Correction

PHYSIOLOGY, BIOPHYSICS AND COMPUTATIONAL BIOLOGY


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Direct visualization of the arterial wall water permeability barrier using CARS microscopy

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The artery wall is equipped with a water permeation barrier that allows blood to flow at high pressure without significant water leak. The precise location of this barrier is unknown despite its importance in vascular function and its contribution to many vascular complications when it is compromised. Herein we map the water permeability in intact arteries, using coherent anti-Stokes Raman scattering (CARS) microscopy and isotopic perfusion experiments. Generation of the CARS signal is optimized for water imaging with broadband excitation. We identify the water permeation barrier as the endothelial basolateral membrane and show that the apical membrane is highly permeable. This is confirmed by the distribution of the AQP1 water channel within endothelial membranes. These results indicate that arterial pressure equilibrates within the endothelium and is transmitted to the supporting basement membrane and internal elastic lamina macromolecules with minimal deformation of the sensitive endothelial cell. Disruption of this pressure transmission could contribute to endothelial cell dysfunction in various pathologies.

Significance

Low water permeability is critical to the pressurized blood conduit function of the artery. Arterial wall permeability is altered in diseases including diabetes and atherosclerosis, in acute shock, and with the aging process. Here we directly map the water permeability and the associated pressure profile across intact pressurized arteries with coherent anti-Stokes Raman scattering microscopy and D2O tracer experiments. We identify the endothelial basolateral membrane as the major barrier to water permeation. The advantageously positioned water barrier permits the direct transfer of arterial pressure to subendothelial macromolecules. The mechanically sensitive endothelial cell is thus protected from static or pulsed-pressure–induced deformation. Disruption of this pressure transmission could contribute to endothelial dysfunction in various disease states.


The authors declare no conflict of interest.

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CARS signal in context with the cellular structures of the biological sample (15, 16). For the remainder of this study all CARS images were generated with fs pulses.

Water/D\textsubscript{2}O Imaging. Intact pressurized rat mesenteric arteries were imaged in vitro with the multimodal microscope (SI Materials and Methods, Pressure Myography and ref. 17). CARS images at the 3,205 cm\textsuperscript{-1} O-H stretch resonance in arteries immersed in water are shown in Fig. 1 together with the macromolecules of the arterial wall. The entire artery cross-section presented in Fig. 1A shows a strong degradation of all of the optical signals near the equatorial plane of the artery (horizontal midplane). Tissue thickness traversed by the excitation lasers is highest at this plane. A z stack of the top of the artery is presented in Movie S1. These spatially varying optical artifacts are caused by tissue-induced aberrations and scattering that degrade the excitation point spread function (PSF). Although this degradation occurs for two-photon excitation fluorescence (TPEF) and sum-frequency generation (SFG) signals, it is particularly apparent for bulk water CARS imaging. CARS imaging additionally suffers from coherent image artifacts resulting from the interference of the resonant and nonresonant signals (18–21). The CARS PSF may be further degraded by tissue dispersion between the significantly different pump and Stokes wavelengths. The CARS signal distortion at the top of the artery is minimal compared with that at the equatorial plane. Imaging across the wall at the top of the artery suffers, however, from the poor optical axial resolution of the microscope. Thus, for imaging curved structures such as a pressurized artery there is a tradeoff between the transmural optical resolution and the signal degradation. For quantitative analysis we selected a plane located at 30° from the equatorial plane (Fig. 1A, Top) that provided a good tradeoff between the signal-to-noise ratio (SNR) and the transmural optical resolution. For reference an electron micrograph (EM) of a rat mesenteric artery is presented in Fig. 1B.

En face artery images recorded during perfusion with H\textsubscript{2}O and D\textsubscript{2}O physiological salt solutions (PSS) (H\textsubscript{2}O-PSS and D\textsubscript{2}O-PSS) are presented in Fig. 1 C and D, respectively. Perfusion with D\textsubscript{2}O-PSS results in a large reduction of the CARS signal in the lumen. D\textsubscript{2}O penetrates the arterial wall while leaving the CARS signal in the bath unaffected (Fig. 1 D and E and Movie S2). The lumen and bath can be considered infinite pools of O-D and O-H bonds, respectively, because their solutions are continuously replaced while imaging. This allows the recording of concentration profiles at steady state for long periods of time (>1 min) to improve the SNR. The relative permeability of the wall layers can in principle be extracted from these images. D\textsubscript{2}O-PSS in the lumen did not have any adverse effects on arterial cells. Arteries perfused with D\textsubscript{2}O-PSS and the dead cell stain Sytox blue (Thermofisher Scientific) did not exhibit any labeling after 30 min. However, lumen D\textsubscript{2}O-PSS resulted in a small thinning of the internal elastic lamina (IEL) and smooth muscle cell layer (SM), as shown in Figs. 1E and 2 A and B, that was more pronounced with decreasing luminal pressure. This phenomenon can be observed dynamically in Movies S2 and S3. Swapping the lumen and bath solutions caused expansion of the IEL-SM region (Fig. S2). We suggest that the change in wall thickness with D\textsubscript{2}O-PSS is due to a slightly lower permeability of the hydro-seal to D\textsubscript{2}O than to H\textsubscript{2}O. This permeability difference is sufficient to have a net volume flow entering the artery and is likely due to the ∼20% difference in their self-diffusion coefficients (22). Similar differences in permeability to H\textsubscript{2}O and D\textsubscript{2}O...
have been reported across water channels such as AQP1 (23). It is the increased volumetric flow rate through the exit pipette and water column tubing that increases luminal pressure. We refer to the change in luminal pressure upon D_2O perfusion as an osmotic pressure $P_{\text{osmo}}$ because it is associated with a concentration gradient. We confirmed that D_2O induces an osmotic pressure by observing a rise in luminal pressure and artery diameter in rabbit internal carotid arteries filled with D_2O and immersed in water (SI Materials and Methods, D_2O-Induced Osmotic Pressure in Arteries) (Fig. S3 and Movie S4). We estimated $P_{\text{osmo}}$ by calibrating the thinning of the vascular wall at different luminal pressures (Fig. 2 C–G). Fitting the data in Fig. 2G with Eq. S6 we estimate that $P_{\text{osmo}} = 12.5 \pm 0.8$ mmHg (SEM) (Table S2). This is consistent with the water selective hydro-seal residing at the luminal surface of the IEL.

Physical disruption of the EC is known to increase the artery wall water permeability (1). Together with our studies demonstrating IEL thinning with D_2O-PSs perfusion, these results suggest that the EC and/or basement membrane contain elements of the hydro-seal as they are the only known structures between the IEL and vascular space. To resolve the EC membranes, typically ~0.25 μm apart, we imaged EC across their nuclei where the ~1-μm distance separating the membranes (Fig. 1B) is well resolved by our system (transverse optical resolution FWHM = 0.3 μm). The raw O-H CARS signal with H_2O-PSs, shown in Fig. 3d, defines the optical artifacts induced by the sample. The normalized CARS signal $I_{\text{CARS}}$ and OH bond mol fraction $x_{\text{OH}}$ images (Materials and Methods) are shown in Fig. 3 B and C, respectively, for luminal D_2O-PSs. Profiles along the dashed line in Fig. 3A are shown in Fig. 3F. Note that the optical artifact correction using the H_2O-PS signal was small over the nucleus and IEL, but became larger farther away from the lumen. Right-sided paired sample t tests yielded $P = 2 \times 10^{-3}$ for both $I_{\text{CARS}}$ and $x_{\text{OH}}$, respectively, and show that the results are significant. These results are consistent with the endothelial basolateral membrane (EBM) or basement membrane being the major component of the artery wall hydro-seal.

**Endothelium Disruption with Digitonin.** To evaluate the composition of the hydro-seal, we aimed to selectively remove the intima lipid material with digitonin perfusion (SI Materials and Methods, Disruption of the Endothelium). Digitonin did not alter the IEL structurally but removed the ECs as well as, surprisingly, the basement membrane of the artery wall (EM in Fig. 3E). We measured the permeability $L_p$ in control and after digitonin treatment (1) (SI Materials and Methods, Permeability Measurements) to be $4.5 \pm 1.1 \text{cm} \cdot \text{s}^{-1} \cdot \text{cm} \cdot \text{H}_2\text{O}^{-1}$ and $28.7 \pm 2.3 \times 10^{-3} \text{cm} \cdot \text{s}^{-1} \cdot \text{cm} \cdot \text{H}_2\text{O}^{-1}$ (SEM), respectively (n = 5, paired t test, $P = 0.001$). The results are reported in Table S3 and demonstrate an increase in permeability by a factor of $8.1 \pm 1.8$ SEM after digitonin treatment. Additionally, digitonin completely removed the wall-thinning effect during D_2O-PSs perfusion. Movie S3B shows the D_2O-PSs transition. After digitonin treatment the $x_{\text{OH}}$ image, shown with its profile along the dashed line in Fig. 3 D and G, respectively, exhibits a dramatically reduced gradient across the cell compared with the control experiment, Fig. 3 C and F, respectively. The maximum amplitude of the profile derivatives in $I_{\text{CARS}}$ and $x_{\text{OH}}$ in Fig. 3G was reduced by nearly 60% compared with the control experiment (Table S4). Gray-scale images in Fig. 3 C and D range from 0 (black) to 1.2 (white). Note the reduced water concentration outside the artery after digitonin treatment compared with that in the control experiment. This is further evidence of the increased wall permeability. These data are consistent with digitonin-soluble lipids playing a major role in the hydro-seal and with the IEL weakly contributing to the overall hydraulic resistance. Given the hydrophilic protein structure of the basement membrane (24, 25), it is reasonable to conclude that the EBM is the major hydro-seal of the arterial wall.

**AQP1 Immunohistochemistry.** We hypothesized that the distribution of the AQP1 water channel protein, previously identified in arterial ECs (26), would be higher in the permeable EAM than in the impermeable EBM. This distribution of AQP1 would also explain why inhibition of AQP1 does not affect water permeation under physiological pressures (14) because it is not involved in the rate-limiting step for water permeation at the EBM. We performed immunohistochemistry of AQP1 in arteries fixed under pressure (SI Materials and Methods, AQP1 Immunohistochemistry and Imaging). Fluorescence images were collected using a stimulated emission depletion (STED) microscope to provide in-plane resolution of at least 100 nm, adequate to differentiate the EAM and EBM. A representative example from these studies is presented in Fig. 4, where the AQP1 fluorescence antibody was found to intensely and selectively label the EAM. The EAM to EBM AQP1 signal ratio measured in three animals was $6.9 \pm 1.4$ (SEM) (9.7, 4.8, 6.3) in the raw STED images and $25 \pm 3.8$ (SEM) ($n = 18, 25, 32$) in the deconvolved STED images. These ratios are significantly higher than unity according to paired right-sided t tests ($P = 0.0027$ and 0.012 for the raw and deconvolved images, respectively).

These data are in agreement with the results of the CARS experiments and are consistent with (i) AQP1 generating the high EAM permeability and (ii) the low permeability of the hydro-seal–forming EBM resulting from the lack of AQP1. We note that factors such as membrane composition could play a role in generating the difference in permeability at the EAM and EBM. Such differences are unknown and would be difficult to determine. Total endothelial plasma membranes are primarily phosphatidylcholine (27) with cholesterol (28) that could generate very low water permeability (29) in the EBM.
Discussion

Because water transport via diffusion and filtration is likely dominated by the same barrier, we conclude that the observed water concentration gradients are directly related to the physical pressure gradients across the arterial wall. Thus, these water/D$_2$O CARS images reflect the location of pressure gradients across the artery wall at subcellular resolution. Consequently, the high permeability of the EAM coupled to the low permeability of the EBM results in a very efficient transfer of the arterial pressure to the immediately adjacent elastin and collagen macromolecule infrastructure without generating stress on the mechanically sensitive EC (30, 31). It is important to note that the low water compressibility [$6 \times 10^{-4}$% volume change/100 mmHg (32)] results in very little water movement to equilibrate the arterial pressure with the endothelium cytosol. Again, this minimizes the impact of arterial pressure waves on the endothelium.

Herein direct evidence is provided that the hydro-seal of the arterial wall is located at the EBM. This conclusion is primarily based on the observation of steep gradients in [H$_2$O] profiles at the EBM, using optimized water CARS imaging in pressurized arteries. The specific localization of AQP1 to the EAM likely contributes to the observed high water permeability of this membrane. Imaging pressure gradients on the subcellular scale is extremely problematic. Because the pressure gradients across the arterial wall would predictably follow the resistance to water permeation, it is proposed that water permeability imaging is a surrogate for pressure gradient localization. Using this rationale, we argue that the EBM is the location of the largest pressure gradient across the arterial wall. By placing the hydro-seal at the EBM the arterial pressure is efficiently transferred to the adjacent elastin and collagen infrastructure without generating volume changes or stress on the mechanically sensitive EC. It is likely that this distribution of water permeability in the arterial wall will play an important role in many disease states as well as different therapeutic approaches. It has recently been reported that AQP1 plays a protective role against hypertension-augmented atherosclerosis in mice (33). This observation supports our hypothesis that AQP1 plays a role in reducing pressure-related strain on the endothelial cells.

Materials and Methods

Multimodal Nonlinear Optical Imaging. CARS microscopy allows vibrational imaging of molecular bonds with subcellular 3D resolution (34–36). For CARS microscopy in biological samples the target resonance bandwidth dictates the excitation pulse width required for a good compromise between chemical...
specificity and signal intensity (37). Thus, CARS microscopy is usually conducted with ps pulses because their spectral bandwidth matches the typical 15 cm⁻¹ resonance bandwidth of many metabolites in biological tissues (18, 38, 39). However, fs pulses provide a better match with the broad Raman resonance of water. Water and D₂O CARS imaging has previously been performed in simple systems, using either ps (34, 40, 41) or fs (42) pulses, although no direct comparison has been presented. We tested both excitation schemes for water imaging and compared their performances.

The coherent pump, probe, and Stokes beams required for CARS microscopy are derived from a dual beam mode-locked ultrafast laser (InSight DeepSee; Spectra-Physics). The degenerate pump-probe beam is tuned to 780 nm such that the 3.205 cm⁻¹ Raman shift with the Stokes beam at 1.040 nm is located at the O-H stretch vibration band and gives the highest CARS contrast between water and D₂O (42). The two beams are collinearly polarized and are combined via a custom dichroic mirror with a cutoff wavelength at 1.020 nm and coupled to a laser scanning upright microscope (SP8; Leica Microsystems). The delay between the two pulses is adjusted with a delay stage to maximize the water CARS signal. The beams are focused by a 25x water immersion, 1.0 numerical aperture (N.A.) objective (PL-IRAPO 25x/1.0 motCorr; Leica Microsystems). A critical feature of this objective for CARS imaging is its chromatic aberration correction between 700 nm and 1,300 nm that allows parfocality between the pump and Stokes wavelengths. The motorized collar allows the plane of focus to translate and maintain the water CARS signal at the center slice of the imaged volume. Epi-detection is used to collect the emitted light. A cold mirror (Newport) was placed underneath the perfusion chamber to reflect some of the CARS signal, which is forward generated in bulk medium (39), back to the objective. The emitted light is separated from the excitation light by a primary dichroic mirror (FF735-Di01; Semrock) and directed to four nondescanned detectors where a second dichroic mirror (Semrock) splits the light between a pair of water CARS detectors (HyD) and a pair of photomultiplier tubes (PMT) (Leica Microsystems). The light directed to the two HyDs is filtered by two IR blocking filters (FF01–72020SP and FF01–7905SP; Semrock), split by a dichroic mirror (FF605-DI02; Semrock) that separates the narrow-band water CARS signal at 624 nm from the broad elastic autofluorescence signal, and then detected after bandpass filtering (FF01–620/14 and FF01–571/72; Semrock). The light directed to the two PMTs is filtered by a hybrid filter (FF605-DI02; Semrock) and split by a dichroic mirror (FF484-FD01; Semrock) and the short-wavelength component is detected with a bandpass filter (FF01–492SP; Semrock) when imaging nuclei stained with Syto 40. This filter was replaced by a narrowband filter (FF01–448/20; Semrock) to specifically image the 446-nm pump-Stokes sum-frequency mixing signal generated at collagen fibers. In the fs excitation scheme the pump and Stokes beams have pulse widths of 110 fs and 280 fs, respectively. The dispersion occurring in the optical path of the pump-probe beam is precompensated in the Insight DeepSee and is therefore transform limited (TL) at the focal plane. The dispersion in the Stokes beam is not compensated for and its pulse width is therefore expected to be slightly broadened at the sample (~300 fs). The CARS spectral resolution, determined by the FWHM of the power spectrum of the optical dispenser (35), is estimated to be ~40 cm⁻¹ for the fs excitation scheme. The optical setup can also be operated in ps excitation mode, using spectral focusing (43, 44). Spectral focusing consists of chirping the pump and Stokes pulses to increase the CARS spectral resolution while maintaining the instantaneous frequency difference (IFD) between the two chirped pulses constant. For identical chirped-pump and Stokes pulses the CARS spectral resolution increases linearly with the pulse stretching factor a = τa/τp, the ratio of the FWHM of the chirped-pulse intensity to that of the TL pulse τp. Pulse compressors using two transmission holographic gratings (Norton Corporation) introduce excess negative group delay dispersion (GDD) to stretch the pump and Stokes pulses to 3 ps and 1.3 ps, respectively (45). The CARS spectral resolution of the ps excitation scheme is ~12 cm⁻¹.

Arterial Wall Macromolecular Structures Imaging. For the acquisition of images shown in Fig. 1, elastin was labeled with Alexa fluor 488 Hydrazide (17) (ThermoFisher Scientific), with scanning rate 700 lines per minute; pixel sizes 99 nm (xy) and 600 nm (z); and field of view (FoV) 347 μm (x), 51 μm (y), and 323 μm (z). For all other experiments elastin was not labeled and its two-photon excitation autofluorescence was recorded instead.

Water CARS Imaging. With the endothelial nuclei plane identified, the O-H signal was measured across the wall during H₂O-D₂O perfusion with 40 3D-image stacks of the same animal. The FoV of D₂O-PSS was then perfused until a steady state was reached and another series of 3D stacks was collected. Images were acquired at 22 frames per second with a line-scan rate of 16 kHz, a pixel size of 96 nm (xy) and 420 nm (z), and a FoV of 60 μm (xy) and 25 μm (z). Image analysis. The water and D₂O images were first registered separately, using the nucleus channel. A phase correlation registration algorithm (46) was implemented in MATLAB to estimate the 3D shifts and redundant registration (47) was then used to improve the robustness of the estimated shifts. The average of the registered water and D₂O images was computed and then registered together. Because of the wall thickness change with luminal D₂O, we chose to register the EC nuclei without trying to register the entire wall. This was done by applying a Tukey window on the EC nuclei to remove the signal originating from the rest of the wall.

Line profiles were measured at four different z planes near the middle nuclei section and at three locations inside the nuclei for each z plane. Line profiles were averaged over 15 consecutive horizontal lines. For each animal (n = 4), all of the profiles were analyzed and these technical replicates were averaged together. The z planes were selected such that the position of the half-maximum of the nucleus signal occurred on average at least 0.5 μm before that of the IEL. This was done to ensure that the two EC membranes were properly resolved in the line profiles. Indeed, if profiles are measured near the top of the nucleus, the IEL appears to occur before the nucleus signal in the line profiles because of the smaller axial optical resolution compared with the transverse resolution. The half-maximum point was chosen because the signal slope there provides the smallest localization error when the thickness of the feature of interest, nucleus or IEL, is not exactly known. For each profile we estimated the spatial separation between the rise in the water CARS signal and the distance as the same between the peaks of the first derivatives of the water CARS and nucleus signals in the intensity profiles. The peak amplitudes of the signal derivatives, denoted gₐₗₜ and gₐₗₜ for Iₑₐₑₚ and Xₑₐₑₚ, respectively, were also recorded. The signal derivatives were calculated as the convolution of the signals with a derivative of Gaussian kernel with a SD of 0.4 μm. The search for the peaks of the derivatives was restricted to locations ranging from the lumen to the end of the IEL. This restriction was imposed because the autocorrelation function of the signal being Fourier filtered in the signal acquisition decreases and the water CARS signal do not reflect water content but are due to optical artifacts. The corrected water signal Xₑₐₑₚ cannot be analyzed beyond the IEL because the 3D translation registration used here cannot account for the wall deformation in the presence of luminal D₂O-PSS.

For presentation purposes, images in Fig. 1A and Movies S1–S3 were filtered in Fiji with the Kalman stack filter plugin. The noise variance and prediction bias parameters of the filter were set to 0.05 and 0.8, respectively.

O-H Bond Mole Fraction Quantification. The raw CARS signal I(r) has a quadratic dependence on the probe concentration (36) and is modulated by a sample induced optical artifacts term A(r). For H₂O-D₂O mixtures we have I(r) = A(r)·[OH(r)]² + B·OH(r) + c, where OH(r) is the O-H bond mole fraction of the solution within the probing volume at pixel location r in the image. The amplitude fluctuation in OH with respect to the background signal from pure H₂O. Its contribution is removed with the normalization Iₑₐₑₚ(r) = I(r)/Iₑₐₑₚ, given by

\[ Iₑₐₑₚ(r) = \frac{[OH(r)]² + B·OH(r) + c}{a + b + c}. \]  

Solving Eq. 1 yields Xₑₐₑₚ. For completeness both Iₑₐₑₚ and Xₑₐₑₚ images were analyzed. The coefficients a, b, and c are obtained from a quadratic fit of the CARS signal recorded in pure D₂O (Fig. S4). Note that the immersion medium must be prevented from mixing with the solution during calibration and experiments when using long working-distance (WD) objectives. This is because small differences between the water and D₂O refractive indexes [0.004 and 0.003 at 780 nm and 1,040 nm, respectively (48)] result in significant spherical aberrations over long WDs (49). These aberrations (i) degrade the pump and Stokes PSF, (ii) induce a significant focal shift between them, and therefore (iii) degrade the CARS signal independently of the probe concentration within the focal volume. We observed these aberrations in the TPEF PSF recorded in pure D₂O. We measured a 2.8-μm axial shift between the pump and Stokes PSF that is in good agreement with that from simulated PSF in D₂O. Parfocality of the pump and Stokes PSF was verified with water immersion. During calibration the immersion medium was separated from the solution by a coverslip. During artery D₂O-PSS perfusion experiments the immersion medium formed by the bath solution was maintained by constantly replacing the bath with H₂O-PSS.
In the above estimation of $\omega_{\text{opt}}$ the optical properties of the tissue are assumed to remain unchanged in $D_2O$ and any motion between $l/D$ and $\omega_{\text{opt}}$ needs to be corrected for, using either the SFG or the TPEF images. This estimation is similar in principle to that in ref. 40 except that here the CAR$S$ signal is fitted with a quadratic function of $\omega_{\text{opt}}$, rather than a power function, in agreement with the theory.

Supporting Information

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SI Materials and Methods

Comparison of fs-CARS and ps-CARS Signals. To assess the suitability of the fs excitation scheme, signals from water and D₂O droplets embedded in oil were recorded for each excitation scheme with matched average power at the sample. The contrast-to-noise ratio (CNR) between the OH and OD CARS signals reported in Table S1 is defined as CNR = (I_{OH} - I_{OD})/σ, where σ = (σ_{OH} + σ_{OD})^{1/2} is the SD of the noise in the CARS signal, and I_{OH} and I_{OD} denote the mean and SD of the CARS signals from molecular bond k, respectively.

We performed a numerical simulation of the CARS signal to evaluate the relative signal intensities to be expected with each excitation scheme and to validate our measurements. We follow the approach taken in ref. 37 and simulate the CARS signals in the time domain. Briefly, the pump–probe and Stokes fields E_p and E_s are described by chirped pulses E_i(t) = 1/√(2π) exp(-(t/τ_i)^2 + jE_i^2 + α(t)), i = (p, s), with temporal pulse width parameter τ_i = τ_i/√2 ln 2, pulse stretching factor α = τ_i/τ_0 = 1 + (β_e/τ_0)^2 (50), temporal chirp coefficient β_e, and angular optical frequency α(t).

Assuming the two pulses have the same temporal chirp coefficient β_e = β, the instantaneous frequency difference between the two pulses is α(2) = α_p - α_s + 2β_0, where τ_0 is the time delay of the Stokes pulse with respect to (wrt) the pump pulse. We simulate the CARS signal for an isotropic Raman impulse response χ(t), which is the sum of a vibrational component χ_v(t) and an instantaneous electronic nonresonant component χ_e(t) = χ_e(0) δ(t).

We consider the case where χ_v(t) consists of a single vibrational component with polarizability μ, angular resonance frequency α, and exponential dephasing time T_2, so that χ_v(t) = -jμ(0) exp[α(t) - j(t/T_2)], with θ(t) the Heaviside step function. The induced Raman polarization is described by Q(t) = χ_v(0) F(t), where F(t) is the convolution operator, F(t) = E_p(t) E_s(t) is the optical driving force, and * indicates complex conjugation. Thus, Q(t) is also the sum of a resonant component Q_v(t) = χ_v(0) F(t) and a nonresonant component Q_e(t) = χ_e(0) F(t)/ α^2 + β^2. Finally, the CARS signal intensity SCARS is obtained by probing the Raman polarization Q(t) with the probe field E_p and spectral selection of the anti-Stokes field, so that after time integration it is written as SCARS = ∫ |Q(t)| E_s(t) dt = ∫ |Q_v(t)| E_s(t) dt + ∫ |Q_e(t)| E_s(t) dt = SC_v + SC_e + SC_{νν}. Hence for a single vibrational component the CARS signal is the sum of purely resonant component SC_v, a purely nonresonant component SC_e, and a heterodyne term SC_{νν} resulting from the interference of the resonant and nonresonant anti-Stokes fields components (36):

\[ SC_v = \int |Q_v(t)| E_s(t) \, dt \] [S1]

\[ SC_e = \chi_e(0) \int |E_s(t)|^2 |E_v(t)|^2 \, dt \] [S2]

\[ SC_{νν} = 2\chi_e(0) \int |E_s(t)|^2 Re \{Q_v(t) E_p(t) E_s(t)\} \, dt \] [S3]

The Raman spectrum of water is complex and exhibits multiple bands with both homogenous and inhomogeneous broadening components (51,52). An accurate modeling of the water CARS signal is beyond the scope of this paper but for illustrative purpose we simulated the CARS signal for a single resonance at α = 3,300 cm⁻¹, with a purely exponential dephasing time of T_2 = 60 fs and μ/χ_v = 1. Because of the dispersive nature of the term SC_v, the maximum CARS signal is reached at a frequency α(2) that is red shifted wrt α_v.

Fig. S1 shows the CARS spectrum and its components for TL pulses, with τ_0 = 110 fs and τ_0 = 280 fs, and for chirped pulses, τ_p = 3 ps and τ_s = 1.3 ps. All spectra are normalized by the purely nonresonant component SC_v(TL) obtained with TL pulses. The total CARS signal with TL pulses SC_v(TL) is two orders of magnitude higher than that obtained with chirped pulses. SC_νν. Spectral focusing is also illustrated in Fig. S1 where the resonant component SC_v(TL) with TL pulses (FWHM = 307 cm⁻¹) is broadened compared with the Lorentzian Raman line width (1/πτ_sc = 177 cm⁻¹), whereas SC_v obtained with chirped pulses is identical to the Raman line width. Note that the unit μ/χ_v ratio was set to provide a ratio SC_v(TL)/SC_v(TL) = 22 that is close to the measured water to D₂O CARS ratio of 24 in pure solutions. We record the maximum value of SC_v(TL) and calculate the ratio R_{νν} = SC_v(TL)/SC_v(TL), between the total CARS signals with the TL and chirped pulses. The ratio R_{νν} = SC_v(TL)/SC_v(TL) is similar to the purely nonresonant signals with both excitation schemes is also informative when imaging water as it represents R_{νν} in the limit of infinitely short dephasing times. We calculate that R_{νν} = 137 and R_{νν} = 233 for the pulse parameters and Raman resonance parameters described above. When T_2 is reduced to 45 fs, corresponding to the full 235 cm⁻¹ bandwidth of water (51), R_{νν} is increased to 165 (R_{νν} unchanged). Turning our attention to the specificity, defined by the purely resonant to purely nonresonant signal ratio R_{νν} = SC_v/SC_e, we calculate that R_{νν} drops only by a factor of 1.8 when using TL pulses compared with the chirped pulses used in the fs excitation scheme. These variations in signal intensity and specificity are in agreement with those predicted by equations 8 and 9 in ref. 37 after replacing the differing pump and Stokes pulse widths by pulses with an identical width τ_0 = √2πτ_νν/√(τ_p^2 + τ_s^2), giving the same CARS spectral resolution.

We note that the water to D₂O signal ratio R = 8.4 reported in Table S1 is higher than its value of 5.0 obtained from the calibration data shown in Fig. S4. These calibration data are used for the calculation of the O–H bond mole fraction. The higher ratio measured here is due to different geometries and optical paths between the H₂O and D₂O droplets. Whereas this does not allow calibrating R, the signals comparison with fs and ps excitations remains valid.

Thus, we conclude that the CARS measurements reported in Table S1 are compatible with the simplified water CARS model presented here. We also conclude that for water CARS imaging the benefits of the two orders of magnitude signal increase with the fs excitation scheme far outweigh a possible drop in specificity by a factor of less than 2.

Pressure Myography. Adult male Sprague–Dawley rats (200–330 g) were euthanized by CO₂ exposure from a compressed source (cylinder or house supply) into a closed chamber. A second assurance of death was made by a thoracotomy to create a pneumothorax. A midline abdominal incision was made through the peritoneum and the mesentery was removed from the animal and placed in ice-cold Mops-buffered solution (composition below). A section of the mesenteric arcade was pinned out and third-order arteries were dissected without side branches. Arteries were cannulated onto heat-polished micropipettes (outer diameter ~250 μm) in a 2-mL chamber (RC-27; Warner Instruments) mounted in a temperature-regulating heating platform (PH-6;
Warner Instruments). After warming to 37 °C, the artery was pressurized to physiological pressure (70 mmHg) using a gravity-fed pressure tower and the artery was straightened. Only arteries that contracted to the α1-adrenergic agonist, phenylephrine (PE; 3 μM, Sigma) and fully dilated to the M3-muscarinic receptor agonist, acetylcholine (Ach; 1 μM, Sigma) were used for experiments. Drugs were added to the superfusion under continuous flow as the microscope setup prevented direct access to the bath.

**Artery perfusion for water/D2O imaging.** In one set of experiments, D2O-O-PSS was perfused at 50 μL/min using a gastight glass syringe (Hamilton Robotics) mounted in a Beehive syringe pump (BASI), during continuous superfusion of H2O-O-PSS at 3 mL/min. In a second set of experiments the two solutions were swapped.

**Physiological salt solutions composition.** Mops-buffered solution, referred to as H2O-O-PSS, consisted of 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl2, 1.17 mM MgSO4, 2.0 mM Mops, 1.2 mM Na2HPO4 or NaH2PO4, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, and 2.75 mM NaOH (pH 7.40 ± 0.02 at 37 °C). The same solution was prepared in D2O instead of water, with pH adjusted to pH 6.99 ± 0.02 (53), referred to as D2O-PSS.

**Disruption of the Endothelium.** In a subset of experiments, the endothelium was disrupted by perfusion of a 0.01% digitonin suspension (D5626-5G; Sigma) at low pressure at 50 μL/min for 5 min, followed by normal H2O-O-PSS for 5 min before repropressurizing. The water concentration profile was measured both before (control) and after exposure to digitonin.

**Permeability Measurements.** Starling’s law relates the volumetric flow rate Jv across the artery wall to the pressure drop P across the wall by Jv = Lp * S * P, where Lp and S are the wall hydraulic conductivity and surface area, respectively. In these experiments the osmotic pressure difference between the lumen and bath is zero because H2O-O-PSS is present in both compartments. We measured the rat mesenteric artery dimensions (using the inner diameter) and volumetric flow rate before and after digitonin treatment at P = 70 mmHg physiological pressure in n = 5 biological replicates. The results are reported in Table S3.

**Electron Microscopy.** Arteries were cannulated and pressurized in H2O-O-PSS as above and imaged using an Olympus FV1200 (Olympus) microscope. The smooth muscle and endothelial visibility was tested using 1-3 μM PE followed by 0.1–1.0 μM Ach, respectively, added directly to a static bath. At 70 mmHg and while heating to 37 °C, arteries were rinsed in a rinse buffer containing 0.1 M sodium cacodylate + 2 mM CaCl2 and then fixed while cannulated for 1 h at 70 mmHg while heating to 37 °C [fixative composition: 1% paraformaldehyde (Electron Microscopy Services), 3% glutaldehyde (TAAB Laboratories Equipment Ltd.), 0.1 M sodium cacodylate, 2 mM CaCl2]. After washing in the rinse buffer three times for 5 min, the artery was removed from the cannulating pipettes, transferred to a vial of Fixative, and agitated on a belly dancer for 1 h at room temperature, before being transferred to a cold room for continued overnight fixation. Arteries were processed for transmission electron microscopy (TEM) processing.

Arteries treated with digitonin were prepared as above, then perfused with 0.01% digitonin for 5 min, rinsed with H2O-O-PSS for 5 min, and then washed with rinse buffer before fixing as above for TEM processing.

**AOPI Immunohistochemistry and Imaging.** IHC was performed on intact pressurized arteries that were sectioned into radial slices for imaging on a STEM microscope. The protocol was performed on n = 3 biological replicates.

**Sample fixation protocol.** Arteries were cannulated, heated to 37 °C, and gradually pressurized to 70 mmHg in H2O-O-PSS. After equilibration at high pressure, arteries were fixed while pressurized at ~37 °C in 2% paraformaldehyde for 10 min, then at ~34 °C in 95% ethanol for 10 min, and then in 100% ethanol for a 5-min period and two 10-min periods. The artery was removed from the cannulating pipettes, manually dipped in 100% ethanol to ensure the lumen was fully dehydrated, and then stored in fresh 100% ethanol. Sample 1 was stored overnight at room temperature in 100% ethanol and then stored at 4 °C before paraffin embedding on the following day (IHC protocol below). Samples 2 and 3 were stored in 100% ethanol at 4 °C before paraffin embedding on the same day.

**IHC protocol.** The sample was dipped twice in 100% Xylene for 10 min at 37 °C before paraffin embedding for 15 min at 65 °C and then in a second paraffin solution for 20 min at 65 °C. Paraffin-embedded sections were dewaxed at 65 °C, and antigen retrieval was performed for 10 min in citrate buffer at pH 6.0, followed by blocking with 10% donkey serum for 20 min at room temperature (RT) before immunostaining. Primary polyclonal rabbit IgG antibodies (20333-1-AP; Protein Tech) were incubated overnight at 4 °C and washed for 10 min, followed by secondary F(ab')2 antibody (donkey anti-rabbit IgG conjugated with Alexa Fluor 594; 711-586-152; Jackson Immuno Research) staining at 1/200 dilution for 90 min at RT. SIR-DNA (Cytoskeleton Inc.) was used to label nuclei (54) at 1:500 dilution for 15 min at RT. The samples were mounted on a slide with Prolong Gold for 24 h and imaged with a 1.5 coverslip. Control experiments in which the primary antibody was either omitted or substituted by nonimmune rabbit IgG were also performed. Kidney sections positive for AQP1 were used for optimizing the immunolabeling protocol.

**Confocal and STED microscopy.** To assess further AQP1 localization at the nanoscale level, superresolution microscopy was performed using the STED methodology. Time-gated STEM images were obtained using a commercial STEM microscope (S800 STEM 3X; Leica Microsystems), equipped with a white-light laser and a pulsed 775-nm STEM depletion laser. A 100×/1.4-N.A. oil immersion objective lens (HCX PL APO STEM white; Leica Microsystems) was used for imaging. For resolution comparison, confocal and STEM images were taken sequentially for AQP1 labeled with Alexa 594 and imaged using 560-nm excitation, a scan speed of 600 lines per second, a 570- to 630-nm emission detection range with gated hybrid detectors, and the 775-nm STEM depletion laser. The pixel size is 25–25 nm (1,024 x 1,024 pixels) and six line averages were performed. Confocal images were collected for the auto-fluorescence signal of the vessel wall with 488-nm excitation (490–540-nm emission range) and for the labeled nuclei (SIR-DNA) via 647-nm excitation and 650–740-nm emission. Z stacks were collected at 0.160-μm depth intervals; images were deconvolved using the classical maximum-likelihood estimation algorithm in Huygens Professional software version 15.10.1 (SVI) and examined and reconstructed using Imaris software version 7.7.2 (Bitplane).

**Quantification of AQP1 signal.** To quantify the relative distribution of AQP1 at the EC membranes the ratio of the apical to the basolateral AQP1 signals was measured in the raw and deconvolved STEM images. For each biological replicate three line profiles across the EC nucleus were traced and the signals at the EAM and EBM recorded. The EAM to EBM signal ratios of the technical replicates were averaged together and are reported for each animal in the main text. The location of the EAM was determined by the AQP1 peak on the luminal side of the EC nuclear signal. The distance between the apical AQP1 peak and the EC nuclear membrane signal was also measured and was on the order of 210 nm. The EAM was estimated to be at the same distance away from the opposite nuclear membrane. We note that the AQP1 signal decays rapidly after the EAM and is essentially at the background level inside the nucleus and farther in the wall (Fig. 4A, Inset). Thus, small shifts in the estimation of the EBM position do not significantly impact the EAM to EBM signal ratio.
Comparison of Diffusional and Hydrostatic Pressure Driving Forces. In these isotopic exchange experiments it is important to understand the various driving forces at play. Water transport can occur via diffusion, driven purely by water and D\textsubscript{2}O concentration gradients, and via filtration, driven by hydrostatic pressure gradients. These processes are quantitatively closely related (55). With pure opposing D\textsubscript{2}O and H\textsubscript{2}O compartments, the diffusion driving force is five orders of magnitude higher than the hydrostatic pressure driving force at 70 mmHg (calculation below). The difference in permeability of the hydro-seal to water and D\textsubscript{2}O suggests a very tight interaction of the water molecules with the hydro-seal that can therefore be of two types: a lipid membrane or a porous membrane with pore sizes on the order of the size of a water molecule. In either case the diffuse and filtration permeability coefficients P\textsubscript{d} and P\textsubscript{r}, respectively, have the same order of magnitude (55) and therefore diffusion is the dominant driving force in these studies. Consistent with this notion, we observed that the water gradient across the wall did not change when the luminal pressure was reduced by 50%.

To compare the water transport driving forces involved during D\textsubscript{2}O perfusion the diffusional component ΔG\textsubscript{d} and filtration component ΔG\textsubscript{f} of the free energy are estimated as (55)

\begin{equation}
ΔG_d = \bar{V} \Delta P
\end{equation}

\begin{equation}
ΔG_f = RT \ln \frac{X_1}{X_2}
\end{equation}

where \(\bar{V} = 18 \text{ cm}^3 \text{ mol}^{-1}\) is the partial molar volume of water, \(\Delta P\) is the hydrostatic pressure difference across the wall, \(R\) is the gas constant, \(T\) is the temperature, and \(X_1\) and \(X_2\) are the O-H bond mole fraction in the lumen and bath, respectively. For \(\Delta P = 70 \text{ mmHg}\), \(ΔG_d = 0.17 \text{ J/mol}\). The purity of the D\textsubscript{2}O solution is \(X_1 = 10^{-3}\) and the natural abundance of D\textsubscript{2}O in water is 0.014% (56) so that \(X_2 = 0.9997\) and at \(T = 298.15\text{ K}\) we have \(ΔG_f = -17 \text{ kJ/mol}\). Thus, we estimate the diffusional driving force to be five orders of magnitude higher than the hydrostatic driving force.

D\textsubscript{2}O-Induced Osmotic Pressure in Arteries. Rat mesenteric arteries. The wall thickness was measured at various luminal pressures during H\textsubscript{2}O-PSS and D\textsubscript{2}O-PSS perfusion to characterize the osmotic pressure induced by luminal D\textsubscript{2}O-PSS. We focused on the wall thickness change with pressure because it plays an important role in the measurement of the water concentration with CARS microscopy. In these experiments, high-resolution 2D images of the arterial wall were recorded at the artery equator plane to minimize vertical motion impact. Images for a given experimental condition were registered together and averaged. The wall thickness was measured as the distance between peaks of the elastin autofluorescence signal and the collagen SFG signal. The luminal pressure was set by adjusting the height of the water column during H\textsubscript{2}O-PSS perfusion and left unchanged during D\textsubscript{2}O-PSS perfusion. Luminal pressures of 17 mmHg, 35 mmHg, 49 mmHg, and 70 mmHg were applied, corresponding to 25%, 50%, 70%, and 100% of the in vivo pressure, respectively. The artery length was set at its near physiological value, as described in SI Materials and Methods, Pressure Myography and was then kept constant. The wall thickness \(T\) was fitted with a quadratic function of the luminal pressure \(P\). We argued that the osmotic pressure induced by luminal D\textsubscript{2}O does not change with luminal pressure. Consequently, we constrained the fitted model to be identical for both the H\textsubscript{2}O-PSS and D\textsubscript{2}O-PSS perfused datasets, except for a shift in luminal pressure \(P_{osmo}\). This was achieved by combining both datasets into a single dataset and using the quadratic model

\[
T = a(P + uP_{osmo})^2 + b(P + uP_{osmo}) + c,
\]

where we introduced the dummy variable \(u\) that takes the values 0 and 1 for the H\textsubscript{2}O-PSS and D\textsubscript{2}O-PSS datasets, respectively. Thus, the least-squares fitted coefficients \(a\), \(b\), and \(c\) are identical for both datasets and only \(P_{osmo}\) differentiates the two models. The nonlinear least-squares fit was solved with the Matlab function fit, using the NonlinearLeastSquares method. The starting point of the fitting algorithm was obtained as follows. First, the coefficients \(a\), \(b\), and \(c\) were estimated by fitting the H\textsubscript{2}O-PSS dataset separately (\(u = 0\)). Then the least-squares solution for \(P_{osmo}\) was calculated for the D\textsubscript{2}O-PSS dataset (\(u = 1\)). Note that because the wall thickness is linearly related to the radial strain \(E = 1 - T/T_0\), where \(T_0\) is the wall thickness at the lowest pressure measured, the quadratic fit above yields the same value of \(P_{osmo}\) for \(T\) and \(E\). The fitted values of \(P_{osmo}\) in different animals can thus be compared.

Rabbit internal carotid artery. Male New Zealand White rabbits (1.53–1.54 kg) were euthanized by intravenous injection of 2 mg/kg potassium chloride while under general anesthesia with isoflurane. Rabbits were injected with 1,000 units of heparin intravenously prior to euthanasia.

Rabbit internal carotid artery segments were dissected, mounted to a cannula as described in ref. 12, and immersed in H\textsubscript{2}O-PSS bath solution. A Hepes PSS solution was used for these experiments only. A pressure column and a pressure transducer (72-4496; Harvard Apparatus) were connected at opposite ends of the artery. The artery and the pressure column were loaded with H\textsubscript{2}O-PSS (control) and then with D\textsubscript{2}O-PSS. For each experiment the artery was disconnected from the pressure column at \(t = 0\) by closing of a three-way valve. The rise in luminal pressure shown in Fig. S3 and the increase in artery diameter shown in Movie S4 demonstrate net volume inflow into the D\textsubscript{2}O-filled artery. This is caused by the lower permeability of the artery wall to D\textsubscript{2}O than to H\textsubscript{2}O. Similar results were obtained in \(n = 3\) biological replicates. Note that Fig. S3 and Movie S4 were recorded in two different arteries. Luminal pressure in the control experiment exhibited a small decay consistent with net volume flow filtering out of the artery.
Simulated CARS spectrum obtained with the fs and ps excitation schemes for a simplified Raman water model. The CARS spectrum and its components for the fs excitation scheme (blue, TL pulses, $\tau_p = 110$ fs, $\tau_s = 280$ fs) are two orders of magnitude higher than those obtained with the ps excitation scheme (red, chirped pulses, $\tau_p = 3$ ps, $\tau_s = 1.3$ ps). A single isotropic resonance ($T_2 = 60$ fs) is used to approximate the Raman water line $I_r$ (black triangles). All spectra are normalized by the purely nonresonant signal $S_{e,TL}$ obtained with the fs excitation scheme.
Fig. S2. Increase in arterial wall thickness during water perfusion with D_2O-PSS in the bath. (A and B) Arterial wall image during H_2O-PSS perfusion with H_2O-PSS bath (A) and D_2O-PSS bath (B). Gray, raw water CARS image; green, elastin TPEF. (Scale bar, 4 μm.)

Fig. S3. Luminal pressure trace of an artery loaded with D_2O-PSS and immersed in H_2O-PSS. The rabbit internal carotid artery was disconnected from the pressure column at t = 0 by closing of a three-way valve and the luminal pressure was monitored with a pressure transducer. The rise in luminal pressure demonstrates net volume inflow caused by the lower permeability of the artery wall to D_2O than to H_2O.
Fig. S4. Calibration of the water CARS signal intensity at 3,205 cm$^{-1}$ vs. water mole fraction $x_{OH}$ in H$_2$O-D$_2$O mixture solutions. The quadratic fit of the data is also shown with its equation.

Table S1. Comparison of O-H and O-D CARS signals in water and D$_2$O with the fs and ps excitation schemes

<table>
<thead>
<tr>
<th>Probe</th>
<th>fs</th>
<th>ps</th>
<th>fs/ps</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-H</td>
<td>309 ± 18</td>
<td>1 ± 0.027</td>
<td>309 ± 20</td>
</tr>
<tr>
<td>O-D</td>
<td>36.9 ± 2.1</td>
<td>0.119 ± 0.003</td>
<td>310 ± 20</td>
</tr>
<tr>
<td>O-H/O-D</td>
<td>8.386 ± 0.005</td>
<td>8.392 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>CNR</td>
<td>14.27 ± 0.03</td>
<td>0.813 ± 0.003</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM. CNR denotes the contrast-to-noise ratio between the O-H and O-D CARS signals.

Table S2. Osmotic pressure $P_{osmo}$ induced by D$_2$O-PSS perfusion

<table>
<thead>
<tr>
<th>$T$, μm</th>
<th>Animal 1</th>
<th>Animal 2</th>
<th>Animal 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P$, mmHg</td>
<td>H$_2$O</td>
<td>D$_2$O</td>
<td>H$_2$O</td>
</tr>
<tr>
<td>17</td>
<td>26</td>
<td>23.0</td>
<td>25.0</td>
</tr>
<tr>
<td>35</td>
<td>20.9</td>
<td>18.2</td>
<td>20.7</td>
</tr>
<tr>
<td>49</td>
<td>17.9</td>
<td>16.2</td>
<td>18.2</td>
</tr>
<tr>
<td>70</td>
<td>15.3</td>
<td>14.3</td>
<td>15.5</td>
</tr>
<tr>
<td>$P_{osmo}$, mmHg</td>
<td>10.9 ± 0.8</td>
<td>12.0 ± 1.5</td>
<td>14.7 ± 1.6</td>
</tr>
<tr>
<td>$R^2_{adj}$</td>
<td>0.997</td>
<td>0.990</td>
<td>0.989</td>
</tr>
</tbody>
</table>

Shown is estimation based on quadratic fits of the wall thickness $T$ vs. luminal pressure $P$ in arteries perfused with H$_2$O-PSS and D$_2$O-PSS and immersed in H$_2$O-PSS. The fitted value for $P_{osmo}$ is reported for each animal with its SE together with the degrees of freedom adjusted $R^2$ of the fits. Averaged over the three animals, $P_{osmo} = 12.5 ± 0.79$ mmHg (paired two-sided t test, $P = 0.0039$).
Table S3. Effect of perfusion with digitonin on rat mesenteric artery permeability

<table>
<thead>
<tr>
<th>Biological replicate no.</th>
<th>Experiment</th>
<th>Artery length, μm</th>
<th>Artery diameter, μm</th>
<th>J/S, 10⁻⁴ cm⁻¹s⁻¹</th>
<th>Lp, 10⁻⁷ cm⁻¹s⁻¹.cm H₂O⁻¹</th>
<th>Lp_dig/Lp_c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>2,046</td>
<td>250</td>
<td>0.75</td>
<td>7.9</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>Digitonin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>3,413</td>
<td>300</td>
<td>0.22</td>
<td>2.3</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>Digitonin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>3,391</td>
<td>250</td>
<td>0.60</td>
<td>6.3</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Digitonin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>3,391</td>
<td>272</td>
<td>0.27</td>
<td>2.8</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>Digitonin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>2,441</td>
<td>304</td>
<td>0.31</td>
<td>3.3</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>Digitonin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*J/S denotes the volumetric flow rate per surface area, Lp the permeability, and Lp_dig/Lp_c the ratio of the permeability after digitonin treatment over the control permeability. Lp_c and Lp_dig are 4.5 ± 1.1 cm⁻¹s⁻¹·cm H₂O⁻¹ and 28.7 ± 2.3 x 10⁻⁷ cm⁻¹s⁻¹·cm H₂O⁻¹, respectively (paired t test, P = 0.001), and Lp_dig/Lp_c = 8.1 ± 1.8. Values are means ± SEM.*

Table S4. Characterization of the water concentration profiles across endothelial nuclei before (control) and after perfusion with digitonin

<table>
<thead>
<tr>
<th>Biological replicate no.</th>
<th>Control</th>
<th>Digitonin</th>
<th>Digitonin/control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g_raw</td>
<td>g_corr</td>
<td>g_raw</td>
</tr>
<tr>
<td>1</td>
<td>0.067</td>
<td>0.098</td>
<td>0.028</td>
</tr>
<tr>
<td>2</td>
<td>0.004</td>
<td>0.119</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.073</td>
<td>0.107</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.078</td>
<td>0.113</td>
<td>0.031</td>
</tr>
<tr>
<td>5</td>
<td>*</td>
<td></td>
<td>0.037</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.076 ± 4 x 10⁻³</td>
<td>0.109 ± 5 x 10⁻³</td>
<td>0.032 ± 3 x 10⁻³</td>
</tr>
</tbody>
</table>

g_raw and g_corr denote the maximum amplitude of the CARS signal derivative along the x axis in l_CARS and x_OH, respectively. *Data excluded because of excessive in-frame motion during acquisition. The mean values of g_raw and g_corr in control and digitonin-treated datasets are statistically different according to a two-sided t test (P = 2 x 10⁻⁴ and P = 1 x 10⁻⁴ for g_raw and g_corr, respectively).*

Movie S1. Z stack of water CARS images at 3,205 cm⁻¹ in intact pressurized mesenteric artery. The z stack ranges from the top of the vessel to the 30° plane selected for image analysis. Color scheme is identical to that in Fig. 1.
Movie S2. Water CARS time-lapse movie in artery during D$_2$O perfusion. Shown is dynamic en face water CARS imaging at 3,205 cm$^{-1}$ in an intact mesenteric pressurized artery perfused with D$_2$O. Note the thinning of the wall upon D$_2$O perfusion due to a difference in the wall permeability to H$_2$O and D$_2$O. Imaging details are in SI Materials and Methods. Color scheme is the same as in Fig. 1. (Scale bar, 20 μm.)

Movie S2
Movie S3. High-resolution water CARS time-lapse movie in artery during D₂O perfusion. (A and B) Dynamic en face water CARS imaging at 3,205 cm⁻¹ in a pressurized intact mesenteric artery (A) and in the same artery treated with luminal 0.01% digitonin (B). The wall thinning upon D₂O perfusion in the intact artery disappears after treatment with digitonin due to the hydro-seal disruption. Further evidence of this disruption is revealed by comparing the drop in CARS signal in the bath upon D₂O perfusion: It is minimal in the intact artery but very significant in the digitonin-treated artery. Color scheme is the same as in Fig. 1. (Scale bar, 4 μm.)

Movie S3
Movie S4. Rabbit internal carotid artery after loading with D$_2$O and immersion in H$_2$O. The increase in diameter together with the luminal pressure trace shown in Fig. S3, recorded in another artery, demonstrates net volume inflow into the artery. This is caused by the lower permeability of the artery wall to D$_2$O than to H$_2$O.

Movie S4