Video Article

Two-photon Imaging of Intracellular Ca^{2+} Handling and Nitric Oxide Production in Endothelial and Smooth Muscle Cells of an Isolated Rat Aorta

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Abstract

Calcium is a very important regulator of many physiological processes in vascular tissues. Most endothelial and smooth muscle functions highly depend on changes in intracellular calcium ($[Ca^{2+}]_i$) and nitric oxide (NO). In order to understand how $[Ca^{2+}]_i$, NO and downstream molecules are handled by a blood vessel in response to vasoconstrictors and vasodilators, we developed a novel technique that applies calcium-labeling (or NO-labeling) dyes with two photon microscopy to measure calcium handling (or NO production) in isolated blood vessels. Described here is a detailed step-by-step procedure that demonstrates how to isolate an aorta from a rat, label calcium or NO within the endothelial or smooth muscle cells, and image calcium transients (or NO production) using a two photon microscope following physiological or pharmacological stimuli. The benefits of using the method are multi-fold: 1) it is possible to simultaneously measure calcium transients in both endothelial cells and smooth muscle cells in response to different stimuli; 2) it allows one to image endothelial cells and smooth muscle cells in their native setting; 3) this method is very sensitive to intracellular calcium or NO changes and generates high resolution images for precise measurements; and 4) described approach can be applied to the measurement of other molecules, such as reactive oxygen species. In summary, application of two photon laser emission microscopy to monitor calcium transients and NO production in the endothelial and smooth muscle cells of an isolated blood vessel has provided high quality quantitative data and promoted our understanding of the mechanisms regulating vascular function.

Video Link

The video component of this article can be found at https://www.jove.com/video/52734/

Introduction

Calcium is a fundamental second messenger within vascular cells such as endothelial and smooth muscle cells. It is the primary stimulus for vascular contraction and plays a major role in vascular dilation, including its effects through NO generation within the endothelium. Due to limitations of imaging technologies, it has been virtually impossible to observe calcium handling within the intact vessel. The development of two photon imaging systems and the creation of new calcium or NO labeling dyes, makes it possible to image at a sufficient depth and resolution to begin to understand calcium dynamics and NO production within the vasculature.

Two photon microscopy has recently been applied in tissue, organs and even whole animal studies because of its superior ability to deeply penetrate tissues with low background fluorescence and high signal sensitivity.[1,2] The narrow spectrum of two photon excitation at the illumination focal point and the use of non-descanned detectors are the reasons why two photon microscopy is superior to traditional confocal microscopy. Confocal microscopy cannot produce high-quality images at the necessary tissue depth due to the auto-fluorescence and the scattering of out-of-focus light into the confocal pinhole. Consequently, we have developed a method using a two photon microscope to measure $[Ca^{2+}]_i$ signaling and NO production in intact, individual blood vessel cells with high resolution and a low signal-to-noise ratio.

Protocol

The experimental procedures described below were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical College of Wisconsin and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

1. Isolation of the Rat Aorta

1. Anesthetize rats with isoflurane (5% induction, 1.5 to 2.5% maintenance) or another IACUC approved method.
2. Place the rat in a supine position. In order to expose the abdominal organs, make a small ventral midline incision through the skin and the subcutaneous abdominal tissue. Using gauze pads gently deflect the intestines to expose the aorta and vena cava as shown in **Figure 1**.
3. Briefly, blunt dissect the aorta from the connective tissue and from the vena cava. Using suture, tie off the aorta as close as possible to the kidney. Dissect about 1 - 2 cm of aorta and place it in a 15 ml conical tube containing normal Physiological Salt Solution (PSS; in mM: 140 NaCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 4.5 KCl, 6 Glucose, 10 HEPES) on ice.
4. Once the aorta is isolated, euthanize the animal according to approved IACUC protocols. At the completion of all non-survival procedures euthanize deeply anaesthetized animals by thoracotomy inducing pneumothorax to ensure the humane demise of the animal.[3]
5. Using a dissection microscope, fine-tipped forceps, and microscissors dissect the fat and connective tissue off of the aorta. Once the aorta is clean, cut the aorta longitudinally and place it in PSS on ice for later.

2. Dye Loading and Incubation

1. Pipet 7 µl of 1 mM Fluo-4 AM dye, which is supplied in DMSO-based solution, into individual 500 µl tubes that have been covered with aluminum foil. Store in the freezer at -20 ˚C to preserve the dye properties over time. Note: these aliquoted solutions should be stable for at least six months.
2. Before use, allow the dye solutions to warm up to RT before opening. Prepare working solutions immediately before use. Do not store the diluted reagent for later use.
3. Add 7 µl of ready-made 1 mM Fluo-4 AM dye dissolved in DMSO solution to 450 ul of PSS containing no calcium. Add 40 µl of non DMSO based 10% Pluronic Acid solution to the loading cocktail to help disperse the acetoxymethyl (AM) esters and improve loading of the calcium dye.
4. Place the dissected aortas into the 500 µl tubes containing the loading cocktail (as described in 2.3) for 1 h. Cover all tubes with foil and place on a rotating shaker at RT.
5. To monitor NO production in the vasculature, use DAF-FM diacetate dye. Incubate the aorta in a solution containing 450 µl PSS, 40 µl pluronic acid and 5 µl of 10 mM DAF-FM diacetate. Similar to the calcium incubation protocol (described in 2.3), place the aorta in an 500 µl tube containing the NO-dye solution covered in foil and place on a rotating shaker for 30 min at 4 ˚C followed by next 30 min at RT.
6. When NO production is measured, use the endothelial NO synthase blocker, L-NAME, to block NO production as a control as shown in **Figure 4B**. For that procedure, add to cocktail (as described at 2.5) 100 nM L-NAME for the last 30 min of incubation (at RT).

3. Laser Scanning Two photon Microscopy Protocol

1. After the 1 h incubation, wash the aorta 2 - 3 times in clean PSS to remove extracellular dye.
2. Transfer the aorta to a silicone-coated dish containing either normal PSS or Ca^{2+}-free buffer (depends on the experimental protocol). Pin the aorta down onto the silicone coating with the adventitia in contact with the silicone (*i.e.,* endothelial lumen exposed to the dish opening) using µ-shaped pins. Alternatively, use a Slice Anchor grid or glue to fix the tissue.
   Note: To reduce stress and possible mechanical artifacts, transfer and fix aorta in Ca^{2+}-free solution and wait 5 - 10 min before starting the experiment.
3. Connect a peristaltic pump or a syringe to the silicone-coated plate for automatic or manual application of drugs or for changing the extracellular solution.
4. Place the dish beneath the nose piece of the upright two-photon microscope, and install and position a 25× (N.A. 1.05 and working distance 2 mm) water-immersion objective lens above the specimen. Note: If this specific lens is not available, use an objective specifically manufactured for two photon imaging, because it can increase signal resolution dramatically compared with a regular objective.
5. Activate the two photon laser using the software (the laser begins to pump and turn on). Tune the laser excitation to 820 nm.
6. Using epifluorescence, locate the aorta and focus the objective on the endothelial surface using coarse adjustment.
7. Switch the microscope into two photon laser scanning mode, ensuring that the excitation laser is mode locked, the non-descanned detectors are engaged and the emission filters appropriate to the expected emission spectra are installed. Note: Here, the FV10-MRL/R (495 to 540 nM) were used.
8. Start live scanning using approximately 5% laser power and finely focus the objective in order to visualize the endothelial cells.
   Note: The endothelial cells will exhibit a strong fluorescent signal when incubated in normal PSS. This will be noticeably weaker when the vessels are incubated in calcium-free buffer. The endothelial cells will be present on the top of the opened aorta and the smooth muscle cells can be seen just below the endothelial cells (see **Figure 2**). Because blood vessels are neither smooth nor even, there will be places where both smooth muscle cells and endothelial cells are present.
9. Once the cells are in focus, program the microscope software to collect sequential images in fast scan mode (raster scan, 512 x 512 pixel window with a frame collection frequency of 1.1 s). In parallel with Fluo-4 AM fluorescence, collect transmitted light images in order to detect changes in vessel contraction and better visualize the experimental conditions.
10. After collection of baseline signal images, change the Ca^{2+} ion concentration in the external solution or slowly apply pharmacological stimulus agents using the peristaltic pump while imaging. Observe the fluorescent signal increase within the loaded cells.
    Note: Endothelial cells respond to changes in flow, thus it is recommended to use low laminar applications and vehicle testing before application of pharmacological activators of Ca^{2+} or NO signaling. To recalculate intracellular calcium to nM concentration values in vessels loaded with Fluo-4 (dissociation constant for Fluo-4 is 345 nM), fluorescence intensity could be recorded at baseline and after addition of ionomycin and $MnCl_2$.[4]

4. Image Processing and Calculations

1. Download and install Fiji image processing package.

2. Open the image file in Fiji. Upon importing the file, split the channels (transmitted light and fluorescent signal) when prompted. Use only the fluorescent signal for the data analysis.
3. Within the Fiji program, click on the analyze tab and scroll down to the tools option. Under the tools option, find the tab that says ROI manager and click on it; a new window will appear.
4. After this, click on set measurements under the analyze tab. Select the specific measurements of interest. For the calcium transient measurements, use the mean gray value option.
5. With the circular trace tool, begin to trace the regions of interest (ROI) and click “add” on the ROI manager screen for every ROI. Once all cells of interest have been traced, in the ROI manager window, select Multi Measure under the “more” tab. Remember to either save the measurements directly or copy and paste all of these measurements into a spreadsheet.
6. Open *.txt file or Excel spreadsheet from Fiji and transfer the numbers to a new Origin worksheet. Note: Alternatively, use any program like Sigma Plot or Prism for further analysis.
7. Within the Origin program, use Plot → Line+Symbol menu to observe an overview of all transient responses. Deselect the unresponsive cells.
8. Recalculate trace number to time scale (X axis), use Column → Set Column Values and multiply the trace number column to the image collection frequency (in our case 1.1s).
9. Use Analysis → Statistics on Rows for all selected recordings to calculate Mean (Y axis) and Standard Error trace. Plot final Graph for current group of cells Plot → Line + Symbol.
10. The mean calcium transient calculation is described as followed.
    1. For total calcium release within the Origin program, click on Graph, then use menu Analysis → Calculus → Integrate. Total integral of area under curve appears in Result Log.
    2. For transient kinetics, click on Graph, then use menu: Analysis → Non-linear Curve Fit → Select Data Set (choose X axis range from maximum to end of transient response). Start Fitting → Select Function → Exponential Decay 1 → Done.
       Note: Exponential fitting appears in Graph window and Result Log. Obtained data (values) represent total amount of calcium released, and kinetics of the channels mediated the transient response.

Representative Results

In order to accurately assess the contribution of calcium to vascular physiology (vasodilation and vasoconstriction), a protocol was designed to load calcium dyes into both endothelial cells and smooth muscle cells in isolated intact aortae. The general experimental set up depicted in **Figure 1,** shows the basic strategy for isolation and preparation of the vessel before imaging. Briefly, after isolation of the aorta from the rat, it should be cleaned of fat and connective tissue and slit longitudinally. The opened aorta should then be placed in a foil-covered 500 µl tube containing the prior prepared loading cocktail as described in the protocol and incubated at RT for 1 hr with light rocking. Following the incubation, the aorta should be washed, pinned down onto a silicone-covered plate with the adventitia nearest the silicone and lumen upwards, and transferred to an upright two photon microscope for imaging.

Once the aorta has been placed on the microscope and the vessel is in focus, the endothelial cells should be easy to locate by their strong fluorescent emission (**Figure 2A)**. The endothelial cells will also be the first object to come into focus because the lumen of the vessel, which consists primarily of endothelial cells, should be facing upward when the vessel is pinned down. In addition to imaging endothelial cells, there will be locations where smooth muscle cells are visible (**Figure 2B**). Thus both endothelial and smooth muscle cells could be identified within the same imaging field (**Figure 2C**). Having the capability to image both vascular cell types within the same imaging field can lead to a better understanding of how these cells jointly, yet independently, respond to different agonists and stimuli.

To demonstrate the utility of this method, measurements of aorta cells responsiveness to the addition of acetylcholine (ACh), a potent vasodilator that specifically causes changes within the endothelium, was determined. As shown in **Figure 3A**, the endothelial cells can be easily seen while incubated in normal PSS. After the addition of ACh (**Figure 3B**), the endothelial cell fluorescence increases indicating that intracellular calcium content is also increasing. The quantification of the emitted fluorescence from the ROIs, or the endothelial cells, is shown in **Figure 3C**. After the addition of ACh, the intracellular calcium content increases rapidly and then slowly returns back to baseline. The increase in calcium within the endothelial cells in response to ACh is consistent with previous reports.[5,6]

Similar to calcium studies, this protocol is suitable for the detection of endothelial NO production. For these experiments, aortic endothelial cells were loaded with DAF-FM diacetate dye as shown on **Figure 4A**. This approach was recently utilized to demonstrate that NO production increases in a Ca^{2+}-dependent manner in response to ACh stimulation.[6] Importantly, after treatment with L-NAME, an endothelial NO synthase inhibitor, this response was blocked (**Figure 4B**).

Another representative example of how this technique can be applied to measure intracellular calcium is shown in **Video 1**, where calcium containing solution was added to a vessel that was incubated in calcium free buffer. When aorta are initially incubated in the calcium free buffer (at the beginning of the video), the endothelial cells are weakly fluorescent indicating low levels of calcium in the cells. However, after the addition of normal PSS, the cells immediately respond by increasing $[Ca^{2+}]_i$ concentration and emitting high intensity fluorescence. This experiment shows that the cells are alive and responsive to external stimuli. This experiment also validates the viability of this method with its yield of high quality data.

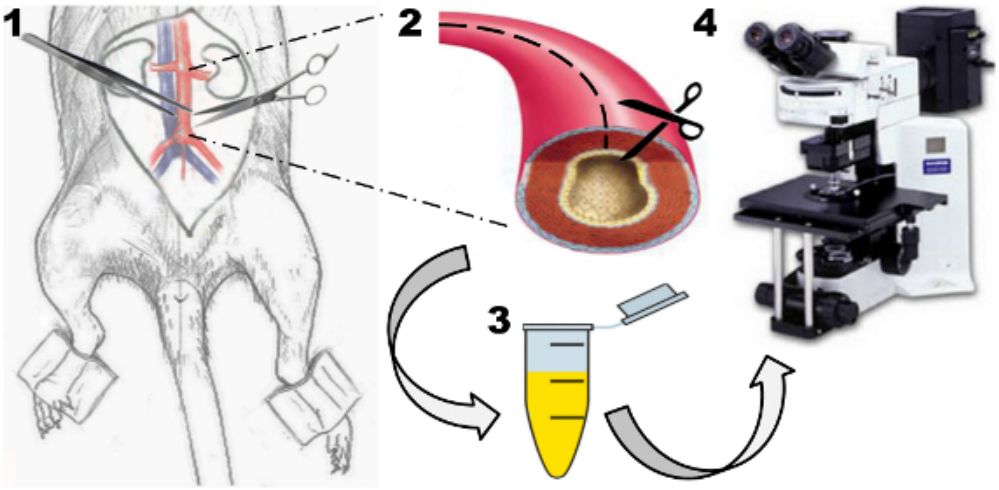


Figure 1. Aorta Isolation and Experimental Set Up. 1) After anesthetizing the rats, make an incision in the abdomen and isolate the aorta from the connective tissue and the vena cava. 2) Following extraction of the aorta, clean the vessel of fat and connective tissue and open the vessel by cutting longitudinally. 3) Incubate the vessel in the Fluo-4 AM dye for 1 hr at RT with slight rocking. 4) Following incubation, wash the aorta, pin it onto a silicone-coated plate, lumen facing upwards, and transfer it to the stage of an upright two-photon microscope. Please click here to view a larger version of this figure.

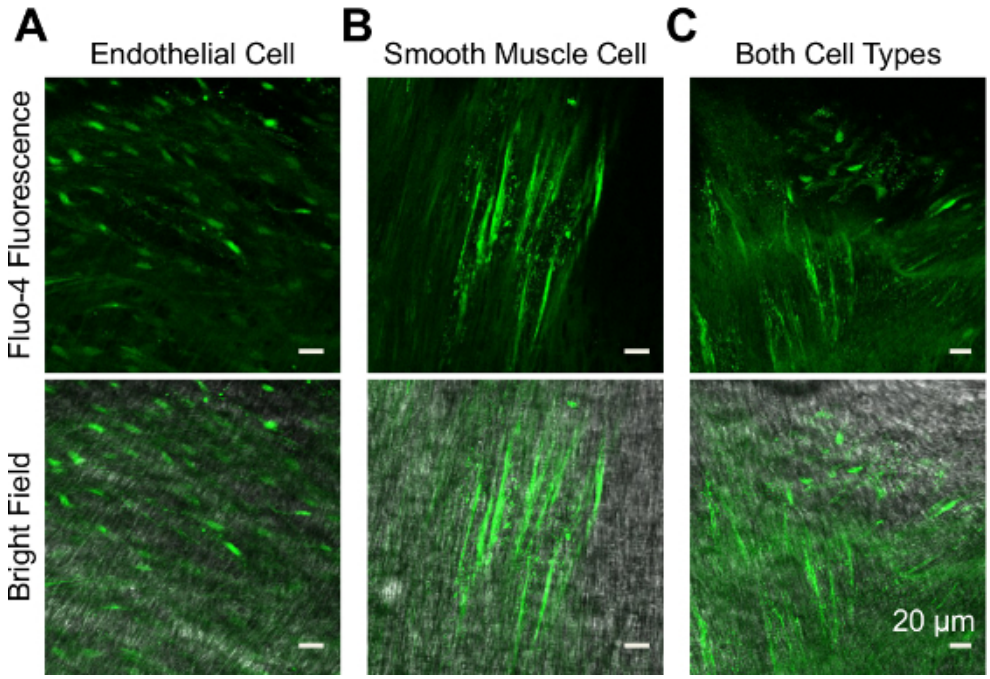


Figure 2. Imaging Smooth Muscle and Endothelial Cells. After sufficient loading of the calcium-dye, it is possible to image both smooth muscle cells and endothelial cells. (**A**) Top, is an example of Fluo 4 fluorescence of endothelial cells in an isolated rat aorta. Bottom, is a fluorescent image merged with transmitted light view (similarly for **B** and **C**). (**B**), is an example of Fluo 4 fluorescence of smooth muscle cells in an isolated rat aorta. (**C**) due to the non-uniform thickness of the aorta, it is also possible to image regions where both smooth muscle and endothelial cells are present. Scale bars are shown. Please click here to view a larger version of this figure.

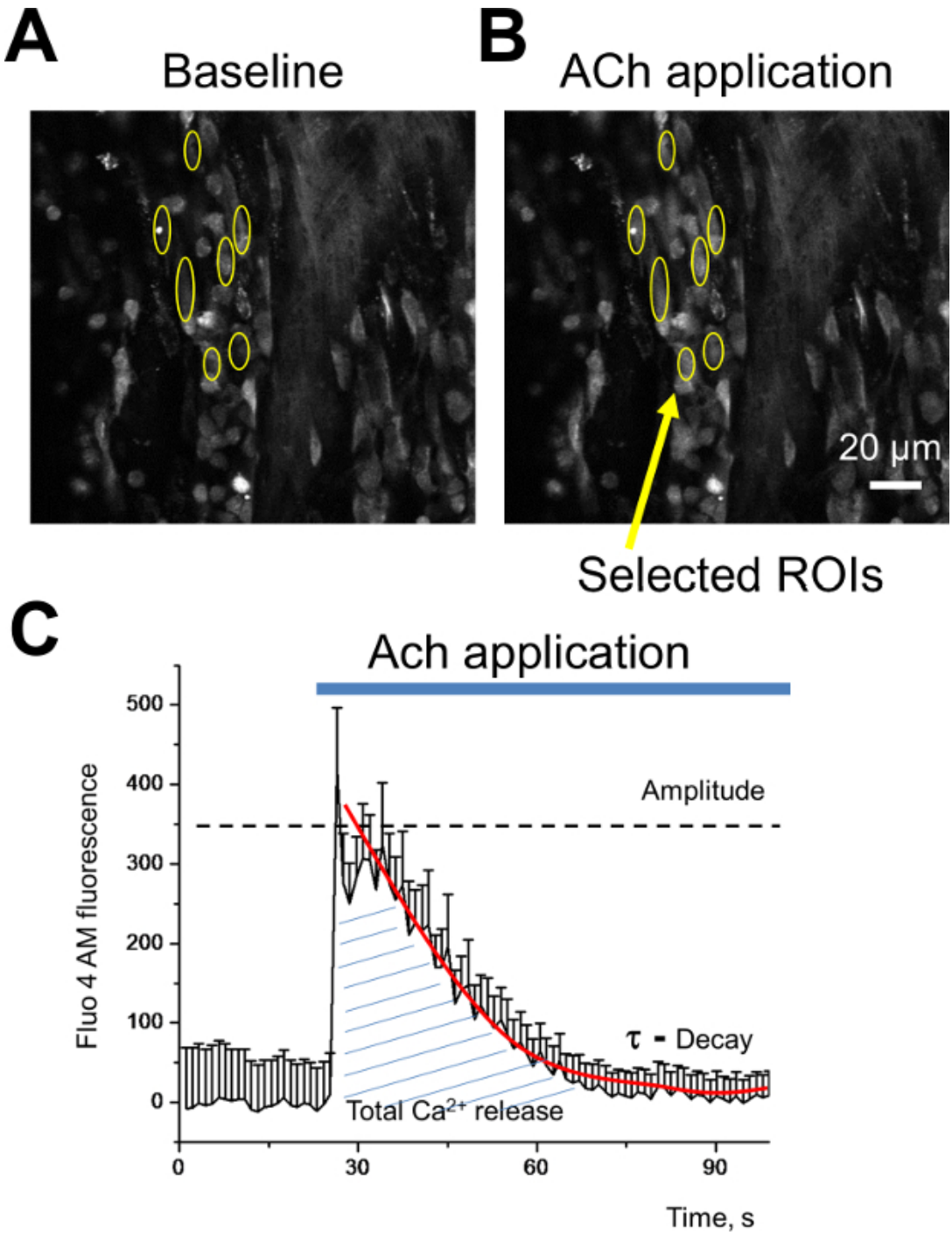


Figure 3. Measuring Calcium Transients in Endothelial Cells in Response to ACh. (**A**) The aorta was initially prepared and incubated in normal PSS buffer for baseline recordings. (**B**) Following addition of acetylcholine (ACh), the fluorescent signal emitted by the endothelial cells (ROIs) enhanced indicating increasing levels of calcium within the cells. Scale bar is shown. (**C**) The calcium transients of the endothelial cells in response to Ach were calculated using Origin software. Please click here to view a larger version of this figure.

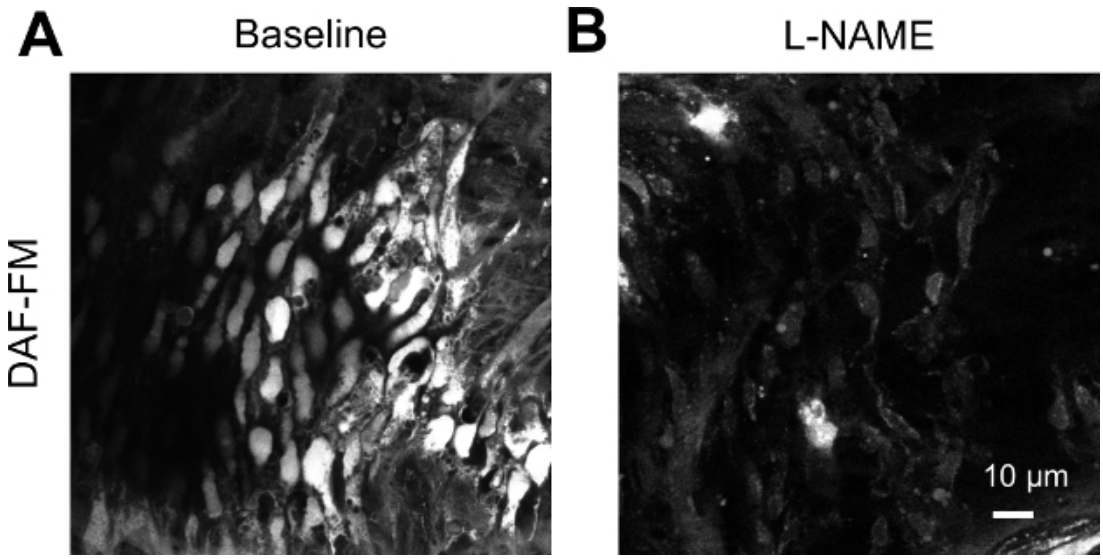
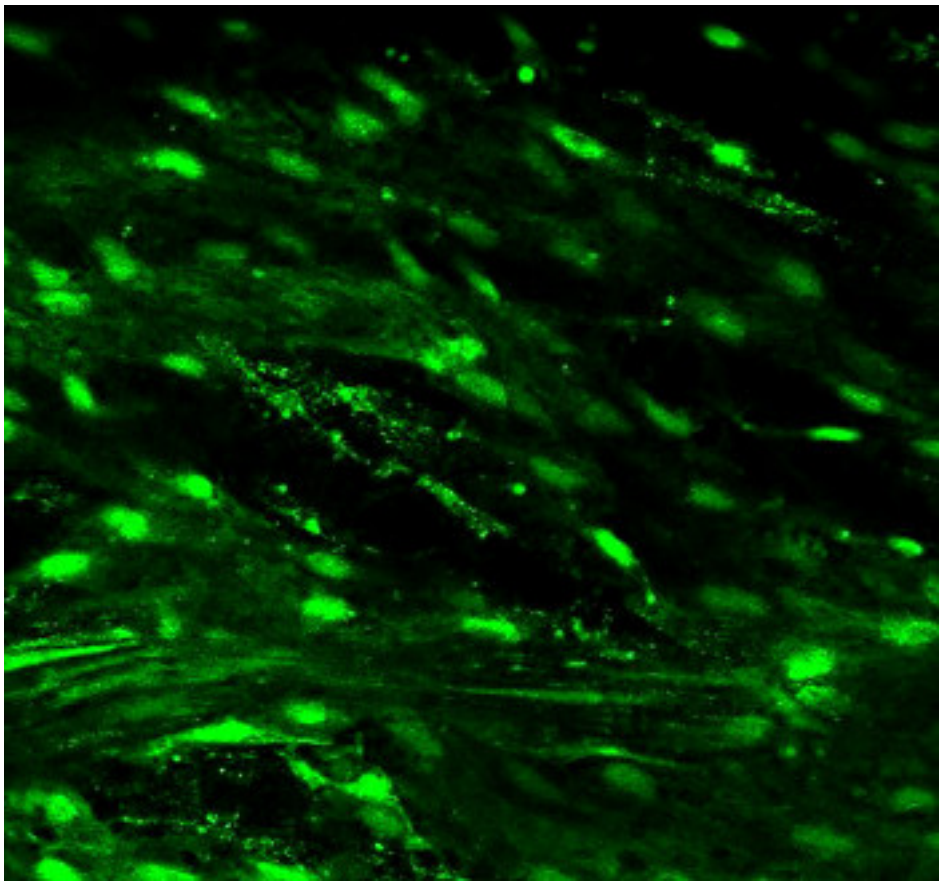
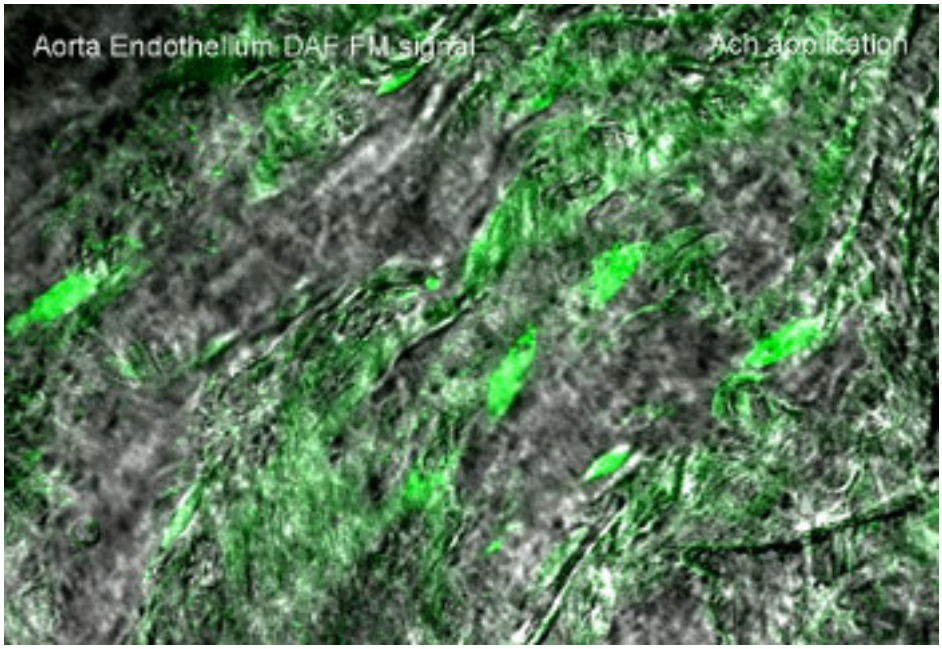


Figure 4. Imaging Nitric Oxide in Endothelial Cells. (**A**) After loading the aorta with the DAF-FM diacetate dye and placing it on the stage of a two photon microscope, it is possible to image NO levels within the endothelial cells using emitted fluorescence. (**B**) Following incubation with the endothelial NO synthase inhibitor, L-NAME, the overall fluorescence decreased within the ROIs, indicating reduced levels of NO within the cells. Scale bar is shown. Please click here to view a larger version of this figure.



Video 1. Example of Endothelial Cell Responsiveness to Changes in Calcium Concentration. Following preparation and incubation in Fluo-4 AM dye, the aorta was washed with calcium-free buffer and placed on the stage of a two photon microscope. When the vessel is placed in the calcium-free buffer, most of the endothelial cells are weakly fluorescent. Following addition of normal PSS, the endothelial cells respond by increasing intracellular calcium concentration and emitting a strong fluorescent signal.



Video 2. Example of NO production in endothelial cells of isoltated rat aorta. Following preparation and incubation in DAF-FM diacetate dye, the aorta was placed on the stage of a two photon microscope. Following the addition of Ach in to bath solution, the endothelial cells respond by increasing intracellular NO production and emitting a strong fluorescent signal. Please click here to view this video.

Discussion

Experimental Overview. To better understand the contribution of calcium and NO to vascular physiology, a novel method was developed for measuring $[Ca^{2+}]_i$ and NO within smooth muscle and endothelial cells of isolated intact aortas. Together, this protocol consists of these critical steps: 1) Mechanical isolation and preparation (not enzymatic digestion) of the vessel. It is important to keep the tissue healthy and intact as much as possible to obtain optimal physiological recordings. 2) Incubation in the calcium-labeling dye or NO-labeling dye with pluronic acid is critical, however, for some other vessels types, a different pluronic acid and incubation time may need to be tested. 3) Proper fixation of the aorta to the silicone-coated dish with the grid and pins will provide a stable view during imaging with the two photon microscope. Because the vessel will constrict and dilate, it is important to make sure that the vessel is properly fixed to the dish to avoid movement artifacts that may arise. 4) Quantification of the results. Please note that transient amplitude may not always be the best parameter to compare the physiological characteristics of the cells. The integral of the transient may show a better difference due to total $[Ca^{2+}]_i$or NO release.

When the experiments are performed correctly, it is possible to monitor the $[Ca^{2+}]_i$ (or NO levels) changes in response to stimuli as shown in **Figures 3** & **4** and **Video 1 & 2**. The combination of an *ex vivo* preparation and the use of two photon imaging can provide better signal resolution and precise measurements of intracellular processes in vascular tissues. Here, these technologies were applied in combination with the classical non-ratiometric Ca^{2+} and NO indicators (Fluo-4 AM and DAF-FM diacetate, respectively), to measure calcium transients and NO production in individual cells of an isolated whole aorta. We believe that this technique will help to forward our knowledge about the mechanisms regulating $[Ca^{2+}]_i$ and NO levels within the different vascular cell types.

Modifications and troubleshooting. The basic principles of measuring Ca^{2+} signaling in single smooth muscle cells with sensitive ionophores was initially published in 1985.[7] Most of the recent methods for imaging Ca^{2+} levels in individual vascular cells have employed a laser-scanning confocal microscope and isolated or cultured preparations. For dynamic tissues like blood vessels, it is difficult to measure calcium or NO levels *in vivo* using microscopy-based techniques.[2] The main limitation here is that nearly all blood vessels on the arterial side of the circulation have an internal elastic lamina that lies between the endothelium and the innermost layer of smooth muscle cells.[8] Furthermore, most of the extracellular material between the smooth muscle and adventitia layer is collagen.[8] Elastin and collagen are a major source of auto-fluorescence with high intensity and a wide range of excitation wavelengths. The strong background fluorescence coupled with increased light scattering during confocal imaging produces low signal resolution and therefore, reduced detection of calcium signaling or NO production. The limiting factor of loading fluorescent probes into vascular cells in sufficient concentrations to make meaningful measurements can be resolved by using an *ex vivo* preparation, a two photon microscope, and applying a new class of ionophores, such as Asante Red, which emits fluorescence at long wavelengths, where there is less interference from the auto-fluorescence of the connective tissue and fibers. However, one of the main limitations of this method is that it is difficult to combine two or more dyes during imaging. The excitation spectrums for most of the available dyes for two photon imaging have wide, polymodal distribution characteristics, which make it difficult for measuring two different ionophores simultaneously. However, low noise hybrid detectors could be used to narrow the detected emission spectrums assuming that the ionophores emit light at different wavelengths. For instance, in this described procedure it is not possible to measure both NO production and Ca^{2+} concentration changes because the emission spectrums overlap.

Vascular endothelial cells that are in direct contact with blood flow are exposed to fluid shear stress and regulate vascular homeostasis.[9] The current method can be also applied to monitor flow-induced calcium transients and NO production in endothelial cells *ex vivo*. Additionally, this method can be successfully applied to studying Ca^{2+} concentration changes in smooth muscle cells of small resistance arteries extracted from

the kidney. In this case, the dissected small resistance arteries should be incubated in Ca^{2+}-free solution to avoid smooth muscle cell damage due to Ca^{2+} overload. This modification can also be applied to the aorta to reduce cell death.

Perspectives. Fluorescence-based imaging has been widely used to study cellular physiology *in vitro*; however, isolated cell models do not always recapitulate physiological processes that occur when the cells are in their native environment. This has limited the applicability of cell lines to translational research.[10] As described here, the use of *ex vivo* preparations has given us the opportunity to monitor cellular behavior in freshly isolated tissue where all local physiological processes and interaction with other cell types are still occurring. With the growing number of genetic engineering technologies available and the development of models that recapitulate human diseases, *ex vivo* preparations will be a useful tool for translational research. For the studies described here, the Dahl Salt-Sensitive (SS/JrHsdMcwi) hypertensive rat strain was used. The SS rat is a naturally occurring inbred genetic model of salt-sensitive hypertension that recapitulates many aspects of progressive human hypertension. This model is being used extensively and has provided key insights into the mechanisms underlying salt-sensitivity and vascular dysfunction.[11-13] With this rat model, researchers have been able to test the contribution of key mechanisms to complex diseases (like hypertension) using ZFNs, TALENs, and CRISPR/Cas-based gene-editing technologies.[6,14-17] Using the experimental procedure described here along with these gene-editing resources, it is now possible to start testing the contribution of calcium and other key factors to salt-sensitive hypertension and vascular dysfunction in many different animal models.

Disclosures

The authors have nothing to disclose.

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