**

Fabrice Dabertrand(1), Christel Krøigaard(1), Adrian D Bonev(1), Joseph E Brayden(1), Anne Joutel(2), Mark T Nelson(1)

(1) University of Vermont College of Medicine, Burlington, VT. (2) INSERM U740, Faculté de Médecine Paris 7, Paris, France

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), caused by dominant mutations in NOTCH3 receptor, is a genetic paradigm of small vessel disease of the brain. Cerebrovascular
dysfunction occurs prior to the appearance of brain lesions but the underlying mechanisms are unknown. Also, it remains unclear why the clinical manifestations are restricted to the brain whereas NOTCH3 is expressed in both cerebral and peripheral arteries. Myogenic tone, the vasoconstriction in response to intravascular pressure, is a resistance vessel fundamental feature, particularly important in the brain where blood flow is constantly and precisely adapted to a highly dynamic metabolism. Here, using a recently developed preclinical mouse model of CADASIL, we examined the consequences of an archetypal CADASIL-associated NOTCH3 mutation (R169C) on the relationship between intravascular pressure and vasoconstriction in cerebral parenchymal arterioles (PAs) and pial arteries as well as in peripheral (mesenteric) arteries. We found that Notch3(R169C) did not affect the myogenic response or smooth muscle membrane potential of PAs, the major site of small vessel disease, at low pressure (20 mm Hg). However, constriction to physiological pressure (40 mm Hg) was greatly reduced and the smooth muscle membrane potential was 10 mV more hyperpolarized in PAs from TgNotch3R169C mice than in those from controls, indicating that the defect emerges as intravascular pressure increases. The Notch3(R169C) mutation did not affect inward rectifier potassium channels, voltage-dependent Ca2+ channels, the contractile apparatus, endothelial-dependent influences on myogenic tone, or responses to BK channel inhibition. Notably, Notch3(R169C) augmented constriction to the preferential KV1 channel blocker, 4-aminopyridine (4-AP), and increased the current density of KV1 by 70%. Similar defect was observed in pial arteries, but not in mesenteric arteries. Remarkably, reduction of Kv current densities using HB-EGF, a modulator of Kv channel expression, restored myogenic response in TgNotch3R169C PA. Our data reveal for the first time an abnormal membrane hyperpolarization due to a cerebro-selective elevation of KV1 channels in smooth muscle cells of cerebral arteries and arterioles, compromising the myogenic response in this animal model of CADASIL, and therefore provides insight into the selective vulnerability of cerebral vessels to CADASIL mutation.

Constitutively active inward-rectifier K+ channels in vascular smooth muscle prevent myogenic tone development in mouse bladder arterioles

Nathan R. Tykocki, Thomas J. Heppner, Adrian D. Bonev and Mark T. Nelson
University of Vermont

Prolonged decreases in bladder blood flow are linked to both overactive and underactive bladder pathologies. However, although the urinary bladder is highly vascularized, the mechanisms that regulate bladder vascular tone are largely unknown. Here, we examined the myogenic and vasoactive properties of mouse bladder feed arterioles to determine how bladder vasculature function is regulated. Unlike similar-sized vessels from other vascular beds, isolated pressurized bladder arterioles did not contract to increases in intraluminal pressure (5-80 mmHg), indicating an absence of myogenic tone. Vessels contracted normally to vasoconstrictor agonists and 60 mM K+, and pre-contracted vessels retained dilatory responses to carbachol and the IK/SK opener NS-309, indicating that the lack of myogenic tone was not attributable to poor vessel viability or endothelial cell damage. The inward-rectifier K+ (KIR) channel blocker BaCl2 caused significant constriction, and electrophysiological recordings in isolated bladder arteriolar smooth muscle cells showed significant Ba2+-sensitive inward currents, suggesting the presence of functional KIR channels. Interestingly, bladder arterioles from smooth muscle-specific KIR-knockout (KIRSMKO) mice exhibited substantial myogenic tone at 60 and 80 mmHg, indicating that elimination of smooth muscle KIR channels “restored” myogenic responses to these arteries. In contrast, arterioles from endothelial cell-specific KIR-knockout mice showed no myogenic response at 60 mmHg and a small myogenic response at 80 mmHg compared with KIRSM KO mice. Taken together, these findings suggest that smooth muscle KIR channels are constitutively active in bladder arterioles and are largely responsible for the absence of myogenic tone. The elevated basal activity of KIR channels in bladder arteriolar smooth muscle cells provides a unique mechanism for opposing myogenic constriction and maintaining blood flow during normal bladder function.

Activation of ATP-Sensitive Potassium (KATP) Channels underlies vasodilation to PACAP, but not CGRP, in Pressurized Rat Middle Meningeal Artery

Arsalan Syed, MASayo Koide, Victor May, George Wellman
University of Vermont
Migraine is a complex neurological disorder that often presents as an intense unilateral headache accompanied by nausea, photophobia and other neurological symptoms. Activation of the trigeminovascular system and/or the sphenopalatine ganglia involving the release of the neuropeptides pituitary adenylate cyclase activating polypeptide (PACAP) and calcitonin gene related peptide (CGRP) has been implicated in vasodilation of the middle meningeal artery (MMA) and the sensation of migraine headache. However, the mechanism by which these two peptides exert their vasodilatory effect on the MMA is unclear. Activation of distinct receptors for PACAP and CGRP have been linked to activation of adenylyl cyclase in vascular smooth muscle. In addition, CGRP receptors have also been identified in vascular endothelial cells. Activation of cyclic AMP-dependent protein kinase has been shown to induce vasodilation via multiple mechanisms including phosphorylation and activation of smooth muscle KATP channels in a variety of vascular beds. In the present study our goal is to determine the role of KATP channels in vasodilation mediated via PACAP and CGRP in rat MMA. In isolated, pressurized MMAs both PACAP and CGRP induced significant vasodilation, although PACAP (EC50 ~ 1 pM) exhibited ~ 1,000-fold greater potency compared to CGRP (EC50 ~ 1 nM). PACAP-induced MMA dilation was completely abolished by the KATP channel inhibitor, glibenclamide (10 µM). In marked contrast, glibenclamide did not influence MMA dilation caused by CGRP. Further, N-Nitro-L-Arginine (L-NNA), a nitric oxide synthase inhibitor, had no effect on dilation caused by PACAP or CGRP. These observations demonstrate that PACAP dilates MMA via activation of vascular KATP channels, while CGRP acts through an alternative pathway. Thus, it appears that PACAP and CGRP contribute to the etiology of migraine via two distinct mechanisms. Therapeutic approaches targeting a combination of both PACAP and CGRP may be more effective than targeting either of these peptides alone in alleviating migraine headache.

TRPM4-dependent Contraction of Retinal Pericytes

Albert L. Gonzales and Mark T. Nelson
University of Vermont

Pericytes are an integral part of the neurovascular unit and likely contribute to the neuronal-dependent regulation of capillary blood flow. Like vascular smooth muscle cells (VSMCs), pericytes are thought to contract through membrane depolarization and activation of voltage-dependent calcium channels which then causes focal constrictions of capillaries. TRPM4, a melastatin transient receptor potential channel, is essential for VSMC membrane depolarization and contraction. Its role in pericyte contraction is not known. We used immunohistochemistry and confocal microscopy to examine the role of TRPM4 channels in pericyte contraction within the mouse retina. We observed TRPM4-specific immunolabeling in the smooth muscle cells within precapillary arterioles (<10 µm) and in a sub-population of pericytes surrounding the capillaries most proximal to the arterioles in the mouse retinal vasculature. In addition, we observed constriction in response to the thromboxane A2 analog, 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F2α (U46619) in pre-capillary arterioles and in distal and proximal pericyte-containing capillaries. This response was blocked by the TRPM4 inhibitor 9-phenanthrol in pre-capillary arterioles and proximal pericyte-containing capillaries, but not in pericyte-containing capillaries distal to feeding arteriole. These findings suggest that TRPM4 plays a role in pericyte constriction within retinal capillary system.

Endothelial dysfunction in rat mesenteric arteries 24 h following traumatic brain injury

University of Vermont

Traumatic brain injury (TBI) has secondary effects outside of the point of injury, but these systemic vascular effects have not been thoroughly studied. The aim of this study is to investigate the effects of TBI on endothelial function in the systemic circulation 24 hours after fluid percussion-induced TBI in rats. Myogenic tone, vasodilation to acetylcholine (ACh), the contribution of nitric oxide (NO), and Ca2+-activated K+ channels (KCa) were studied in pressurized mesenteric resistance arteries from TBI and control rats. At 80 mm Hg, myogenic tone (% tone) was increased in TBI arteries compared to controls (33 ± 4 %, n=11 vs 27 ± 2 %, n=10, P<0.05, respectively). The maximum ACh-induced vasorelaxation was significantly attenuated in TBI compared to controls (59 ± 9%, n=6 vs 100 ± 1%, n=5; P<0.0001). In the presence of the NO
synthase (NOS) inhibitor \( \omega \)-Nitro-L-arginine (L-NNA, 100 µM), ACh-induced dilations were attenuated in the control group (58 ± 12%, n=7, P<0.0001), but not in the TBI group (60 ± 7%, n=6, n.s.) suggesting the preservation of the endothelial-derived hyperpolarizing factor (EDHF). To elucidate the contribution of the NO component, ACh-induced dilations were performed in the presence of Ca²⁺-activated SK (apamin, 300 nM), BK and IK (charybdotoxin, 200 nM), and BK (paxilline, 1 µM) channel blockers. The remaining NO component of the ACh-induced dilation was diminished after TBI by 50% (n=8, P<0.05). These results suggest that TBI impairs NO signaling capability and endothelial function in tissue beds remote from the point of injury.

**Effects of extracellular histones on vascular endothelium are mediated by TRPV4 and TLR4 pathways in mouse mesenteric resistance arteries**


*Departments of Surgery & Pharmacology, University of Vermont; **Departments of Biochemistry & Molecular Biology and Pathology, University of Oklahoma Health Sciences Center

Histones released into the bloodstream from cells damaged in trauma and sepsis may contribute to endothelial dysfunction, multi-organ failure and death. Extracellular histones interact directly with the plasma membrane of vascular endothelial cells (EC) leading to calcium influx. Activation of transient receptor potential vanilloid 4 (TRPV4) cation channels and inflammatory pathways through toll-like receptors (TLR) in the endothelium also cause calcium overload and cell death. We hypothesized that extracellular histones act through both TRPV4 and TLR4 pathways on ECs leading to calcium influx and vasodilation. The effect of increasing concentrations of unfractionated histones (5; 10; 20 µg/mL) on diameter and EC calcium signals was tested using pressure myography and spinning disc confocal microscopy, respectively. Arterial lumen diameter was expressed as percent change in baseline diameter. Histones at 20 µg/mL caused endothelial-dependent dilations (22±7 %, n=7) in pressurized arteries (80 mm Hg). These dilations were abolished in TRPV4 knockout mice (-3±2 %, n=5), and attenuated in TLR4 knockouts (2±4 %, n=5). Histones at all concentrations, applied to intact mesenteric artery endothelium from mice expressing a calcium biosensor (GCaMP2 mice) in the presence of cyclopiazonic acid (30 µM) induced localized and propagating signals. These local events were partially blocked by a TRPV4 antagonist (HC-067047, 5 µM) and TLR4 gene ablation. This study shows for the first time a vasoactive effect of histones in the systemic circulation, involving both TRPV4 channel activation and TLR4 signaling. The pathways activated by circulating extracellular histones may provide therapeutic targets for pathological states including trauma and sepsis.

**Time course of the inversion of neurovascular coupling and altered spontaneous Ca²⁺ activity in astrocytic endfeet after subarachnoid hemorrhage**

Anthony C. Pappas, Masayo Koide, George C. Wellman

*University of Vermont*

Dysfunction of the intra-cerebral microcirculation may contribute to the development of delayed ischemic neurological deficits following aneurysmal subarachnoid hemorrhage (SAH). Neurovascular coupling (NVC), which links focal increases in neuronal activity with local arteriolar dilation, is essential for proper brain function and metabolism. Recently, we reported an inversion of the NVC response in brain slices obtained from SAH model animals (Koide et. al. PNAS 2012). Rather than dilate, brain parenchymal arterioles constrict following neuronal activation. The evidence suggests that higher amplitude spontaneous Ca²⁺ events in astrocytic endfoot set the stage for the inversion of NVC by increasing the basal perivascular K⁺ concentration. While this study determined a mechanistic link between altered Ca²⁺ activity of perivascular astrocytes and impaired neurovascular communication, it only examined animals 4 days after SAH induction. Using combined infrared differential interference contrast microscopy and 2-photon laser microscopy to image acute cortical brain slices, we examined the impact of SAH on NVC and spontaneous Ca²⁺ activity in astrocytic endfeet at six time-points after SAH. Our results show that the onset of the inversion of NVC occurs within 24 hr of SAH and coincides with an emergence of higher amplitude spontaneous Ca²⁺ events in astrocytic endfeet. Further, all time-points showing
inversion of NVC also show a greater proportion of high amplitude spontaneous Ca2+ events. These data support a model in which altered Ca2+ signaling of astrocytic endfeet contributes to the NVC deficits observed after experimental SAH.

**AKAP150-dependent cooperative TRPV4 channel gating is central to endothelium-dependent vasodilation and is disrupted in hypertension**


*University of Vermont, Burlington, VT; Cornell University, Ithaca, NY; University of Washington, Seattle, WA*

Endothelial cell (EC) dysfunction is a hallmark of hypertension. We recently discovered that local calcium influx through clusters of functionally coupled TRPV4 channels (detected optically as “TRPV4 sparklets”) drives physiological vasodilation. Here, we show that stimulation of EC muscarinic receptors activated TRPV4 sparklets exclusively at discrete sites at myoendothelial projections (MEPs)—specialized regions of ECs that contact adjacent smooth muscle cells. This activation was dependent on protein kinase C (PKC) and was absent in mice lacking the PKC-anchoring protein AKAP150 (A-kinase anchoring protein 150), which was localized predominantly to MEPs. Cooperative gating of TRPV4 channels within a cluster amplified calcium influx at MEPs by more than 2-fold. This cooperativity was largely absent at non-MEP sites and was virtually eliminated by chelation of intracellular calcium or AKAP150 knockout, suggesting AKAP150-dependent potentiation of TRPV4 activity by calcium influx via adjacent channels. Notably, MEP-localization of AKAP150 was disrupted in angiotensin II-induced hypertension, leading to a complete loss of muscarinic activation of TRPV4 channels, much weaker coupling among TRPV4 channels at MEPs, and approximately an 80% reduction in carbachol-induced vasodilation. Our results support the concept that endothelial-dependent vasodilation of resistance arteries is enabled by MEP-localized AKAP150, which ensures the proximity of PKC to TRPV4 channels and coupled channel gating necessary for efficient communication of the endothelium to the smooth muscle cells in arteries—a molecular configuration that is disrupted in hypertension.

**Probing allostery and signaling through novel, PKG1α-specific activators**

Thomas M. Moon, Jessica L. Sheehe, Matthew J. Tavares, Joseph E. Brayden, Wolfgang R. Dostmann

*University of Vermont*

The cGMP-dependent protein kinase (PKG) serves as an integral component of second messenger signaling in numerous biological contexts including cell motility, synaptic plasticity and vasodilation. In smooth muscle, the PKG isoforms Iα and Iβ integrate the nitric oxide (NO) and natriuretic peptide (NP) mediated signal transduction pathways by modulating intracellular Ca2+, cell contractility and ultimately blood flow. Understanding the regulatory mechanism of the PKG holoenzyme in relation to the maintenance of smooth muscle tone is central to guiding pharmacological discoveries relevant to prevention and treatment of cardiovascular diseases. Direct pharmaceutical targeting of PKG Iα has remained unsuccessful largely due to the enzyme’s complex, multi-domain architecture and, consequently, the field’s rudimentary understanding of the molecular mechanisms involved in PKG Iα activation. Recently, we solved the first crystal structure of the regulatory domain of PKG Iα (AA: 78-355, PDBID: 3SHR). This structure allowed us to identify a helical domain, unique to type I PKG, which we termed the switch helix (SW). Here, we present evidence that the SW helix activates PKG in vitro and promotes downstream BK channel activity in cerebral artery myocytes.