Integration and Modulation of Intercellular Signaling Underlying Blood Flow Control

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Key Words

Arterioles · Calcium-activated potassium channel · Conducted dilation · Endothelium · Exercise · Gap junctions · Microcirculation · Resistance arteries · Sympathetic nerves · Vascular smooth muscle · Ascending vasodilation

Abstract

Vascular resistance networks control tissue blood flow in concert with regulating arterial perfusion pressure. In response to increased metabolic demand, vasodilation arising in arteriolar networks ascends to encompass proximal feed arteries. By reducing resistance upstream, ascending vasodilation (AVD) increases blood flow into the microcirculation. Once initiated, e.g. through local activation of K⁺ channels in endothelial cells (ECs), hyperpolarization is conducted through gap junctions along the endothelium. Via EC projections through the internal elastic lamina, hyperpolarization spreads into the surrounding smooth-muscle cells (SMCs) through myoendothelial gap junctions (MEGJs) to promote their relaxation. Intercellular signaling through electrical signal transmission (i.e. cell-to-cell conduction) can thereby coordinate vasodilation along and among the branches of microvascular resistance networks. Perivascular sympathetic nerve fibers course through the adventitia and release norepinephrine to stimulate SMCs via α -adrenoreceptors to produce contraction. In turn, SMCs can signal ECs through MEGJs

to activate K⁺ channels and attenuate sympathetic vasoconstriction. Activation of K⁺ channels along the endothelium will dissipate electrical signal transmission and inhibit AVD, thereby restricting blood flow into the microcirculation while maintaining peripheral resistance and perfusion pressure. This review explores the origins and nature of the intercellular signaling that governs blood flow control in skeletal muscle with respect to the interplay between AVD and sympathetic innervation. Whereas these interactions are integral to daily activity and athletic performance, determining the interplay between respective signaling events provides insight into how selective interventions can improve tissue perfusion and oxygen delivery during vascular disease.

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Introduction

It is both humbling and a tremendous honor to receive the Malpighi Award from the European Society for Microcirculation. My studies of the microcirculation began

S.S. Segal is the ESM Malpighi Awardee for 2013. This paper was written to complement the lecture he presented at the International Union of Physiological Societies (IUPS) Meeting in July 2013 in Birmingham, UK.

formally in 1984 at the University of Virginia, as a post-doctoral fellow under the mentorship of Professor Brian Duling, to whom this article is dedicated in memoriam. With now over 3 decades of sharing the search, such recognition by the Society truly reflects the efforts and achievements of the outstanding individuals with whom I have the privilege of sharing this passion in my laboratory. The relationships presented here are developed with respect to how the phenomenon of 'propagated vasodilation' in the microcirculation has evolved into a multifaceted field of investigation. According to the ability of vasomotor responses to spread along and among the branches of the resistance vasculature, such coordinated regulation among the arterioles and their proximal feed arteries has become recognized as 'conducted', 'spreading' and 'ascending' vasodilation with respect to the context in which it has been studied. In light of my focus on understanding the control of muscle blood flow during exercise, concepts are presented with controversies addressed. Given the nature of the Malpighi Award, the perspective conveyed herein reflects the development and evolution of my laboratory and research program, which I respectfully submit as an example of how studying the microcirculation can open many doors.

The underlying theme of this article is career development, so I am addressing primarily young investigators studying the microcirculation as it is they (you!) who represent the future of our discipline. Indeed, my decision to pursue advanced training stemmed from the desire to be able to have trainees of my own one day. Through my own extraordinary mentors at the University of California, Berkeley, the University of Michigan and the University of Virginia, I developed a sense of how this could be done. Since its inception (in 1987) in the Noll Laboratory for Human Performance Research at Penn State, my laboratory has focused on studies of microvascular signaling and exercise hyperemia. However, this article is not intended as a review of either topic. Rather, my goal is to convey key personal experiences as a new field of investigation has been defined and has matured. I shall refer to each of these as a 'Career-Defining Moment' (CDM) in the hope that others may reflect on their own. I also wish to convey a bit of a philosophical perspective garnered along the way.

Our publications serve as scientific 'currency', and the impact of individual papers typically wanes over time, with but a select few earning recognition as 'classics' (e.g. [1–3]). In the current environment, young investigators (and those who judge them) are often more concerned with the quantity rather than the quality and originality

of their work. One of the greatest rewards as a principal investigator and mentor is to facilitate the development of eager trainees into new investigators who are prepared to identify and implement original inquiry. Given the pressures to remain abreast of developments in one's own area of investigation, typically less attention is given to papers that were published more than a few years ago. In addition, when compared to many of the articles that I read as a graduate student, the writing style in current publications tends to be much more focused on specific relationships under investigation. To hark back to earlier days when research articles encompassed more of the 'story' of how particular questions evolved and why findings were interpreted in a particular way, I have attempted to present this article in such a fashion. Though I may appear to digress occasionally, such is the case when one deviates from their intended path to gain perspective. My hope is that rather than detracting from the overall presentation, these complementary facets of information will help to convey relationships that are integral to the story being told.

Background and Perspective

The decision to focus on studying the microcirculation for a career evolved from my undergraduate and early graduate work in exercise physiology. Inspiration came from the desire to understand how the human body is able to support extreme levels of energy turnover during intense physical activity. My first research project (my Master's thesis at Berkeley) involved human subjects (fellow graduate students and willing friends!) to address a classic problem in human energetics: What governs oxygen consumption, particularly during the recovery period while it remains elevated despite the cessation of external work? We found that lactic acid, produced in proportion to exercise intensity and long purported to regulate recovery oxygen consumption, actually had no discernable effect! [4]. Perusal of such correlations in the 'classic' literature that had been used to substantiate these relationships revealed them to be a coincidental matter of when samples were collected and the availability of a precursor (e.g. muscle glycogen)... So much for cause-and-effect! As a doctoral student at Michigan, my research focused on skeletal muscle performance and how it was affected by injury and physical training [5, 6]. As with athletic performance, each of these relationships involves a key role for the microcirculation. Thus, my fate was sealed: to focus on blood flow regulation in light of the coupling be-

tween metabolic demand and oxygen delivery during exercise. These interactions underscore a most impressive response of the vascular supply.

According to the span of its energetic requirements, the dynamic range of skeletal muscle blood flow when transitioning from rest to maximal perfusion during exercise (i.e. functional hyperemia) is greater than in any other tissue in the body. Expressed relative to tissue mass, muscle blood flow can range from <5 ml/min/100 g at rest to well over 250 (and perhaps >400) ml/min/100 g during intense running and cycling [7, 8]. Thus, blood flow to skeletal muscle can vary 50- to 100-fold, and it is the resistance vasculature (i.e. arterioles and their feed arteries) that governs this incredible range of tissue perfusion [9]. In turn, with skeletal muscle typically comprising 30–50% of total body mass, the ability of its vascular supply to dilate and reduce total peripheral resistance is so great that vasodilation must be restricted to prevent a fall in arterial perfusion pressure [10]. As discussed later in this article, this regulatory role is served primarily by the sympathetic nervous system [10, 11].

Classic studies of functional vasodilation in response to contractile activity had focused on the regulation of tissue oxygen pressure [2, 3] and the ability of ‘vasodilator substances’ produced by active skeletal muscle fibers to relax vascular smooth-muscle cells (SMCs) [12, 13]. In practice, functional vasodilation entails the ability to override (i.e. ‘escape’) sympathetic vasoconstriction [10, 11, 14] through mechanisms that – remarkably – are still being determined. A year after beginning my doctoral studies, the role of the endothelium in governing the relaxation of vascular smooth muscle was identified in the rabbit aorta [1], the principal systemic artery. Ensuing research confirmed and extended endothelium-dependent dilation throughout the resistance vasculature [15–18]. In light of integrating vasodilation (i.e. SMC relaxation) via the endothelium and vasoconstriction (i.e. SMC contraction) through sympathetic nerves, an underlying goal of this review is to identify and integrate respective roles in blood flow control.

When learning to study the microcirculation – rather than the consequences of what may have been done to it while preparing it for study – my strongest impression was the absolute requirement for punctiliousness. Indeed, as I try to explain to prospective trainees, one must be prepared for dedicating the time and effort requisite to collecting meaningful data. As manifested in the works of Marcello Malpighi (1628–1694), it is revolutionary to reveal the secrets of nature by studying things that are too small to be seen by the naked eye. For example, Malpighi

was the first (in 1661) to observe red blood cells coursing through the pulmonary capillaries of the frog [19], thereby establishing the functional link between arteries and veins that had been inferred by William Harvey (1578–1657) in his treatise ‘*De Motu Cordis*’ (‘On the Motion of the Heart and Blood’) [20]. Such insightful, definitive observations were integral to Malpighi’s founding of the science of microscopic anatomy and pioneering the use of histology towards understanding the structural organization of tissues and organs in plants and animals.

Professor Duling advised (and experience has confirmed) the need for caution when viewing through the microscope, to remain objective and to be attentive to what nature is trying to tell you. August Krogh was a master when studying the microcirculation of animals [2, 3] in order to gain insight into his studies of human performance [21]. A take-home message from all of this is that one should not be afraid to make mistakes during the process of discovery! Thus, a favorite adage of mine is that any experiment worth doing well is worth doing half-assed first. In other words, if you have an idea, try it out as best you can with what you’ve got ... See if something is there that warrants further study! The hard part then lies in designing and executing the definitive experiment(s) to test your hypothesis rigorously, while recognizing that one’s findings are only as good as one’s controls. As exemplified by Malpighi’s discoveries, a most rewarding challenge for an investigator is the implementation of a novel scientific approach that enables new and definitive insight into the workings of nature; this is especially true when it involves microcirculation!

Ascension of Propagated Vasodilation

When beginning to study the microcirculation in 1984, I was directed to the phenomenon of ‘propagated vasodilation’ and was told (by Professor Duling) that it was ‘an idea whose time may have come’. The sole reprint request from his 1970 article [22] had been mounted over his desk for years ... This paper described the spread of vasodilation along arterioles of the hamster cheek pouch in response to stimulating a discrete site with an appropriate stimulus. Thus, vasoactive agents were delivered as a brief (<1 s) pulse from a micropipette positioned with its tip adjacent to the wall of an arteriole (diameter approx. 30 μ m), thereby confining the stimulus to within 20–40 μ m [22]. In response to acetylcholine (ACh), dilation spread (termed ‘propagated vasodilation’) bidirectionally along the arteriole over distances encompassing several hun-

Fig. 1. AVD in a microvascular resistance network. **a** Illustration depicts a feed artery (FA) arising from a large conduit artery. The FA carries blood into the tissue, e.g. skeletal muscle (dotted line) and gives rise to primary (1A) arterioles, branches *a* and *b*. These each give rise to second-order (2A) arterioles, which branch into third-order (3A) and then terminal (TA) arterioles, with each TA giving rise to groups of capillaries (postcapillary microvessels omitted for clarity). **b** Vasodilation originating in 2A and 3A (in branches *c* and *d*) ‘ascends’ into the parent 1A (branch *a*) but not

into the proximal FA. At the same time, branch *b* may receive little or no dilator signal from its daughter 2A and 3A (not shown) supplying an inactive region of the muscle. Under these conditions, lack of AVD into the FA limits the increase in tissue blood flow. **c** With greater dilation of its daughter 2A and 3A arterioles, vasodilation ascends into branch *b* which is summed together with signals along branch *a*, in the FA. Dilation of the FA reduces the proximal resistance restricting tissue blood flow. Total blood flow into the microcirculation increases dramatically.

dred micrometers. In contrast, other vasodilators (e.g. papaverine and eledoisin) produced dilation only within the vicinity of their direct effect. The ability to inhibit propagated vasodilation using local anesthetics (i.e. lidocaine or procaine) was interpreted as suggesting neural activation as the underlying mechanism [22]. However, transmission along vascular smooth muscle was also considered, in agreement with Hilton’s conclusions regarding ascending vasodilation (AVD) in the cat hindlimb [23]. A few years later, experiments performed on sheep carotid arteries implicated the activation of K^+ channels as a mechanism by which procaine (as well as lignocaine) affected electrical activity in the vessel wall [24]. Nevertheless, definitive studies had resolved the neural control of arterioles in tissues of frogs and hamsters [25]. Indeed, the original observation of spreading vasodilatation in the web of the frog hindlimb was attributed to a local axon reflex [26]. While defining regional variation in the nature of innervation among vascular beds and animal species, ensuing publications have confirmed cholinergic, nitroxidergic,

purinergic and sensory as well as adrenergic (i.e. sympathetic) innervation of the microvasculature, albeit with regional variation (review [27]).

In the late 1970s, using preparations dissected from the small intestine of guinea pigs, Hirst and Neild [28] employed dual intracellular microelectrodes to demonstrate that current injected into a submucosal arteriole (diameter 30–75 μm) at one site evoked changes in membrane potential (V_m) at remote sites along the vessel and its contiguous branches. These studies thereby demonstrated that an electrical signal could spread for at least 2–3 mm along the arteriolar wall. In light of studies that advanced electrical conduction in smooth muscle [29, 30], signaling over such distances was attributed to electrical transmission between neighboring SMCs. While Hirst and Neild [28] were the first to define the cable properties of electrical conduction along arterioles, their preparations were neither pressurized nor perfused and electrical signal transmission was evaluated in the absence of vasomotor responses. Though the endothelium was not disrupted, its

role in governing vascular smooth muscle function had not yet been considered. Reflecting on ‘the idea whose time had come’, the role of propagated vasodilation in blood flow control was ripe for investigation – particularly in light of AVD during exercise [23, 31].

As illustrated in figure 1, the functional significance of AVD is that events originating in distal microvessels spread upstream into the proximal arterial supply in order to attain maximal blood flow [32]. In the absence of AVD, any constriction of feed arteries upstream from arteriolar networks effectively restricts tissue blood flow into the microcirculation, even when arterioles within the tissue dilate maximally [32, 33]. Such regional differences in vasomotor control within resistance networks [12, 31, 34] provide an effective mechanism for maximizing the capillary surface area for oxygen extraction, particularly when the total blood flow into the microcirculation is limited by proximal vasoconstriction [31]. Regional differences in the nature of vasomotor control within vascular networks also points to opportunities for developing therapeutic interventions targeted to increase total blood flow into a tissue or tissue region (e.g. feed arteries and proximal arterioles) versus expanding the surface area for gas and solute exchange (e.g. distal arterioles and capillary networks) with the tissue parenchyma.

My early experiments as a postdoctoral fellow focused on the feed arteries that supply the arteriolar networks of the cremaster muscle in anesthetized hamsters. As this model enabled imaging of resistance networks during blood flow control, we identified AVD in response to the contraction of striated muscle fibers [32]. Determining that feed arteries external to the cremaster muscle (and confirmed using the gracilis muscle) dilate in concert with their arteriolar networks during muscle contraction substantiated earlier observations of AVD in cats [23, 31]. Our focus then turned to determining the mechanism underlying how vasodilation actually traveled along the vessel wall. Several possibilities were apparent. First was a role for vasodilator nerves (above). Second, increased flow through the lumen of arteries had been proposed as a mechanism of vasodilation [35, 36]. Thus, when vessels dilate downstream, the resulting flow increase through upstream vessels could promote vasodilation [37]. A third mechanism entails myogenic relaxation, whereby the increased flow velocity through the feed artery reduces transmural pressure (according to Bernoulli’s principle), resulting in vasodilation. To perform a definitive experiment, we needed a way to eliminate respective variables!

The hamster cheek pouch preparation enables access to relatively long unbranched arterioles while they re-

main perfused by the systemic circulation [38]. We developed a protocol whereby a single arteriole (diameter approx. 25 μm) was occluded simultaneously at 2 sites using micropipettes separated from each other by several hundred microns. This maneuver created a ‘sealed segment’ (fig. 2) of constant volume (and diameter), devoid of blood flow and occluded at each end. With tetrodotoxin added to block neural propagation, the arteriole was stimulated with ACh from a micropipette positioned proximal to the first occluder while the diameter was observed at a remote site distal to the second occluder. Using this paradigm, the first CDM for me in studying the microcirculation came late one afternoon. With respective micropipettes positioned as in figure 2, the ACh stimulus was delivered at the proximal site and – to my absolute amazement – vasodilation occurred along the arteriole beyond the distal occluder despite no vasomotor response within the occluded segment! It should be recognized that even if SMCs relaxed within the sealed segment, it could not change in volume (or diameter) while occluded at each end. This paradigm served as a direct test (and confirmation) of the hypothesis that a vasodilator signal could travel along the arteriolar wall independent of nerves or mechanical signaling [39].

My initial (half-assed) experiment to determine whether vasodilation could spread along an occluded arteriole (where blood flow could not change) involved a single occluder. When presenting these preliminary findings at a conference, I was challenged by an astute observer who recognized that even though the signal traveled through the site of occlusion (where neither transmural pressure nor shear stress could change), the arteriole beyond the occluder filled with blood as it dilated. Thus, changes in shear stress or in wall stress could not be excluded as mechanical signals for vasodilation. Sealing a segment between dual occluders solved this limitation by eliminating changes in blood flow (or diameter) within the segment. Remarkably, the signal conducted through both sites of occlusion along the sealed segment. As a positive control for the effects of damaging cells, if a pipette was rubbed across the arteriole with sufficient vigor, the propagated response was lost. Thus, nearly 2 years after beginning my postdoctoral training, a definitive experiment set our thinking (and ensuing research efforts) on a fundamentally new course: instead of responding to nerves or changes in transmural pressure or blood flow (i.e. shear stress), we proposed that intercellular signaling (i.e. cell-to-cell conduction) through gap junctions is the mechanism underlying propagated vasodilation in arterioles [39, 40]. En-
suing experiments demonstrated that simultaneous stim-

Fig. 2. Conducted vasodilation in vivo. A sealed arteriolar segment (approx. 500 μm long) was created in the hamster cheek pouch using a pair of occlusion micropipettes. The stimulus micropipette containing ACh was positioned to the left of the proximal occluder with its tip (1 μm) adjacent to the arteriolar wall. In response to

a 200-ms pulse of ACh, vasodilation was measured to the right of the distal occluder (i.e. within the broken circle) despite no change in diameter or volume within the occluded segment. Scale bar = 100 μm . Original (unpublished) image from experiments performed in Segal and Duling [39].

ulation of paired daughter arterioles downstream evoked vasomotor responses that summed in their parent arterioles upstream [41]. These findings illustrated that information arising at discrete locations on distal branches of a resistance network was integrated to determine the response of proximal branches. According to earlier findings [28], electrical signaling was implicated as a mechanism for conducting vasomotor responses along the arteriolar wall [40]. Complementary studies from independent laboratories indicated that signals initiated from capillaries could govern arterioles that controlled their perfusion [42, 43]. Nevertheless, early (half-assed?) experiments were unable to determine a definitive role for gap junctions or discern whether the endothelium (vs. the smooth muscle) was integral to signal transmission. These studies also did not preclude a role for the perivascular nerves!

New Beginnings

As a young investigator attempting to establish independence, one of the greatest (of many) difficulties entails determining which avenue of investigation to develop as

'your own'. I began by exploring a role for conducted vasodilation (CVD) in the microcirculation of striated muscle using the hamster cremaster muscle [44, 45]. A key experiment focused on terminal arterioles having high levels of resting vasomotor tone, such that no red blood cells were flowing through their lumen. As none of the capillaries supplied by these arterioles was perfused, I tested the hypothesis that CVD underlies microvascular recruitment and capillary perfusion with red blood cells. The key finding was that, in response to an ACh stimulus delivered at the distal end of a terminal arteriole, dilation traveled upstream (i.e. ascended) into the parent arteriole, resulting in perfusion of the terminal arteriole and the capillaries that it supplied [46]. As the response was unaffected by tetrodotoxin (i.e. it was not mediated by nerves), AVD within the microcirculation was shown to promote arteriolar recruitment and capillary perfusion along striated muscle fibers [46]. Complementary experiments using the cremaster preparation illustrated that contracting bundles of striated muscle fibers could evoke a similar response [47, 48], confirming a functional role for arteriolar conduction in matching local perfusion with metabolic demand. It should be recognized that although used

widely as an intravital preparation to study the microcirculation [44–50] and as a source of isolated microvessels for in vitro studies [51], the cremaster muscle does not attach to the skeleton nor does its contraction promote locomotion. Moreover, it serves a functional role only in males. Alternative approaches were thus required to study the microcirculation of limb muscles that are recruited during physical exercise, irrespective of gender.

Referring to classic studies of the soleus and extensor digitorum longus hindlimb muscles of rats as representative of ‘slow-twitch’ and ‘fast-twitch’ muscle fiber types, respectively [52, 53], we developed the rat hindlimb as a preparation for intravital imaging [54]. Perfusion pressure measured at the distal end of feed arteries (fig. 1, just above the dotted line) was 20–30% lower than systemic arterial pressure, thereby indicating that feed arteries indeed function as proximal sites of resistance to skeletal muscle blood flow. Remarkably, feed arteries of both soleus and extensor digitorum longus dilated in response to muscle contraction, confirming AVD as a physiological response in true skeletal muscles [54]. Such findings helped to lay the foundation for questions that have since driven the research in several laboratories including my own: What is the cellular pathway for signal transmission along the vessel wall? What are the vasoactive signals and how do they travel? Are these signals under physiological regulation and if so, how? As developed below, addressing these relationships has given rise to more focused questions, as ‘conduction’ has evolved and matured into a field of investigation in its own right.

Cellular Pathways for Signal Transmission

The two principal cellular elements of the microvessel wall are SMCs and endothelial cells (ECs) [55]. In light of classic studies of the ‘nexus’ and electrical conduction in smooth muscle [29, 30, 56] and of electrical conduction along strips of guinea pig taenia coli [57] and rabbit aorta [58], the first studies of electrical signaling along the arterioles were interpreted to reflect intercellular coupling between SMCs [28]. These experiments were performed on isolated preparations that enabled access to the arteriolar wall with microelectrodes. As vessels were approached from the abluminal surface, it was assumed that recordings were from the SMCs. However, one of the limitations of intracellular recording from multicellular preparations is the uncertainty as to which cell it was actually being recorded from. Inclusion of a fluorescent dye in the recording microelectrode had been used by neurophysiologists

Fig. 3. Dye labeling during intracellular recording. In an isolated pressurized feed artery of the hamster cheek pouch retractor muscle, Lucifer yellow dye (green; MW = 457 Da) was retained within an SMC and did not spread to adjacent cells. Note cytoplasmic distribution as SMC encircles the vessel. In contrast, propidium iodide (red; MW = 668 Da) microinjected into the central EC spread readily to surrounding ECs, thereby labeling the respective nuclei. Scale bar = 10 μ m. Image taken from Emerson and Segal [84] (used with permission from Wolters Kluwer Health).

to identify projections of individual neurons [59]. Furthermore, as gap junctions enable passage of molecules ≤ 1 kDa, it was assumed that dye transfer from the injected cell to neighboring cells indicated effective coupling through gap junctions [60, 61]. For example, Lucifer yellow (molecular weight, MW = 457 Da [62]) and propidium iodide (MW = 668 Da) are dye tracers that we have used for such a purpose [63, 64] (fig. 3). Nevertheless, it should be recognized that the absence of dye coupling does not preclude the existence of electrical coupling [59], as the size and charge characteristics of dye molecules may impede their diffusion through gap junction channels [65] to a far greater extent than the diffusion of ions (e.g. K^+ and Cl^-) during electrical charge transfer.

We had advanced the hypothesis that cell-to-cell signaling was the basis of propagated vasodilation in the hamster cheek pouch [39, 40]. A key advantage of the cheek pouch preparation is that microvessels supplying this thin epithelial tissue can be readily accessed by micropipettes (fig. 2). In 1991, a travel award from the Microcirculatory Society Inc. supported my visit to the University of Geneva to collaborate with Jean-Louis Bény, who had begun using dye labeling during intracellular recording from cells in isolated strips of pig coronary arteries

Fig. 4. Structural and functional arrangements of microvascular SMCs and ECs. **a** Schematic 2-dimensional depiction of how ECs and SMCs are oriented to each other in arterioles and resistance arteries. ECs align along the vessel axis in the direction of blood flow. In contrast, SMCs are oriented perpendicular to the flow stream and encircle the intima within the intact vessel (see fig. 8a). Note corresponding orientations of EC versus SMC nuclei. The IEL lies between respective cell layers with holes for endothelial projections (see fig. 5). **b** Schematic longitudinal section along top and bottom edge of vessel wall depicting adjacent SMCs (in cross-section) in contact with the abluminal surface of consecutive ECs.

Along the downstream segment, hyperpolarization (shaded) has conducted longitudinally and radially into the surrounding SMCs (arrows) to coordinate vasodilation. The signal is shown spreading into the upstream segment; note longitudinal gradients in hyperpolarization and SMC relaxation. Neighboring cells are coupled to each other via gap junctions (not shown), which are robust between neighboring ECs (arrows depicting longitudinal current flow), sparse between adjacent SMCs and restricted to discrete MEGJs between ECs and SMCs (arrows depicting radial current flow; see inset).

[61]. In his laboratory, we performed the first intracellular recordings from cheek pouch arterioles *in vivo* with Lucifer yellow dye in the microelectrode [66]. Following recording, preparations had to be transferred from the electrophysiology rig to a fluorescence microscope to confirm electrode placement. Anxiously fiddling with knobs in the dark, another CDM occurred when we observed robust dye transfer along the endothelium following recording from an EC. Conversely, we found no evidence of dye transfer when recording from (circumferential) SMCs,

nor was there any between ECs and SMCs. Thus, the endothelium appeared to be the most robust cellular pathway for signal transmission along the arteriolar wall. Ensuing studies of EC morphology in arterioles from several tissues, including the hamster cheek pouch, supported this conclusion [67]. Being oriented along the vessel axis in the direction of blood flow, individual ECs lining arterioles are able to contact numerous SMCs while each SMC may contact nearly as many ECs as they encircle the intima (fig. 4). Indeed, this functional morphology suggested

a key role for the endothelium in coordinating SMC relaxation along branches of resistance networks.

Several years elapsed before intracellular recording with dye labeling was integrated with intravital microscopy to observe vasomotor responses concomitant with electrical signaling in vivo [68]. During this time, a controversy developed as to whether arteriolar ECs and SMCs are electrically coupled to each other, particularly in the intact system. Experiments performed on cheek pouch arterioles isolated for in vitro studies found that V_m and depolarization to phenylephrine (PE), an agonist selective for the α_1 -adrenoreceptor (α_1 AR), evoked depolarization of ECs in addition to SMCs [69]. Because α_1 ARs are expressed on the SMCs (but not the ECs) of arterioles [70], these findings implied that respective cell layers were coupled electrically to each other through myoendothelial gap junctions (MEGJs). In striking contrast, when cheek pouch arterioles were studied in vivo, PE was found to depolarize SMCs, and depolarization (with ensuing vasoconstriction) was conducted along the smooth muscle layer without affecting the V_m in the endothelium [68]. Furthermore, although hyperpolarization was conducted along either the endothelium or smooth muscle, respective cells had a distinct response waveform [68]. Thus, while either ECs or SMCs could provide a pathway for conduction and did so according to the stimulus, respective cell layers did not appear to be electrically coupled to each other when arterioles were controlling blood flow in vivo. To find my laboratory at odds with that of my mentor with regard to such a fundamental question was one of the most difficult periods of my career. However, controversy in science is often the incentive for developing new experimental paradigms!

In the preceding experiments, respective cell layers of the arteriolar wall remained intact. Thus, an alternative approach was needed to determine the role of smooth muscle versus that of endothelium, as well as to test for myoendothelial coupling. Those familiar with fluorescence imaging know well that the excitation of a fluorochrome can damage cells through the production of reactive oxygen species; fluorescein is especially phototoxic in this regard [71]. Early studies investigated the role of the endothelium in governing reactivity of pial arterioles on the surface of the mouse brain [72]. Sodium fluorescein was injected into the tail vein during illumination with intense green light. The interaction of light with dye ('light-dye treatment', LDT) damaged the endothelium as confirmed by loss of vasodilation to ACh or bradykinin, while vasodilation to papaverine (a smooth muscle relaxant) was preserved [72]. A similar approach in the arteri-

oles of the rat cremaster muscle was found to eliminate the dilator response to an acute increase in blood flow velocity, thereby implicating a role for shear stress exerted on the endothelium as a mediator of vasodilation [73]. In view of these findings, we attempted to use fluorescein dye in the hamster cheek pouch to establish the role(s) of endothelium versus smooth muscle in the conduction of vasodilation and vasoconstriction. However, the sodium salt of fluorescein (MW = 332) is hydrophilic and readily escapes from the vascular compartment. Accordingly, following dye injection into the femoral vein, our initial (half-assed?) experiments in the cheek pouch found inconsistent disruption of both ECs and SMCs (assessed by loss of dilation to ACh and of constriction to PE delivered from micropipettes) in the region of LDT [74]. A better experimental approach was needed!

We reasoned that conjugating the fluorochrome to albumin (MW approx. 65 kDa) should promote its retention within the vascular compartment when injected intravenously. In practice, this modification to the LDT protocol enabled selective damage to the endothelium [74]. In addition, using a micropipette to apply albumin-conjugated fluorescein to the abluminal surface of an arteriole selectively disrupted SMCs while the endothelium remained intact [74]. Damaging SMCs eliminated the conduction of vasoconstriction through the treated segment without affecting the conduction of vasodilation [74]. Although damaging ECs prevented ACh from initiating vasodilation at the site of EC damage, ACh delivered onto the arteriole beyond the site of EC damage resulted in conduction past the site of EC injury. Thus, as implied by our earlier findings, once initiated from an intact vessel region, vasodilation could travel along either the SMC or the EC layer of these arterioles in vivo. Ensuing experiments, in which SMCs and ECs were damaged selectively at different sites along the same arteriole, illustrated that, depending upon the nature of the stimulus, either SMCs or ECs could serve as the cellular conduction pathway. However, the loss of conducted responses when SMCs and ECs were damaged at separate sites along the same arteriole indicated that respective cell layers were not directly coupled to each other in vivo [75] – a conclusion that was consistent with our earlier findings [68]. Attenuation of CVD during the inhibition of nitric oxide (NO) synthase further suggested that a 'wave' of NO was being released along the arteriole during the conducted response [75], but it would be several years before the underlying Ca^{2+} signaling was understood. Ensuing experiments from an independent laboratory using intracellular recordings from arterioles in the mouse cremaster

muscle also indicated that arteriolar ECs and SMCs are not electrically coupled to each other *in vivo* [76].

Given the presence of MEGJs in cheek pouch arterioles as well as feed arteries [77], one explanation that may resolve the controversy surrounding myoendothelial coupling *in vitro* versus *in vivo* is that these discrete sites of heterocellular coupling through MEGJs can be modulated, e.g. through phosphorylation and/or nitrosylation of connexin (Cx) subunits [78–80]. Thus, one hypothesis that remains to be resolved is that MEGJs may be functionally ‘closed’ under physiological conditions *in vivo* and ‘open’ during the isolation of vessels for *in vitro* studies. Once the ability to isolate, cannulate and pressurize individual arterioles was established [51], this experimental approach (i.e. pressure myography) became central to research efforts in many laboratories. In studying such preparations, it should be recognized that preparing isolated vessels involves loss of blood flow and transmural pressure, chilling the excised tissue to near-freezing temperatures (typically 4°C), cutting the vessel free from surrounding tissue and the network in which it has interacted with other vessel branches [12, 81] – all during immersion and perfusion with artificial solutions while being chilled, rewarmed and pressurized... before performing the actual experiment! That these preparations remain viable and provide valuable information is not the concern. Rather, it should be recognized that all reports of myoendothelial coupling are based upon isolated vessel preparations studied *in vitro* [69, 82–91] or their constitutive ECs and SMCs when grown in culture [92]. A controversy that remains to be resolved is whether MEGJs in a given vessel are effectively closed under physiological ‘homeostatic’ conditions and then open during ‘stress’ from insult or injury.

Functional Morphology: Myoendothelial Coupling

While lining the entire cardiovascular system, the endothelium exhibits regional variability in structure and function [93, 94]. Throughout the resistance vasculature, ECs are coupled functionally to each other through gap junction channels that are comprised primarily of Cx37, Cx40 and Cx43, albeit with regional variation [95, 96]. While present in SMCs, the expression of gap junctions between ECs is far greater [77, 95, 97]. An important feature of resistance vessels is the intimate association of SMCs with their underlying ECs (fig. 5). Nearly 50 years ago, classic studies of the cellular ultrastructure along arteriolar networks in the fascia of rabbit skeletal muscle revealed discrete sites at which ECs extended projections

through the internal elastic lamina (IEL). At that time, the presence of ‘myoendothelial junctions’ in electron micrographs was interpreted to suggest that ‘humoral transmitter substances’ (e.g. catecholamines) taken up from the bloodstream by luminal ECs were made more readily available for diffusion to the surrounding SMCs [55]. As functional studies became more sophisticated, the role of the endothelium in mediating smooth muscle relaxation via the release of autacoids (e.g. NO) was identified [1]. However, in rat mesenteric arteries, endothelium-dependent vasodilation was found to prevail when NO production was inhibited, and this was associated with smooth muscle hyperpolarization, leading to the recognition of endothelium-dependent hyperpolarizing factor (EDHF; see [98] for review). While EDHF gained recognition as a cytochrome p450 metabolite of arachidonic acid [99] and was found to be effective in the hamster microcirculation [100, 101], complementary studies invoked a role for the K⁺ ion as an EDHF [102]. Moreover, findings that hyperpolarization initiated via ECs can lead to the hyperpolarization and relaxation of SMCs along arterioles in the absence of electrical coupling between respective cell layers *in vivo* [68, 76] implicated a physiological role for EDHF in the microcirculation.

Electrophysiological studies of arterioles had typically relied upon a single microelectrode for intracellular recording. Approaching vessels from the abluminal surface ensured that the first cell to be penetrated was an SMC. Opening a vessel to expose the lumen (‘en face’) [61, 85] or manipulating a microelectrode through the hole remaining from a severed branch [69] are strategies that have been used to record from the endothelium while it remains associated with the vessel wall. However, such preparations no longer experience transmural pressure, which otherwise evokes depolarization and constriction via the myogenic response [103, 104]. When vessels are unpressurized, V_m is typically more negative (–60 to –75 mV) [28, 69] than obtained in pressurized vessels (–30 to –40 mV) [68, 84]. Thus the baseline and operating range for electrical signaling varies under respective conditions. With this difference in mind, and in deference to the earlier studies using paired microelectrodes [28], we performed dual simultaneous intracellular recordings in isolated pressurized feed arteries (diameter 50–70 µm) of the hamster retractor muscle. Another CDM occurred when we found ECs and SMCs to be electrically coupled to each other during conducted vasomotor responses [84]. A brief pulse of ACh from a micropipette (as in fig. 2) or the intracellular injection of negative current evoked the conduction of hyperpolarization and vasodilation [84]. Si-

Fig. 5. Immunolabeling and fluorescence imaging of vessel wall morphology. Optical sections (thickness, 1 μm) progressing through mouse mesenteric artery. **a** SMCs (red, labeled for smooth-muscle α -actin) with slender, transversely oriented nuclei (blue, stained with TOPRO-3) and collagen fibers (green autofluorescence). **b** IEL (green autofluorescence) containing small holes (i.e.

'fenestrae') enabling EC projections to contact SMCs for myoendothelial coupling. EC nuclei (blue, TOPRO-3) are oval and oriented along the vessel axis. Note nuclei of ECs and SMCs within the same 1- μm optical section as IEL holes, documenting the intimate association of the respective cell layers. Scale bars = 50 μm . Immunolabeling and imaging were performed by Dr. Erika Boerman.

multaneous recording from an EC (confirmed by axial orientation and robust dye transfer to neighboring ECs) and an SMC (circumferential orientation with little or no dye transfer; fig. 3) demonstrated superimposable electrical responses in both cell layers. Further, current injection into an EC produced a corresponding electrical response in SMC and vice versa, along with the conduction of vasodilation during hyperpolarization or vasoconstriction during depolarization [84]. Electron micrographs confirmed the localization of gap junctions at myoendothelial contacts [77, 84]. Thus, functional MEGJs were identified in resistance microvessels, and their role in intercellular signaling between endothelial and smooth muscle layers has evolved into a vigorous area of investigation [69, 82–91, 105–107]. Whether and, if so, how, the role of EDHF versus direct electrical coupling through MEGJs may change under experimental conditions (i.e. in vivo vs. isolated vessels) remains poorly understood [108] and requires further investigation.

In isolated feed arteries of the retractor muscle, selective LDT of the endothelium abolished conducted responses (initiated by ACh) beyond the region of damage,

whereas LDT of SMCs did not impair conduction through the region of SMC damage [18]. These findings illustrated that the endothelium served as the principal cellular pathway for signal transmission (fig. 3, 4). As an 'exercise guy', it was essential for me to determine whether such a role for the endothelium prevailed in the intact system. Thus we performed experiments using rhythmic contractions of the cheek pouch retractor muscle in anesthetized hamsters. Remarkably, feed arteries dilated upstream from the contracting muscle and blood flow increased >4-fold [33]. Performing LDT to disrupt the endothelium midway along the feed artery prevented AVD in response to muscle contraction and abolished CVD in response to local stimulation with ACh (as observed for the same vessels in vitro). Associated with the loss of AVD was an approximately 50% attenuation of the blood flow response to muscle contraction, thereby confirming the critical role of feed artery dilation in attaining maximal perfusion during metabolic demand [33]. Thus, inhibiting conduction along the endothelium (and thereby AVD) effectively restricts blood flowing into the microcirculation. In a recent study, the endothelium of the vascular supply to

Fig. 6. Perivascular sympathetic innervation. Whole-mount preparation of superior epigastric artery from mouse abdominal skeletal muscle. **a** Immunolabeling for tyrosine hydroxylase (green), a limiting enzyme in catecholamine synthesis. Dotted lines indicate vessel edges. **b** Immunolabeling for protein gene product 9.5

(PGP9.5; red), a neuronal marker. **c** Overlay (yellow) of preceding panels confirming sympathetic origin for perivascular innervation of skeletal muscle. Scale bars = 50 μ m. Immunolabeling and imaging were performed by Dr. Erika Boerman.

the rat brain was found to perform a similar role in response to neuronal activity, i.e. AVD along the endothelium was integral to the hyperemic response during neurovascular coupling [109]. In light of endothelial dysfunction associated with vascular aging and disease [110, 111], such findings imply that impaired AVD in skeletal muscle and the brain may contribute, respectively, to diminished physical activity and impaired cognitive function.

Coming from the Other Direction

The sympathetic nervous system is activated according to the intensity of physical exertion [112]. A key outcome is the redistribution of cardiac output away from inactive regions (e.g. the internal organs) to supply oxygen and nutrients to active skeletal muscles [113]. In skeletal muscle, sympathetic innervation extends along resistance arteries (fig. 6) to encompass arteriolar networks [114]. A classic relationship in exercise physiology centers on the interaction between sympathetic nerve activity (SNA) and dilation of the vascular supply in response to muscle contraction [113, 115, 116]. The overriding of sympathetic vasoconstriction by the metabolic demands of exercising skeletal muscle came to be recognized as ‘functional sympatholysis’ [11, 14]. However, if vasodilation were to attain maximal levels throughout the resistance vasculature of skeletal muscle (considering

that it comprises 30–50% of total body mass), total peripheral resistance would no longer be sufficient to maintain arterial perfusion pressure – even during maximal cardiac output [10]. This dynamic interaction was well-demonstrated in chronically instrumented dogs running on treadmills, where intra-arterial infusion of prazosin (to inhibit α ARs) increased hindlimb blood flow in proportion to running intensity as well as the level of α AR inhibition [117, 118]. The take-home message here is that vasoconstriction and blood flow restriction by the sympathetic nervous system increase with the intensity of exercise and the mass of muscle engaged in the activity.

In addressing where SNA was exerting its effect on the vascular supply, Folkow’s classic studies of perfusion pressure and blood flow in the hindlimb of anesthetized cats determined that vasoconstriction was maintained in proximal arteries while vasodilation was manifest downstream within the muscle [31]. More recent intravital studies of the hamster retractor muscle revealed that dilation of feed arteries increased with the level of motor unit recruitment and decreased with the intensity of SNA, even when arterioles within the contracting muscle remained fully dilated [116]. These graded relationships along the resistance network (i.e. regional differences in vasomotor control, alluded to earlier) ensure that feed arteries remain sufficiently constricted to maintain arterial perfusion pressure while dilation of arterioles within the active muscle maximizes capillary perfusion and the sur-

face area for oxygen extraction – particularly as blood flow is limited by proximal resistance (fig. 1). Experiments performed in the hamster cremaster muscle had shown that stimulating perivascular sympathetic nerves suppressed CVD along arterioles [119]. Ensuing studies of retractor muscle feed arteries in vivo [120] and in vitro [121] demonstrated that increasing the level of SNA progressively inhibited CVD, with additive roles for α_1 AR and α_2 AR activation. Thus SNA can modulate AVD in a graded manner, and do so by more than one signaling pathway. Nevertheless, we needed to ascertain whether sympathetic modulation of CVD was manifested constitutively, i.e. without being induced by exercise, perivascular nerve stimulation or pharmacological activation of α ARs.

In human subjects, responses of leg blood flow to graded stationary cycling were reduced by nearly 1/3 in older (approx. 63 years) compared to younger (approx. 26 years) males [122]; however, the mechanism responsible was unknown. Shortly thereafter, it was reported that under resting conditions, blood flow through the femoral artery was attenuated by 25–30% in older versus younger males in conjunction with elevated SNA and α AR-mediated vasoconstriction [123, 124]. To investigate these relationships in the microcirculation of a skeletal muscle integral to locomotion, we developed the gluteus maximus muscle as a preparation for intravital microscopy in the C57BL/6 mouse [125]. In males, our first clue that age was having an effect was an approximately 50% attenuation of CVD in old (aged approx. 20 months) versus young (aged approx. 4 months) mice. Ensuing studies found that rapid onset vasodilation in response to single tetanic contractions was impaired in old male mice but not in their female counterparts [126]. Inhibiting α ARs with phentolamine restored rapid onset vasodilation in the old males; while treating young mice of either sex with norepinephrine (1 nM) had no effect on resting diameter, it suppressed rapid onset vasodilation to resemble that of old males [126]. Furthermore, while there was no difference in steady-state dilation between age groups during rhythmic muscle contractions, arteriolar red blood cell velocity and blood flow were depressed by >50% in the old versus the young males [126]. This outcome is consistent with elevated SNA impairing AVD to restrict muscle blood flow with advanced age. Moreover, these relationships that we first defined in the microcirculation of mouse skeletal muscle were found to prevail in human subjects [127], thereby validating the use of rodents for more invasive studies of the mechanisms that underlie the effects of aging in the microcirculation of humans.

The gluteus maximus muscle is comprised of two distinct regions, each governed by its respective motor nerve and vascular supply [128]. Microvascular networks of respective regions are often joined by anastomotic arterioles and venules. Taking advantage of these anatomical relationships in male mice [129], we tested whether the contraction of one muscle region could initiate vasodilation that spread into the inactive muscle region. Under control conditions, dilation of the anastomotic arteriole was constrained to the active muscle region. However, following inhibition of α ARs with phentolamine, vasodilation spread from the active muscle region into the inactive muscle region [129]. These findings were the first to suggest that a constitutive level of α AR activation effectively restricts the spread of vasodilation between arteriolar networks. Thus, sympathetic neuromodulation can direct blood flow and oxygen delivery to where it is needed most: the active muscle fibers during exercise!

Complementary and Bidirectional Signaling Pathways

In the late 1980s, studies of CVD initiated by ACh in hamster cheek pouch arterioles suggested that these dilations consisted of two components: an initial ‘fast’ response, whereby the vessel appeared to ‘pop’ open within the first couple of seconds, and a slower increase in diameter that progressed over the next few seconds [39–41]. Thus, multiple signaling pathways mediating smooth muscle relaxation were apparent. During the same time frame, the activation of K^+ channels concomitant with a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) was identified as a mechanism of EC hyperpolarization in response to stimulating muscarinic receptors with ACh [130]. Ensuing studies identified essential roles for small- and intermediate-conductance Ca^{2+} -activated K^+ channels (SK_{Ca} and IK_{Ca}) as the basis of EC hyperpolarization to ACh (see [131] for an historical perspective on the definitive studies defining these relationships). We therefore reasoned that SK_{Ca} and IK_{Ca} activation may be involved in the rapid component of CVD. This hypothesis was tested in pressurized feed arteries of the hamster retractor muscle. Treating the site of ACh stimulation with apamin + charybdotoxin (to block the initiation of hyperpolarization via K_{Ca} channel activation) eliminated the ‘fast’ dilator response and thereby revealed the secondary ‘slow’ component of conduction [132]. Ensuing experiments in which ECs were loaded with a Ca^{2+} -sensitive dye, Fura-2, revealed a Ca^{2+} ‘wave’ traveling along the en-

Fig. 7. Endothelial calcium wave contributes to AVD in vivo. Sequential images of an arteriole within the cremaster muscle of transgenic mice expressing GCaMP2 in ECs to illustrate the initiation and spread of a Ca^{2+} wave and vasodilation in response to local stimulation with a brief pulse of ACh (according to fig. 2). **a** Initiation: note local rise of $[\text{Ca}^{2+}]_i$ in ECs across the intima (red

and yellow regions). Arrowhead corresponds to ACh micropipette; arrow indicates direction of blood flow. **b** Transmission: the Ca^{2+} wave has spread bidirectionally along the arteriolar endothelium. **c** Ensuing dilation along upstream segments. Scale bars = 50 μm . Complementary images from experiments performed in Talini et al. [134].

endothelium that corresponded with the ‘slow’ component [132]. This was a CDM in identifying distinct yet complementary signaling pathways that contributed to CVD. Although a Ca^{2+} wave had been implicated during intravital studies of cheek pouch arterioles [75], actually recording this component of the conducted response was a most rewarding breakthrough. Ensuing experiments employed confocal imaging to determine the rise in endothelial $[\text{Ca}^{2+}]_i$ that resulted in the lowering of SMC $[\text{Ca}^{2+}]_i$ [133]. The latter effect was explained by the closure of voltage-gated (L-type) Ca^{2+} channels as hyperpolarization spread through MEGJs into the surrounding SMCs. To investigate these relationships in vivo, collaborative efforts enabled intravital imaging of the microcirculation in transgenic mice expressing a Ca^{2+} -sensitive protein (i.e. GCaMP2) selectively within arteriolar ECs [134], thereby establishing the functional significance of this signaling pathway in vivo (fig. 7). Collectively, these experiments illustrated that rapid (i.e. electrical) signaling coordinates arteriolar dilation over several millimeters (e.g. across daughter and parent branches; fig. 1), while the ensuing

endothelial Ca^{2+} wave can further ‘tune’ the magnitude and duration of dilation along several hundred microns, i.e. along vessel segments closer to the stimulus origin.

A functional role for Ca^{2+} signaling between SMCs and ECs had been implicated in arterioles isolated from the hamster cheek pouch [83]. Using the Ca^{2+} indicator dye Fluo-3, stimulating the arteriole with PE was found to increase Ca^{2+} within SMCs that, in turn, gave rise to elevated Ca^{2+} within underlying ECs [83]. The rise in EC Ca^{2+} was reported to stimulate NO production through activating endothelial NO synthase (fig. 8), thereby providing negative feedback to attenuate vasoconstriction [83]. Ensuing experiments expanded the role of endothelial feedback in modulating SMC contraction to include the activation of endothelial K_{Ca} channels [89]. These respective findings inferred that $[\text{Ca}^{2+}]_i$ increased globally, throughout the cytoplasm of affected cells. However, new insight into the molecular organization of MEGJs (fig. 8), complemented by refined experimental design and confocal imaging of Ca^{2+} signaling microdomains, has provided alternative interpretations [90, 91, 135].

Fig. 8. Bidirectional myoendothelial calcium and electrical signaling. Schematic illustration of: intact vessel segment illustrating luminal ECs, surrounding SMCs and perivascular nerve plexus with varicosities containing NE (**a**); enlarged edge of vessel wall (**b**); EC projection through IEL showing MEGJs underlying bidirectional signaling between EC and SMC with key steps enumerated (**c**). ACh binds to G_q protein-coupled muscarinic (M₃) receptor (1) resulting in the liberation of IP₃, which binds to its receptor IP₃R in the ER, releasing Ca²⁺ to activate K_{Ca} channels (2); the efflux of K⁺ through the plasma membrane (3) hyperpolarizes the cell interior. With SMCs, norepinephrine (NE) released from perivascular sympathetic nerve terminals (PVS) binds to the G_q protein-coupled α₁ adrenergic receptor (4), also liberating IP₃, which activates IP₃R in the sarcoplasmic reticulum (SR) to release Ca²⁺ into the cytosol (5). Elevation of IP₃ and Ca²⁺ within SMCs promotes their diffusion through MEGJs (6 and 7) at endothelial projections

through holes in the IEL (see fig. 5). In turn, local elevation of second messengers may stimulate Ca²⁺ release from the ER within the EC projection (8). Elevation of Ca²⁺ within the endothelial projection can activate IK_{Ca} (9), with K⁺ efflux and hyperpolarization providing negative feedback through the MEGJ (10) to attenuate Ca²⁺ entry into the SMCs (11) through voltage-gated Ca²⁺ channels (VGCC). At the same time, the local rise in extracellular K⁺ can stimulate the electrogenic Na⁺/K⁺ ATPase (12) and inward-rectifying K⁺ channels (not shown), further contributing to SMC hyperpolarization. Influx of Ca²⁺ through transient receptor potential (TRP) channels (13) can augment IK_{Ca} activation in endothelial projections, further promoting SMC relaxation via the myoendothelial transmission of hyperpolarization. Concomitant activation of NO production by endothelial NO synthase (eNOS) further promotes vasodilation (14).

With endothelial-dependent hyperpolarization (EDH) explained by the activation of SK_{Ca} and IK_{Ca}, subsequent to the rise in EC [Ca²⁺]_i, electrophysiological studies of isolated rat mesenteric arteries implicated distinct roles for respective ion channels. Under resting conditions

(V_m, approx. -50 mV), ACh induced EDH (to approx. -75 mV) and relaxation of SMCs, with both responses attributed to the activation of SK_{Ca}. However, when SMCs were first depolarized (to approx. -40 mV) with PE, their repolarization to approximately -50 mV upon stimula-

tion with ACh was attributed to the activation of IK_{Ca} , with SK_{Ca} contributing the remaining approximately -25 mV hyperpolarization [136]. These findings led to the hypothesis that respective K_{Ca} channels, which appeared to have distinct roles in EDH, may be differentially localized in the endothelium [137]. Confocal microscopy and ultrastructural immunohistochemistry illustrated that IK_{Ca} were localized within punctate holes of the IEL in association with EC projections; localization of Cx37 and Cx40 to these same sites implicated association of IK_{Ca} with MEGJs (fig. 8). In contrast, SK_{Ca} channels were found in association with Cx37, Cx40 and Cx43 that formed gap junctions at EC borders [137]. Thus, differentially activated components of EDH appear to be related to the topological separation of SK_{Ca} and IK_{Ca} . Novel experiments using mouse ECs and SMCs grown in coculture addressed the contribution of Ca^{2+} versus inositol 1,4,5-trisphosphate (IP_3) diffusion and the role of respective Cx isoforms in myoendothelial signaling [92, 138, 139]. While providing critical new insight into the directionality and effects of Ca^{2+} versus IP_3 flux through MEGJs under highly controlled conditions, it should be recognized that cocultures lack the presence of an IEL, exhibit a far greater number of EC-SMC contacts than occur in actual resistance vessels and have never experienced blood flow or transmural pressure. Nevertheless, and as supported by more recent studies, conclusions based upon coculture experiments have been strengthened by complementary *in vivo* paradigms [140].

To observe Ca^{2+} signaling of native endothelium while avoiding optical interference from SMCs, mesenteric arteries from transgenic mice expressing $GCaMP2$ in ECs were opened for *en face* fluorescence imaging. Thereby, Ca^{2+} signaling microdomains were identified that corresponded to EC projections through the IEL [85]. Spatially fixed Ca^{2+} release events characterized as ‘pulsars’ were mediated by IP_3 receptors (IP_3Rs) localized to the endoplasmic reticulum (ER) within EC projections. While spontaneously active under basal conditions, stimulation with ACh (and the ensuing IP_3 production) increased pulsar activity several-fold. Consistent with earlier findings [137], immunostaining for IK_{Ca} was localized to holes in the IEL, as was staining for IP_3Rs [85]. Thus, local release of Ca^{2+} from IP_3Rs was associated with activation of IK_{Ca} within EC projections located at holes in the IEL. When activated locally, these channels produce hyperpolarization, and when coupled with MEGJ localization within these microdomains [137], a mechanism was identified for Ca^{2+} -dependent signaling of hyperpolarization from ECs to SMCs. Further insight into these spatial

and functional relationships was gained through recent studies of feed arteries of the hamster retractor muscle [88]. Ultrastructural tomography revealed EC projections through the IEL that directly contacted overlying SMCs. These projections contained ER membranes expressing IP_3Rs in conjunction with IK_{Ca} . Following loading with a Ca^{2+} -sensitive dye (Fluo-4), confocal imaging revealed that stimulating SMCs with PE evoked discrete Ca^{2+} events in ECs (termed ‘ Ca^{2+} wavelets’) that were attributed to the activation of IP_3Rs within the EC projections. As inhibiting IK_{Ca} or endothelial IP_3Rs enhanced vasoconstriction to the α_1AR agonist PE, the bidirectional signaling pathway entailed activation of SMCs with the generation of IP_3 and Ca^{2+} as second messengers, which may then diffuse through MEGJs to activate IP_3Rs of the ER within EC projections (fig. 8). The ensuing activation of IK_{Ca} provided negative feedback to attenuate SMC depolarization and vasoconstriction [88]. Thus, rather than ECs responding to global changes in $[Ca^{2+}]_i$ as initially proposed [89, 141], more detailed studies have revealed endothelial projections as discrete microdomains of heterocellular signaling through MEGJs that modulate the contractile status of SMCs [82, 86, 88, 90, 91, 135, 142]. The take-home message for bidirectional signaling through MEGJs is that, in response to activation of αARs on SMCs, negative feedback from ECs modulates vasoconstriction whereas activation of muscarinic receptors on the endothelium drives SMC relaxation. In both scenarios, myoendothelial signaling is integral to vasomotor (and thereby blood flow) control in the resistance vasculature. It should also be recognized that the activation of αARs and muscarinic receptors with physiological agonists in isolated preparations enables the resolution (and thereby the study) of functional relationships that may otherwise be difficult to ascertain in the intact system.

Simplifying the Model and Putting Things Together

As experimental approaches to investigate the nature of signaling between SMCs and ECs continued to evolve, we turned our attention towards understanding the biophysical properties of native intact microvascular endothelium. Professor Jackson’s laboratory had developed methods for isolating arteriolar smooth muscle from its endothelium to study ion channel signaling events intrinsic to microvascular SMCs and ECs of the rat and hamster [143, 144]. We refined these procedures to isolate intact endothelial ‘tubes’ from mouse microvessels [145], thereby eliminating MEJs and the influence of SMCs on endo-

thelial function. The superior epigastric artery of the mouse was selected as a feed artery from abdominal skeletal muscle because it enables the isolation of intact segments 1–3 mm long and 50–60 μm wide. This experimental model is well-suited for studying the intrinsic properties of electrical conduction and Ca^{2+} signaling along native intact microvascular endothelium in the absence of transmural pressure or blood flow. Control experiments evaluated Ca^{2+} and V_m in freshly isolated endothelial tubes during exposure to graded levels of stimulation with ACh. Remarkably, the response curves for respective signaling events corresponded to the response curve for vasodilation [146], confirming that endothelial tubes retained their integrity for functional signaling. In light of earlier studies that had implicated the endothelium as the cellular pathway for conducting vasodilation, pairs of intracellular microelectrodes were used to inject current into one EC while simultaneously recording V_m in a remote EC located at defined separation distances (typically 500–2,000 μm , corresponding to approx. 15–60 ECs placed end-to-end). A key finding was that the amplitude of the V_m response in the remote EC increased or decreased in a linear manner with the amplitude and polarity of the injected current [63, 146]. This passive behavior as an electrical resistor confirmed the lack of voltage-gated ion channels in the endothelium. Furthermore, the V_m response decayed exponentially with the distance between electrodes, indicating that the electrical signal conducted from cell to cell in a manner consistent with what had been observed for the endothelium of intact feed arteries [18].

Intercellular conduction of electrical signals requires effective coupling among neighboring cells as enabled through gap junctions. Indeed, disruption of gap junctions (e.g. by deleting the Cx40 subunit) impaired conducted responses along the arterioles of the mouse cremaster muscle [147, 148]. Though perhaps less apparent, the high electrical resistance of plasma membranes is also integral to signal transmission from cell to cell. Thus, we questioned whether opening ion channels in the plasma membrane would make ECs electrically ‘leaky’. The endothelial tube preparation proved to be an ideal model for these experiments, as SK_{Ca} and IK_{Ca} are highly expressed in these cells [111] and the pharmacological tools for manipulating these channels are well-defined [131, 149]. Moreover, as there are no other cell types or hemodynamic stimuli that could intervene, the intrinsic biophysical properties of native microvascular endothelium could be defined. A key finding from these experiments was that the activation of SK_{Ca} and IK_{Ca} along the endothelium

resulted in the loss of the electrical signal at remote recording sites. In such manner, opening ion channels in the plasma membrane inhibited electrical signal transmission while gap junctions remained fully patent, as confirmed by robust dye transfer from an injected EC to its surrounding neighbors [63]. Thus, using a novel approach, these experiments revealed that intercellular signal transmission along the endothelium could be modulated independently of gap junctions, by manipulating ion channels in the plasma membrane [63].

In light of earlier discussion, our studies of endothelial tubes provide a foundation from which to reconsider myoendothelial signaling. In particular, how the stimulation of αARs can attenuate the conduction of electrical signals and suppress AVD during exercise. As shown in mouse mesenteric arteries, SNA increased the activity of Ca^{2+} pulsars observed within ECs, indicating that signals initiated on SMCs (where the αARs are activated during SNA) enhanced Ca^{2+} signaling in the endothelium [86]. In turn, vasoconstriction during SNA was shown to be enhanced by inhibiting IK_{Ca} [86]. These findings are consistent with the activation of αARs on SMCs giving rise to localized Ca^{2+} signaling (pulsars) and hyperpolarization (via IK_{Ca} activation) in endothelial projections to provide negative feedback that attenuates sympathetic vasoconstriction. During SNA, norepinephrine is released from perivascular nerves that course along feed arteries and arteriolar networks (fig. 6). Thus, secondary to αAR stimulation, IK_{Ca} (along with nearby SK_{Ca}) would also be activated along the endothelium (via MEGJs; see fig. 8). In turn, dissipating the electrical signal through ion channels in plasma membranes reduces the effective distance of signal transmission along the endothelium [150]. This interpretation explains how SNA inhibits CVD (via αAR activation) along feed arteries studied in vitro [121] and of AVD into feed arteries supplying skeletal muscle in vivo [116]. In a reciprocal manner, the increase in muscle blood flow during exercise can be explained by inhibiting sympathetic vasoconstriction, exemplified in running dogs by the increase in hindlimb blood flow upon pharmacological blockade of αARs [117, 118]. It should also be recognized that the activation of ion channels in SMC membranes can inhibit intercellular signal transmission and the conduction of vasomotor responses [121, 151, 152]. Thus, in light of direct coupling between ECs and SMCs through MEGJs, increasing the loss of electrical charge through membranes in either cell layer can effectively dissipate conducted vasomotor responses including AVD.

Summary and Translation

Aging and vascular disease are accompanied by enhanced SNA, endothelial dysfunction in association with oxidative stress and impaired tissue perfusion [110, 153–155]. Hydrogen peroxide is a common reactive oxygen species and was recently found to activate K_{Ca} channels in the endothelium of feed arteries [111]. This effect of oxidative stress inhibits intercellular conduction in a manner complementary to the actions of SNA described above. Taken together, these findings help to explain why blood flow is restricted with aging. Thus, combining enhanced SNA with oxidative stress can lead to the opening of ion channels in cell membranes, thereby dissipating electrical signals and impairing conduction [150]. With AVD thereby compromised, blood flow into the microcirculation is restricted [33, 126]. Attributing such effects of aging and SNA to specific membrane proteins points to targeting selective therapeutic interventions to restore tissue perfusion. Nevertheless, whereas the activation of K_{Ca} channels can be an effective strategy to promote vasodilation and improve tissue blood flow [156, 157], potentially adverse effects on the ability to conduct electrical signals should also be considered. For example, the coordination of capillary perfusion within and between respective branches of resistance networks [129] (fig. 1) may be compromised with ‘global’ dilation of arterioles secondary to excessive activation of K_{Ca} channels and loss of vasomotor tone.

In presenting the evolution and application of research on ‘vascular conduction’ from the perspective of my laboratory, it should be recognized that complementary findings and lines of investigation have been and continue to be developed by a growing body of investigators contributing to this field as represented by those whose works are cited herein. In addition to the moment-to-moment coordination of vasomotor control emphasized here, vascular conduction also appears to be integral to intercellular communication that underlies the remodel-

ing of vascular networks [158]. Beyond our interest in skeletal muscle, cell-to-cell conduction in vascular resistance networks has been implicated as being integral to tubuloglomerular feedback among nephrons in the kidney [159], neurovascular coupling in the brain [109, 160] and the matching of ventilation and perfusion in the lung [161]. Nearly every cell, tissue and organ in the body relies upon the microcirculation to maintain homeostasis. In turn, with aging, disease and organ dysfunction, it is implicit that the microcirculation is involved. Elucidating the role of intercellular signaling within and between the endothelium and smooth muscle, how these events are modulated by nerves and how they may be influenced by surrounding tissue, blood flow and environmental factors represent unlimited opportunities for microvascular research in the future, particularly in translating what we observe and discover through the microscope towards the treatment of patients and optimizing their quality of life.

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Disclosure Statement

The author declares no conflicts of interest.

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