Correction


The authors note that the following statement should be added to the Acknowledgments: “The authors would like to acknowledge Dr. Sidney Simon for discussions on water movement across membranes as well as the support of the British Heart Foundation (BHF) (Grants FS/13/16/30199 and IG/13/5/30431), and K.A.D. acknowledges support from the Oxford BHF Centre of Research Excellence (RE/13/1/30181).”

www.pnas.org/cgi/doi/10.1073/pnas.1707526114
Direct visualization of the arterial wall water permeability barrier using CARS microscopy

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Edited by Peter Agre, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, and approved March 7, 2017 (received for review December 6, 2016)

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 elastic 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.


This article is a PNAS Direct Submission. Freely available online through the PNAS open access option. See Commentary on page 4574.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1620008114/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1620008114

PNAS | May 2, 2017 | vol. 114 | no. 18 | 4805–4810
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\(^{-1}\) 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. 1C and D, respectively. Perfusion with D\textsubscript{2}O-PSS results in a large reduction of the CARS signal in the lumen of 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 2A 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 D₂O perfusion as an osmotic pressure \( P_{\text{osmo}} \) because it is associated with a concentration gradient. We confirmed that D₂O induces an osmotic pressure by observing a rise in luminal pressure and artery diameter in rabbit internal carotid arteries filled with D₂O and immersed in water (SI Materials and Methods, D₂O-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 \( P_{\text{osmo}} = 12.5 \pm 0.8 \text{mmHg (SEM) (Table S2).} \) 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 D₂O-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 that the EC and/or basement mem-

**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. 5E). We measured the permeability \( L_p \) in control and after digitonin treatment (1) (SI Materials and Methods, Permeability Measurements) to be 4.5 ± 1.1 cm s⁻¹ cm H₂O⁻¹ and 28.7 ± 2.5 × 10⁻⁷ cm s⁻¹ cm H₂O⁻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 ± 1.8 (SEM) after digitonin treatment. Additionally, digitonin completely removed the wall-thinning effect during D₂O-PSs perfusion. Movie S3B shows the D₂O-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 ICARS 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 ± 1.4 (SEM) (9.7, 4.8, 6.3) in the raw STED images and 25 ± 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 a lack of AQP1. We note that other 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 been recently 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 (SPI; 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 25×/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 for parfocality correction and adjusts 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 hybrid 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 (FF01-7220SP 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 other HyD is filtered by a hybrid filter (FF484-FD01; Semrock) and the short-wavelength component is detected with a bandpass filter (FF01 4922SP; Semrock) when imaging nuclei stained with Syto 40. This filter was replaced by a narrowband filter (FF01-44820; 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 distortion function (35), is estimated to be ~300 fs 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 α = t/τ₀, the ratio of the FWHM of the chirped-pulse intensity to that of the TL pulse τ₀. Pulse compressors using two transmission holographic gratings (Newport 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.

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 nucleus being acquired for each series of 3D stacks was then performed 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 corresponding distance as the distance between the peaks of the first derivatives of the water CARS and nuclei signals in the intensity profiles. The peak amplitudes of the signal derivatives, denoted gₘw and gₘ₀, for Iₜₙₑₜₛ and Oₚₛₛ, 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 arterial wall is structurally acoustically dispersive (48). Indeed, these two excitations do not reflect water content but are due to optical artifacts. The corrected water signal xₘₙₑₜₛ 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 l(r) has a quadrature dependence on the probe concentration (36) and is modulated by a sample induced optical artifacts term A(r). For H₂O-D₂O mixtures we have l(r) = lₘ(r) + A(r)δ(r – c), where x₀(r) = H₂O-D₂O molar fraction of the solution within the probing volume at pixel location r in the image. The measured optical artifacts term is given by A(r) = l(r) – l₀(r), where l₀(r) is the image acquired in pure H₂O. Its contribution is removed with the normalization I₉ₜ₈ₙₑₜₛ(r) = l(r)/lₒ(r), given by

\[ I_{CARS}(r) = \frac{a_{CW}(r)^2 + b_{CW}(r) + c}{a + b + c}. \]  

Solving Eq. 1 yields x₀(r). For completeness both I₉ₜ₈ₙₑₜₛ and x₀(r) images were analyzed. The coefficients a, b, and c are obtained from a quadratic fit of the CARS signal recorded in pure H₂O (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 indices [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 \( \chi_{\text{opt}} \) the optical properties of the tissue are assumed to remain unchanged in \( D_2O \) and any motion between \( \bar{l}(r) \) and \( \bar{\rho}_s(r) \) needs to be corrected for, using either the SFQ or the TPEF images. This estimation is similar in principle to that in ref. 40 except that here the CARS signal is fitted with a quadratic function of \( \chi_{\text{opt}} \), rather than a power function, in agreement with the theory.

18. Murday JS, Cotts RM (1970) Self-diffusion in liquids - \( H_2O \) and any motion between \( \bar{l}(r) \) and \( \bar{\rho}_s(r) \) needs to be corrected for, using either the SFQ or the TPEF images. This estimation is similar in principle to that in ref. 40 except that here the CARS signal is fitted with a quadratic function of \( \chi_{\text{opt}} \), rather than a power function, in agreement with the theory.

**Animal Care.** In the conduct of this study all animals were treated according to an approved animal protocol under the Animal Care and Use Committee at the National Heart Lung and Blood Institute, National Institutes of Health.

**ACKNOWLEDGMENTS.** We are grateful to Dr. Han Wen for fruitful discussions on transmembrane water transport.

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}^2 + σ_{OD}^2)^{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 + iβ_i(t^2 + ot_i)), i = (p, s), with temporal pulse width parameter τGi = τi/√2ln2, pulse stretching factor αi = τi/τ0 = 1 + (β2_iτ0^2)^2, (S0), temporal chirp coefficient β_i, and angular optical frequency ω_i. Assuming the two pulses have the same temporal chirp coefficient β_i = β, the instantaneous frequency difference between the two pulses is ωD = ω_s − ω_p = 2β0 where l0 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_e0 δ(t). We consider the case where χ_v(t) consists of a single vibrational component with polarization r, angular resonance frequency ω_e, and exponential dephasing time T_2 so that χ_v(t) = −iε(t) μ exp(iω_0 t −i/T_2), with (θ) the Heaviside step function. The induced Raman polarization is described by Q(t) = χ_F(t)F(t), where χ 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_e(t) = χ_e F(t) and a nonresonant component Q_e(t) = χ_e F(t)/√(r^2 + b^2). Finally, the CARS signal intensity S_CARS is obtained by probing the Raman polarization Q(t) with the probe field E_s and spectral selection of the anti-Stokes field, so that after time integration it is written as S_CARS = ∫ |Q(t)E_s(t)|^2 dt = ∫ |Q_e(t)E_s(t)|^2 dt = S_e + S_c + S_{e-c}. Hence for a single vibrational component the CARS signal is the sum of purely resonant component S_e, a purely nonresonant component S_c, and a heterodyne term S_{e-c} resulting from the interference of the resonant and nonresonant anti-Stokes fields components (36):

S_e = 2χ_e0 ∫ |E_s(t)|^2 |E_p(t)|^2 dt

S_c = χ_{e0} ∫ |E_s(t)|^4 |E_p(t)|^2 dt

S_{e-c} = 2χ_{e0} ∫ |E_s(t)|^2 Re[Q_e(t)E_p(t)E_s(t)] dt.

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 ω_0 = 3,300 cm^{-1}, with a purely exponential dephasing time of T_2 = 60 fs and μ/χ_{e0} = 1. Because of the dispersive nature of the term S_{e-c}, the maximum CARS signal is reached at a frequency ω_0D that is red shifted wrt ω_e.

Fig. S1 shows the CARS spectrum and its components for TL pulses, with τ_0p = 110 fs and τ_0s = 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 S_{e-TL} obtained with TL pulses. The total CARS signal with TL pulses S_{tot-TL} is two orders of magnitude higher than that obtained with chirped pulses, S_{e-TL}. Spectral focusing is also illustrated in Fig. S1 where the resonant component S_{e-TL} with TL pulses (FWHM = 307 cm^{-1}) is broadened compared with the Lorentzian Raman line width (1/πT_2c = 177 cm^{-1}), whereas S_C obtained with chirped pulses is identical to the Raman line width. Note that the unit μ/χ_{e0} ratio was set to provide a ratio S_{e-TL}/S_{e-TL} = 22 that is close to the measured water to D_{2}O signal ratio of 24 in pure solutions. We record the maximum value of S_C for and calculate the ratio R_{tot} = S_{tot-TL}/S_{tot} between the total CARS signals with the TL and chirped pulses. The ratio R_{tot} = S_{e-TL}/S_{e} between the purely nonresonant signals with both excitation schemes is also informative when imaging water as it represents R_{tot} in the limit of infinitely short dephasing times. We calculate that R_{tot} = 137 and R_{tot} = 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, we measured R_{tot} = 165 (R_e unchanged). Turning our attention to the specificity, defined by the purely resonant to purely nonresonant signal ratio ρ = S_e/S_c, we calculate that ρ drops only by a factor of 1.8 when using TL pulses compared with the chirped pulses used in the ps 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_0D = √2τ_pτ_s/√(τ_p^2 + τ_s^2), giving the same CARS spectral resolution.

We note that the water to D_{2}O 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. 4. 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 H_{2}O and D_{2}O 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 CO_2 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;
When warmed to 37 °C, the artery was pressurized to physiological pressure (70 mmHg) or fed by gravity and the artery was straightened. Only arteries that contracted to the α 1-adrnergic agonist, phenylephrine (PE; 3 μM, Sigma) and fully dilated to the M 1-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 D-O imaging.** In one set of experiments, D 2-O-PSS was perfused at 50 μl/min using a gastight glass syringe (Hamilton Robomote) mounted in a Beehive syringe pump (BASI), during continuous superfusion of H 2-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 H 2-O-PSS, consisted of 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl 2 , 1.17 mM MgSO 4 , 2.0 mM Mops, 1.17 mM NaH 2 PO 4 or NaH 2 PO 4 , 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, and 2.75 mM NaOH (pH 7.40 ± 0.02 °C). The same solution was used in D 2-O instead of water, with pH adjusted to pH 6.99 ± 0.02 (53), referred to as D 2-O-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 H 2-O-PSS for 5 min before repressurizing. The water concentration profile was measured both before (control) and after exposure to digitonin.

**Permeability Measurements.** Starling’s law relates the volumetric flow rate J v across the artery wall to the pressure drop P across the wall by J v = L p S P, where L p 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 H 2-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 H 2-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 CaCl 2 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 CaCl 2]. 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 at 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 H 2-O-PSS for 5 min, and then washed with rinse buffer before fixing as above for TEM processing.

**AQP1 Immunohistochemistry and Imaging.** IHC was performed on intact pressurized arteries that were sectioned into radial slices for imaging on a STED 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 H 2-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 ImmunoResearch) 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 1.5x 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 STED images were obtained using a commercial STED microscope (SP8 STED 3X; Leica Microsystems), equipped with a white-light laser and a pulsed 775-nm STED depletion laser. A 100x/1.4-N.A. oil immersion objective lens (HCX PL APO STED white; Leica Microsystems) was used for imaging. For resolution comparison, confocal and STED 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 STED depletion laser. The pixel size is 20–25 nm (1,024 × 1,024 pixels) and six line averages were performed. Confocal images were collected for the autofluorescence 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 deconvoluted STED 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 profiles across the EC nucleus were then determined by the AQP1 peak on the luminal side of the EC membrane along with STED images. For each biological replicate three line profiles across the EC nucleus were traced and the signals at the EAM and EBM recorded.
**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 D₂O concentration gradients, and via filtration, driven by hydrostatic pressure gradients. These processes are quantitatively closely related (55). With pure opposing D₂O and H₂O 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 D₂O 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 \( P_\text{d} \) and \( P_\text{f} \), 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 D₂O perfusion the diffusional component \( \Delta G_\text{d} \) and filtration component \( \Delta G_\text{f} \) of the free energy are estimated as (55)

\[
\Delta G_\text{f} = \bar{V} \Delta P \\
\Delta G_\text{d} = RT \ln \frac{X_1}{X_2}
\]

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 \( \Delta P = 70 \text{ mmHg} \), \( \Delta G_\text{f} = 0.17 \text{ J mol}^{-1} \). The purity of the D₂O solution is \( X_1 = 10^{-5} \) and the natural abundance of D₂O in water is 0.014% (56) so that \( X_2 = 0.9997 \) and at \( T = 298.15 \text{ K} \) we have \( \Delta G_\text{d} = -17 \text{ kJ mol}^{-1} \). Thus, we estimate the diffusional driving force to be five orders of magnitude higher than the hydrostatic driving force.

**D₂O-Induced Osmotic Pressure in Arteries.**

**Rat mesenteric arteries.** The wall thickness was measured at various luminal pressures during H₂O-PSS and D₂O-PSS perfusion to characterize the osmotic pressure induced by luminal D₂O-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 H₂O-PSS perfusion and left unchanged during D₂O-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 D₂O does not change with luminal pressure. Consequently, we constrained the fitted model to be identical for both the H₂O-PSS and D₂O-PSS perfused datasets, except for a shift in luminal pressure \( P_{\text{osmo}} \). This was achieved by combining both datasets into a single dataset and using the quadratic model

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

where we introduced the dummy variable \( u \) that takes the values 0 and 1 for the H₂O-PSS and D₂O-PSS datasets, respectively. Thus, the least-squares fitted coefficients \( a, b, \) and \( c \) are identical for both datasets and only \( P_{\text{osmo}} \) differentiates the two models. The nonlinear least-squares fit was solved with the Matlab function \( \text{fit} \), using the \text{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 H₂O-PSS dataset separately (\( u = 0 \)). Then the least-squares solution for \( P_{\text{osmo}} \) was calculated for the D₂O-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 \( P_{\text{osmo}} \) for \( T \) and \( E \). The fitted values of \( P_{\text{osmo}} \) 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 H₂O-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 H₂O-PSS only. A pressure column and a pressure transducer (72-4496; Harvard Apparatus) were connected at opposite ends of the artery. The rise in luminal pressure shown in Fig. S3 and the increase in artery diameter shown in Movie S4 demonstrate net volume flow filtrating out of the artery.
Fig. S1. 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$_2$O-PSS in the bath. (A and B) Arterial wall image during H$_2$O-PSS perfusion with H$_2$O-PSS bath (A) and D$_2$O-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$_2$O-PSS and immersed in H$_2$O-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$_2$O than to H$_2$O.
**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></td>
<td></td>
<td></td>
</tr>
<tr>
<td></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/V, 10⁻⁴ cm·s⁻¹</th>
<th>Lp, 10⁻⁷ cm·s⁻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>230</td>
<td>210</td>
<td>2.51</td>
<td>26.3</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>250</td>
<td>210</td>
<td>2.30</td>
<td>24.2</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>210</td>
<td>210</td>
<td>2.39</td>
<td>25.1</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>230</td>
<td>230</td>
<td>3.07</td>
<td>32.3</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>217</td>
<td>340</td>
<td>3.40</td>
<td>35.7</td>
<td></td>
</tr>
</tbody>
</table>

J/V 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·s⁻¹·cm H₂O⁻¹ and 28.7 ± 2.3 × 10⁻⁷ cm·s⁻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>0.084</td>
<td>0.119</td>
<td>*</td>
</tr>
<tr>
<td>3</td>
<td>0.073</td>
<td>0.107</td>
<td>0.031</td>
</tr>
<tr>
<td>4</td>
<td>0.078</td>
<td>0.113</td>
<td>*</td>
</tr>
<tr>
<td>5</td>
<td>*</td>
<td>*</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Mean ± SEM 0.076 ± 4 × 10⁻³ 0.109 ± 5 × 10⁻³ 0.032 ± 3 × 10⁻³ 0.048 ± 3 × 10⁻³ 0.43 ± 0.04 0.44 ± 0.04

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 × 10⁻⁴ and P = 1 × 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 S1
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 D$_2$O 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 D$_2$O 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 D$_2$O 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.