Evidence for Cholinergic Regulation of Microvessel Hydraulic Conductance During Tissue Hypoxia

Vicky L. Tucker and Virginia H. Huxley

Cholinergic regulation of single-vessel hydraulic conductivity ($L_p$) during normoxia and hypoxia was tested in single mesenteric vessels of pithed frogs (Rana pipiens). Capillaries were cannulated in situ and perfused with frog Ringer’s solution containing 10 mg/ml albumin and human erythrocytes while the mesentery was continuously superfused with frog Ringer’s solution (15°C). $L_p$ was first measured under normoxic (room air equilibrated) conditions by the modified Landis microocclusion method. Repeated measurements of filtration coefficient under control conditions, for periods up to 80 minutes, demonstrated that $L_p$ did not change with time in normoxic vessels ($n=18$). After initial control measurement ($L_{p0}$), perfusion with 1 μM acetylcholine increased $L_p$ by 4.6±1.0-fold (mean±SEM, $n=6$). The response to acetylcholine was antagonized by the addition of 10 μM atropine to the perfusate ($L_p^*/L_{p0}=1.8±0.4$). Perfusion with atropine alone reduced $L_p$ in three of six capillaries ($L_p^*/L_{p0}=0.56±0.04$); $L_p$ in the remaining three vessels was unaffected. Tissue hypoxia was simulated by exposing the mesentery to deoxygenated superfusate (PO$_2$≤10 mm Hg) for 10–15 minutes. Tissue hypoxia had no effect on $L_p$ in atropine-treated vessels ($n=8$). Without atropine, tissue hypoxia increased $L_p$ by 2.3±0.7-fold, whereas the addition of atropine completely antagonized this response ($n=5$). In contrast to the inhibitory action of atropine during tissue hypoxia, $L_p$ rose 5.2±1.6-fold ($n=4$) in vessels simultaneously exposed to deoxygenated perfusate. We conclude that cholinergic stimulation elevates $L_p$ in frog mesenteric capillaries and that enhanced water conductivity during tissue but not intraluminal hypoxia involves a cholinergic-muscarinic mechanism. (Circulation Research 1990;66:517–524)

Although recent studies have begun to characterize the dynamic nature of the microvascular barrier, the mechanisms responsible for the modulation of permeability remain largely unknown. Hydraulic conductivity ($L_p$), a reciprocal measure of the transcapillary resistance to water flow, is influenced by a number of endogenous compounds and, in some microvascular beds, by oxygen (O$_2$) supply. We have previously demonstrated a reversible elevation of $L_p$ in response to hypoxia in individually perfused microvessels of in situ frog mesentery. Water conductivity increased by fivefold in capillaries exposed to local intraluminal oxygen deprivation. Interestingly, we also observed a rise in $L_p$ (=twofold) when tissue (extravascular) hypoxia was simulated by removal of O$_2$ from the superfusate solution bathing the mesentery. Microvascular sensitivity to superfusate oxygen tension (PO$_2$) in the presence of adequate perfusate O$_2$ could implicate a tissue-derived mechanism for increasing permeability.

We postulated that extravascular nerves are responsible for sensing and initiating a microvascular response to low tissue PO$_2$. To test this, we chose to investigate a putative role for cholinergic modulation for $L_p$ in response to reduced PO$_2$, because norepinephrine or other catecholamines appear to exert an inhibitory effect on permeability. Responses to the cholinergic agonist acetylcholine (ACh) and the muscarinic receptor agonist atropine were examined under normoxic and hypoxic conditions. Muscarinic receptor blockade antagonized the rise in $L_p$ observed during exposure to either ACh or tissue hypoxia. In contrast, exposure to luminal hypoxia

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Supported by National Institutes of Health grants HL-34872 and HL-07047.
Address for correspondence: Vicky L. Tucker, Department of Human Physiology, School of Medicine, University of California, Davis, Davis, CA 95616.
Address for reprints: Virginia H. Huxley, Department of Physiology, University of Missouri School of Medicine, Columbia, MO 65212.
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elevated water conductivity by a separate noncholinergic mechanism. The results indicate that cholinergic-muscarinic agents are capable of modulating $L_p$.

These data are consistent with the hypothesis that nerves and/or other extravascular cells can act as extrinsic modulators of microvascular permeability.

Materials and Methods

$L_p$ of single perfused microvessels was obtained in situ from the mesentery of North American leopard frogs (Rana pipiens). The animals (male, 2–3 years of age, 5–7.5 cm body length) were supplied weekly (J.M. Hazen, Alburg, Vermont) and were housed in a cold room (15°C) in 75×50×30-cm³ tanks providing both deep water and accessible dry areas.

Preparation

Each frog was anesthetized by cerebral pith. Cotton was placed within the brain cavity to ensure separation of the brain stem and the intact spinal cord. Animals were rinsed with tap water, dried, and secured in dorsal recumbency on a cork-lined tray. The abdominal viscera were exposed through a right lateral incision and kept moist with frog Ringer’s solution. The ileal mesentery was gently positioned over a polished quartz pillar (Haerus-Amersil, Sayerville, New Jersey) and tacked to a silicone ring with small insect pins. The surface of the exposed tissue was continuously superfused with frog Ringer’s solution at a rate of 5 ml/min. Temperature was maintained at 12°–15°C at the tissue surface.

Microscopy

The mesentery was transilluminated and viewed with an inverted light microscope at ×6 (Diavert, Leitz, Rockleigh, New Jersey). At a total magnification of ×75, a tissue area of 2 mm² was viewed. A video image of the experimental vessel and about 300 mm² of surrounding tissue was recorded on videotape by a black and white video camera (model 650, Dage-MTI, Michigan City, Indiana).

Solutions

The superfusate fluid was a buffered frog Ringer’s solution of the following composition (mM): NaCl 111, KCl 2.4, MgSO₄ 1.0, CaCl₂ 1.1, glucose 5.0, NaHCO₃ 2.0, N’-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and Na-HEPES 5.0 (230 mosm, pH 7.4 at 15°C). The superfusate fluid was bubbled with air and dispensed from glass aspirator bottles via glass and Tygon gas-impermeable tubing. A glass heat-exchange coil placed within the superfusate line was used to cool the superfusate.

The perfusate was a solution of 10 mg/ml dialyzed bovine serum albumin (BSA, crystallized and lyophilized; A-8763, A-4378, Sigma Chemical, St. Louis, Missouri) in frog Ringer’s solution. The BSA was extensively dialyzed against frog Ringer’s solution to reduce low molecular weight contaminants as previously described. The perfusate solution also contained a 5% (vol/vol) suspension of human erythrocytes, washed in frog Ringer’s solution, for use as flow markers.

The superfusate and perfusate solutions were deoxygenated by equilibration with 100% nitrogen gas (N₂, medical grade). The perfusate was deoxygenated in a glass equilibration chamber. The gas was first bubbled through frog Ringer’s solution before contact with the perfusate. Osmolality of both solutions was rechecked after gas equilibration. Oxygen tensions of the solutions were measured with a polarographic electrode (Diamond Electrical, Ann Arbor, Michigan). The electrode was calibrated in frog Ringer’s solution equilibrated with 100% N₂ and air. Superfusate P O₂ and perfusate P O₂, after transfer from the equilibration chamber to a glass syringe pregassed with N₂, averaged 10 mm Hg or less. Micropipettes were gassed with N₂ and filled with perfusate by use of a stainless steel 22-gauge 2½-in. needle. Equilibration of the superfusate solution with surrounding room air was minimized by the high superfusate flow rate (5 ml/min) and by the maintenance of a thick layer (1 mm) of superfusate above the mesentery.

Microperfusion

Figure 1 is a diagrammatic cross-sectional view of a frog mesentery illustrating the approximated dimensions of a vessel and surrounding tissue. Microvessels ranging from 15 to 40 μm in diameter were cannulated with glass micropipettes filled with perfusate solution. Vessels were classified as arteriolar, true, or venular capillaries on the basis of flow pattern.

Microvessels with white cell rolling, slow or intermittent flow, or an interbranch distance of 800 μm or less were not used. In addition, microvessels close (within 300 μm) to oxygen-supplying arterioles were avoided.

Measurement of $L_p$

The calculation of $L_p$ was based on the following relation:
**Lp=(Jv/S)[Pc−αΔP]**  
(1)

where \(Jv/S\) is fluid filtration per unit area, \(Pc\) is microvessel hydrostatic pressure, and \(αΔP\) is the effective osmotic pressure gradient. \(Jv/S\) and \(Pc\) were measured by the modified Landis micro-occlusion technique. Detailed descriptions and reviews of this method are available. Equation 1, derived from Starling's hypothesis, describes the dependency of convective water movement on the balance between plasma-interstitium hydrostatic and osmotic pressure gradients. The low perfusate BSA concentration (10 mg/ml) used in the present study minimized \(αΔP\). A fixed \(Pc\) corresponding to an effective osmotic pressure gradient of 4 cm H2O, was included in linear regression analysis of \(Jv/S\) on \(Pc\) as previously described.

Microvessels were occluded at least 600 \(μm\) below the cannulation site with a glass rod. This stopped perfusate flow and equalized vessel pressure with delivery pressure. Filtration across the vessel wall during occlusion was estimated from the initial rate of marker cell movement in the closed vessel segment \(((dl/dt))\). The first second of filtration was omitted to reduce compliance artifact. Vessel area was calculated from the vessel length \(L\) between the marker cell and occlusion site, and vessel radius \(r\), assuming a right cylindrical geometry (i.e., volume/surface area \(=πr^2\)). Multiple measurements of \(Jv/S\) were made at two to four hydrostatic pressures for each determination of \(Lp\). The pressure levels that were used ranged from 15–40 cm H2O. An example is shown in Figure 2. Introduction of error due to vessel injury at the occlusion site was minimized by moving the occluder upstream every three or four occlusions.

**Protocols**

Experiments were conducted on one microvessel per animal. All experiments were paired: \(Lp\) was first determined during an initial or control period and again during one or more treatment periods. Control solutions were equilibrated with room air (21% \(O_2\)). Tissue hypoxia was induced by reducing oxygen tension of the superfusate layer only. Perfusate (intraluminal) hypoxia involved recannulation of the vessel with a pipette containing deoxygenated solution. All drug treatments were applied via the perfusate; that is, the vessel was recannulated with a drug-containing pipette.

**Sham treatment.** To evaluate the stability of \(Lp\) over time, repeated measurements of \(Lp\) were made under control conditions. Two subsets of sham vessels were examined, one at the beginning and one at the end of the study. \(Lp\) were determined at approximately 20-minute intervals, and depending on the initial vessel length, up to five \(Lp\) measurements (80 minutes of perfusion) were obtained on a single vessel.

**Drug treatment.** After a control \(Lp\) measurement (elapsed time, 3–10 minutes), microvessels were recannulated and perfused 10–15 minutes with 1 \(μM\) ACh, and a second \(Lp\) was obtained. Some of the vessels were recannulated a third time with perfusate containing 10 \(μM\) atropine in addition to ACh. A 25-minute perfusion period was allowed before the third \(Lp\) determination. In an additional group of vessels, the control period was immediately followed by 10–15 minutes of 10 \(μM\) atropine perfusion. These vessels were used for the hypoxia experiments described below.

**Hypoxia treatment.** \(Lp\) was measured after a 10–15-minute exposure to superfusate hypoxia in the presence of atropine. A group of these vessels was recannulated with deoxygenated perfusate (containing atropine), and a final \(Lp\) was determined during exposure to combined superfusate plus perfusate hypoxia. In an alternate protocol, vessels were exposed to superfusate hypoxia before atropine application. \(Lp\) was measured during 1) control, 2) superfusate hypoxia, and 3) superfusate hypoxia in the presence of atropine. In the latter case, atropine was perfused for 20 minutes before \(Lp\) measurement.

**Drugs**

ACh chloride and atropine free base were purchased from Sigma Chemical. Stock solutions (1 mM) were prepared by dissolving ACh in cold deionized water and atropine in frog Ringer's solution. The stock solutions were diluted to their final concentration with the perfusate solution (Ringer-BSA). Drug solutions were discarded after 12 hours.

**Statistical Analysis**

Linear regression analysis was used to calculate \(Lp\) from the slope of \(Jv/S\) versus \(Pc\). Measures of \(Jv/S\) were obtained at two to four pressure levels for each \(Lp\) calculation. The number of pressure levels was
TABLE 1. Average Hydraulic Conductivities of Sham-Treated Microvessels

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>20–35</th>
<th>36–50</th>
<th>51–65</th>
<th>66–80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lp/Lp⁰</td>
<td>1</td>
<td>1.2±0.1</td>
<td>1.0±0.2</td>
<td>1.2±0.2</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
<td>10</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are fractional increase (mean±SEM) of hydraulic conductivity (Lp) relative to initial control (t=0). Lp⁰, control measurement; n, number of microvessels represented in the time period.

Results

The data from a total of 39 vessels are reported in this study. The mean Lp⁰ was 5.0±0.8×10⁻⁷ cm/(sec cm H₂O) (range, 0.3–30×10⁻⁷ cm/[sec cm H₂O]).

Stability of Lp Over Time

In 18 microvessels, mean Lp did not change significantly during extended exposure to control conditions (Table 1). A variation of 20% or less was observed between time periods; the average coefficient of variation for individual Lp values was also approximately 20%. The largest deviations observed during sham treatment were a 100% elevation (two-fold increase) and a 70% reduction from the initial Lp value. Recannulation of the microvessels during the sham treatment did not produce a consistent or significant effect on the measured Lp. There was no difference in Lp variability between sham experiments performed at the beginning (n=9) and at the end (n=9) of the study.

Lp During Exposure to ACh and/or Atropine

Exposure to ACh for 10 minutes significantly increased Lp 4.6±1.0-fold over control (n=6, Figure 3). In three of these vessels, atropine was added to the perfusate during ACh perfusion (Figure 4). By 25 minutes after application of atropine, Lp was significantly lower than the level reached during ACh alone (Lp/Lp⁰=1.8±0.4, n=3). Figure 5 illustrates the response of six vessels exposed to atropine alone. Lp during atropine is plotted as a function of control Lp for individual vessels. The mean response to atropine was not significantly different from control (Lp/Lp⁰=0.8±0.1); however, a trend toward lower Lp with atropine was noted. Lp comparisons in individual vessels indicated a significant reduction in Lp during atropine in three of six vessels.

Response to Hypoxia in the Presence of Atropine

In contrast with our previous studies demonstrating a 2.4-fold elevation in water conductivity, superfuse hypoxia in the presence of atropine had no demonstrable effect on Lp (n=8, Table 2). However,
when both the superfusate and perfusate were deoxygenated, \( L_p \) increased significantly to 5.2±1.6-fold over the atropine baseline (n=4). This response was indistinguishable from our previous observation\(^9\) of fivefold increase in \( L_p \) during perfusate hypoxia alone.

The results of experiments in which vessels were exposed to tissue hypoxia before application of atropine are illustrated in Figure 6. In these vessels, tissue hypoxia increased \( L_p \) by 2.3±0.7-fold in the absence of atropine. The effect of hypoxia was completely reversed by addition of atropine to the perfusate (\( L_p^0 \)/Lp=0.8±0.2). In two cases, \( L_p^0 \) measured during hypoxia in the presence of atropine were significantly lower than control conductivity.

### Discussion

The studies presented in this paper clearly demonstrate a sensitivity of single-vessel water conductivity to cholinergic stimuli. \( L_p \) increased from control levels when microvessels were perfused with ACh. The addition of atropine to the perfusate antagonized this response, resulting in the return of \( L_p \) toward baseline values. We have observed elevated conductivity in response to superfusate and/or perfusate deoxygenation in previous studies.\(^5,10\) Atropine perfusion, although having no effect on baseline \( L_p \), abolished the rise in \( L_p \) during superfusate hypoxia in the present study. Without atropine, \( L_p \) increased during exposure to superfusate hypoxia as previously observed. Subsequent addition of atropine reduced \( L_p \) to control levels. These results provide strong evidence that the increase in hydraulic permeability during superfusate hypoxia involves a cholinergic-muscarinic event. During combined exposure to superfusate plus perfusate hypoxia, atropine did not prevent a rise in microvessel \( L_p \). We postulate that tissue deoxygenation during superfusate hypoxia increased microvessel permeability by an indirect pathway involving cholinergic stimulation, whereas intraluminal hypoxia elevated \( L_p \) by a direct noncholinergic mechanism.

The ACh sensitivity observed in our study contrasts with that of Smaje,\(^17\) who did not observe an ACh-induced \( L_p \) change in single venules of cat mesentery. Reasons for this discrepancy are not clear. The venules studied by Smaje were autoperfused with blood, and ACh was applied topically via the superfusion solution. In contrast, the present study involved examination of capillaries artificially perfused with ACh applied to the perfusate solution. Thus, possible explanations for the lack of ACh sensitivity in Smaje’s study are as follows: 1) The animal’s blood contains elements responsible for altering ACh sensitivity. 2) Venules are not responsive to ACh. 3) Topically applied ACh does not

### Table 2. Influence of Tissue and Intraluminal Hypoxia on Hydraulic Conductivity After Equilibration With Atropine

<table>
<thead>
<tr>
<th>Number</th>
<th>Vessel</th>
<th>Type</th>
<th>D (μM)</th>
<th>( L_p ) (cm/sec cm H₂O)×10⁻⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>2-25</td>
<td>AC</td>
<td>22</td>
<td></td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>2-26</td>
<td>VC</td>
<td>20</td>
<td></td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>2-28</td>
<td>VC</td>
<td>25</td>
<td></td>
<td>6.3±1.7</td>
</tr>
<tr>
<td>2-29</td>
<td>AC</td>
<td>25</td>
<td></td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>2-30</td>
<td>VC</td>
<td>20</td>
<td></td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>2-31</td>
<td>VC</td>
<td>30</td>
<td></td>
<td>32.1±4.6</td>
</tr>
<tr>
<td>2-32</td>
<td>AC</td>
<td>30</td>
<td></td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>2-33</td>
<td>VC</td>
<td>18</td>
<td></td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>2-34</td>
<td>TC</td>
<td>25</td>
<td></td>
<td>5.3±0.6</td>
</tr>
</tbody>
</table>

Values are hydraulic conductivity±SEM (\( L_p \)). D, diameter; A, 10 μM atropine; \( N₅(S) \), superfusate hypoxia-atropine present; \( N₅(S)/A \), fractional change in \( L_p \) during superfusate hypoxia plus atropine relative to the \( L_p \) during atropine perfusion alone; \( N₅(S+P) \), superfusate plus perfusate hypoxia-atropine present; \( N₅(S+P)/A \), fractional change in \( L_p \) during superfusate and perfusate hypoxia plus atropine relative to the \( L_p \) during atropine perfusion alone; AC, arteriolar capillary; VC, venular capillary; TC, true capillary. Values in parentheses are mean±SEM for fractional changes.
supply a stimulus culminating in an elevation of \( L_p \). The method used by Smaje to measure \( L_p \) assumes a constant capillary hydrostatic pressure during the period of measurement and an albumin reflection coefficient (\( \sigma \)) equal to one. The possibility remains that alterations in these variables interfered with the sensitivity of the \( L_p \) measurement.\(^{11}\) Alternatively, the lack of sensitivity in Smaje’s study could reflect a difference between mammalian and amphibian microvasculature. ACh-induced increases in macromolecule permeability, as measured by protein or dye extravasation, have been reported for mammalian cerebral microvasculature\(^{18,19}\) and gut mucosa.\(^{20,21}\) These findings argue against ACh sensitivity being limited to an amphibian model.

The ability of atropine to antagonize the rise in \( L_p \) in response to ACh indicates the involvement of muscarinic receptors. Although location of these receptors cannot be ascertained from this study, several possibilities exist. Cholinergic muscarinic receptors have been characterized on large vessel\(^{22,23}\) and microvesSEL\(^{24,25}\) endothelium. A functional role for these receptors in the release of endothelium-derived relaxing factor during ACh-induced vasodilation is well documented.\(^{26}\) Recently Oda et al.\(^{20}\) demonstrated ACh-induced cytoskeletal contraction in cultured microvessel endothelium. Busse et al.\(^{27}\) have reported a rise in intracellular free calcium in isolated aortic endothelial cells exposed to ACh. For both of these studies, muscarinic receptor antagonists were shown to inhibit the ACh-induced responses. Thus, one explanation for the increase in \( L_p \) observed in the present study is an ACh-induced elevation in cytosolic calcium leading to contraction of adjacent endothelial cells. The opening of para-cellular pathways via calcium-mediated endothelial contraction has been proposed as a mechanism for regulating capillary permeability.\(^{28,29}\)

As opposed to a direct action on endothelial cells, ACh may have stimulated receptors located on cells lying outside of the microvascular wall. Isolated rat

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Bar graphs showing atropine reversal of capillary response to tissue hypoxia. Bars represent hydraulic conductivity (\( L_p \), mean±SEM) for individual vessels. \( L_p \) was determined during normoxia (open bars), 10–15-min exposure to deoxygenated superfusate (hatched bars), and a 20-min perfusion with 10 \( \mu \)M atropine (solid bars) in addition to tissue hypoxia.
observed, and capillaries are reported to change shape after mesenteric nerve stimulation.

A role for muscarinic receptor stimulation during tissue hypoxia was supported by the observed increase in tissue perfusate deoxygenation, indicating a dissimilarity in response mechanisms of perfusate versus superfusate hypoxia. In our previous studies, the combination of superfusate and perfusate hypoxia resulted in an average 10-fold elevation in \( L_p \). The fractional increase reported here for atropine-treated vessels is fivefold, which is more comparable with our previous estimates for superfusate hypoxia alone. These observations, taken together, suggest that atropine specifically blocked the response to superfusate hypoxia while leaving the perfusate effect on permeability intact.

A reduction in luminal \( O_2 \) during superfusate hypoxia could have a direct effect on the microvascular endothelium. For superfusate hypoxia, a more likely possibility would be the activation of a mechanism originating in the extracellular tissue, because low oxygen superfusion can have a significant effect on tissue \( O_2 \) tension even during adequate perfusion. Hypoxia-induced release of ACh from nerve terminals as a mechanism for elevating \( L_p \) would be consistent with our results. Another possibility would be stimulation of an atropine-sensitive neuronal reflex. Antidromic stimulation of primary sensory fibers (C) fibers leads to vasoconstriction and increased protein leakage in mammalian skin and other peripheral tissues. The neurotransmitter believed to mediate these responses is substance P. With respect to the present study, it is noteworthy that atropine reduces plasma extravasation after either topical application of substance P or antidromic stimulation of sensory fibers, thus implicating a cholinergic component in this afferent reflex pathway. Visceral sensory fibers can be stimulated by hypoxia, however, the participation of oxygen-sensitive afferents in neurogenic plasma extravasation has not been examined. Release of either substance P or ACh during superfusate hypoxia could elevate capillary water conductivity by any of the mechanisms discussed earlier. We have obtained preliminary evidence that \( L_p \) elevation during superfusate hypoxia can be inhibited by verapamil or by the removal of extracellular \( Ca^{2+} \). These findings are consistent with the idea for a central role for \( Ca^{2+} \) in the regulation of capillary permeability.

In summary, the results of the present study support the existence of a permeability-regulating mechanism sensitive to tissue oxygen tension. We have shown that mesenteric exchange microvessels respond to cholinergic stimuli and that superfusate hypoxia stimulates a cholinergic-mediated increase in capillary water conductivity. Speculations were made concerning possible mechanisms including the potential role of tissue-located nerves in mediating the response to superfusate hypoxia. In addition to vaso-dilation, changes in capillary permeability may be an important compensatory mechanism during low oxygen states. Potentiation of transvascular water movement increases convective transport of molecules and dilutes solutes (e.g., metabolites and vasoactive compounds) in the interstitial space. These events may function as compensatory mechanisms during ischemia by forming an ‘alternative circulation’ for nutrient delivery and metabolite clearance.

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