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Corresponding Author:	Yu Wang University of Hong Kong HK Island, HK HONG KONG
Corresponding Author's Institution:	University of Hong Kong
Corresponding Author E-Mail:	yuwanghk@hku.hk
Order of Authors:	Danny Konja
	Cuiting Luo
	Wai Yan Sun
	Kangmin Yang
	Andy WC Man
	Amin Xu
	Paul M Vanhoutte
	Yu Wang
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TITLE:

Assessment of Vascular Tone Responsiveness using Isolated Mesenteric Arteries with a Focus on Modulation by Perivascular Adipose Tissues

AUTHORS AND AFFILIATIONS:

Daniels Konja[1], Cuiting Luo[1], Wai Yan Sun[1], Kangmin Yang[1], Andy W. C. Man[1], Aimin Xu[1], Paul M. Vanhoutte[1], Yu Wang[1]

[1]The State Key Laboratory of Pharmaceutical Biotechnology and the Department of Pharmacology and Pharmacy, the University of Hong Kong, Hong Kong SAR, China

Corresponding Author:

Paul M. Vanhoutte (vanhoutt@hku.hk)
Yu Wang (yuwanghk@hku.hk)

Email addresses of co-authors:

Daniels Konja (kdaniels-1@outlook.com)
Cuiting Luo (cuiting@hku.hk)
Wai Yan Sun (kiwisun@hku.hk)
Andy WC Man (an_dybot@hotmail.com)
Aimin Xu (amxu@hku.hk)

KEYWORDS:

Endothelium, vascular smooth muscle, vasodilatation, vasoconstriction, perivascular adipose tissue

SUMMARY:

The protocol describes the use of wire myography to evaluate the transmural isometric tension of mesenteric arteries isolated from mice, with special consideration of the modulation by factors released from endothelial cells and perivascular adipose tissues.

ABSTRACT:

Altered vascular tone responsiveness to pathophysiological stimuli contributes to the development of a wide range of cardiovascular and metabolic diseases. Endothelial dysfunction represents a major culprit for the reduced vasodilatation and enhanced vasoconstriction of arteries. Adipose (fat) tissues surrounding the arteries play important roles in the regulation of endothelium-dependent relaxation and/or contraction of the vascular smooth muscle cells. The cross-talks between the endothelium and perivascular adipose tissues can be assessed ex vivo using mounted blood vessels by a wire myography system. However, optimal settings should be established for arteries derived from animals of different species, ages, genetic backgrounds and/or pathophysiological conditions.

INTRODUCTION:

Dilatations and constrictions of arteries are achieved by relaxations and contractions, respectively, of their vascular smooth muscle cells. Changes in vascular responsiveness of small arteries contribute to the homeostatic regulation of arterial blood pressure by autonomic nerves and hormones present in the blood (e.g., catecholamines, angiotensin II, serotonin, vasopressin). At the local level, the vascular responses of smooth muscle cells are modulated by signals from both the endothelial cells of the intima and the adipose tissue surrounding the arteries (**Figure 1**).

The endothelium is not only a passive barrier, but also serves as a surface to exchange signals between the blood and the underlying vascular smooth muscle cells. By releasing various vasoactive substances, the endothelium plays a critical role in the local control of vascular tone responses[1]. For example, in response to acetylcholine, endothelial nitric oxide synthase (eNOS) is activated in the endothelium to produce nitric oxide (NO), which induces relaxation of the underlying vascular smooth muscle by activating soluble guanylyl cyclase (sGC)[2]. Other vasoactive substances include the products of cyclooxygenases (e.g., prostacyclin and thromboxane A_2), lipoxygenase (e.g., 12-hydroxyeicosatetraenoic acids, 12-HETE), and cytochrome P450 monooxygenases (HETEs and epoxyeicosatrienoic acids, EETs), reactive oxygen species (ROS), and vasoactive peptides (e.g., endothelin-1 and angiotensin II), and endothelium-derived hyperpolarizing factors (EDHF)[3]. A delicate balance between endothelium-derived vasodilators and vasoconstrictors maintain the local vasomotor tone[4,5].

Endothelial dysfunction is characterized by the impairment in endothelium-dependent vasodilatation[6], a hallmark of vascular aging[7]. With age, the ability of endothelium to promote vasodilatation is progressively reduced, due largely to a decreased NO bioavailability, as well as the abnormal expression and function of eNOS in the endothelium and sGC in the vascular smooth muscle cells[8-10]. Reduced NO bioavailability potentiates the production of endothelium-dependent vasoconstrictors[11,12]. In aged arteries, endothelial dysfunction causes hyperplasia in the media, as reflected by the marked increases in wall thickness, number of medial nuclei, which are reminiscent of the arterial thickening in hypertension and atherosclerosis observed in human patients[13,14]. In addition, pathophysiological conditions such as obesity, diabetes or hypertension accelerate the development of endothelial dysfunction[15,16].

Perivascular adipose tissue (PVAT) releases numerous adipokines to regulate vascular structure and function[17]. The anti-contractile effect of PVAT is mediated by relaxing factors, such as adiponectin, NO, hydrogen peroxide and hydrogen sulphide[18-20]. However, depending on the location and pathophysiological condition, PVAT also can enhance contractile responses in various arteries[21]. The pro-contractile substances produced by PVAT include angiotensin-II, leptin, resistin, and ROS[22,23]. In most of the studies on isolated blood vessels, PVAT has been considered as a simple structural support for the vasculature and thus removed during the preparation of blood vessel ring segments. Since adipose dysfunction represents an independent risk factor for hypertension and associated cardiovascular complications[24], the PVAT surrounding the blood vessels should be considered when investigating the vascular responsiveness of different arteries.

The multi wire myograph systems have been widely used to investigate the vasomotor functions of a variety of blood vessels, including the aorta, mesenteric, renal, femoral, cerebral and coronary arteries[25,26]. The protocols described herein will use wire myography to evaluate vascular responsiveness in mesenteric arteries isolated from genetically modified mouse models, with a special focus on the modulation by PVAT.

PROTOCOL:

All animals used for the following study were provided by the Laboratory Animal Unit of the Faculty of Medicine, The University of Hong Kong. Ethical approval was obtained from the departmental Committee on Use of Laboratory Animals for Teaching and Research (CULATR, no.: 4085-16).

1. Preparations

1.1. Preparation of drugs

1.1.1. Store drugs appropriately as stated in the Material Safety Data Sheet (MSDS) immediately after receiving them. Dissolve the drugs in powder form in solvents as high-concentration stock solutions and then aliquot for storage at -20 °C.

NOTE: Most drugs are dissolved in distilled water to prepare the stock solutions; heating or sonication may be required for some drugs. If drugs do not completely dissolve in water, a drop of 1 M NaOH can be added, while for basic drugs a drop of 1 M HCl can be used. Hydrophobic drugs can be dissolved in dimethylsulfoxide (DMSO) or absolute ethanol. In the latter cases, the final bath concentration (in M) should be known and appropriate controls should be performed to rule out the effects of the solvents.

1.1.2. Prior to experiment, dissolve the drug aliquots (**Table of Materials**) in Krebs-Ringer Bicarbonate solution (Krebs) containing 115 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.17 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM D-glucose and 0.01 mM EDTA, pH 7.4.

1.1.3. For cumulative concentration-response curves, prepare the stocks and working solutions of different drugs by serial dilutions (**Table 1**).

1.2. Setting up the instrument

1.2.1. Calibrate the force transducer for all channels before using the myograph system on each day, or every time the system has been moved.

NOTE: The detailed calibration procedure varies depending on the model. In general, a two-gram weight is applied to the jaws and the corresponding force should 9.81 ± 0.1 mN. If the reading is off by more than 0.1 mN, the transducer should be re-calibrated. For the system used in the present protocol (see the **Table of Materials**) the operating values for the force transducer during

calibration should be between 3000 and 3500. If the value of the transducer is higher or lower, the force transducer must be replaced.

1.2.2. Adjust and align the mounting supports in each chamber. Continuous and repeated usage of the myograph chamber may cause some misalignment of the mounting support, which needs occasional adjustment before experiments to ensure that the jaws are properly aligned.

NOTE: Special attention is needed when adjusting the mounting supports as the force transducers are very sensitive and fragile.

1.2.3. Switch on heaters and gas (95% O_2 and 5% CO_2) at least 30 min before the experiment to allow the chambers and buffers to be warmed up to 37 ± 0.1 °C and equilibrated with the gas mixture.

1.2.3.1. Check the temperature on the thermometer to ensure the accuracy of the heater. Temperature can be modified to run cooling or warming experiments. If the temperature is not correct as set, apply the offset function of the machine to increase or decrease the settings to reach the required temperature.

1.2.4. At the end of the experiment, clean all chambers and turn off the heater as well as the gas running to the setup.

1.2.4.1. Do not turn the gas off before all the liquid in the chamber has been sucked out of the system, otherwise the acid/distilled water may regurgitate and reach the organ-chamber during the next use.

1.2.4.2. To clean the chambers of the wire myograph, the most effective way is to perform acid-wash using a diluted acetic acid solution. Clean the edge and the inside of the chambers with a cotton swab.

1.2.4.3. After washing, rinse the chambers thoroughly with distilled water. Wipe the outside of the chambers with a wet cloth to remove dried salt. Ethanol can also be used if hydrophobic drugs have been used during the experiment.

1.2.4.4. An example of the washing procedure is as follows. Fill the chambers with 8% acetic acid solution and incubate for 2 min. Use a cotton-tipped applicator to mechanically clean the steel chamber surface. Avoid any contact with the aluminum part of the myograph.

1.2.4.5. Aspirate the acetic acid and wash the myograph chamber and supports several times with distilled water and dry the surfaces using absorbent paper or cotton-tip applicators.

1.3. Dissection of the mesenteric arterial rings

NOTE: Animals used for the current study were high-fat diet fed male Adipo-SIRT1 mice and wild type littermates as controls. Each animal weighed approximately 45 g at the time of the experiments.

1.3.1. Euthanize the mouse by intraperitoneal injection of pentobarbital sodium (25 mg/kg).

1.3.2. With surgical scissors and forceps, perform a mid-line laparotomy to reveal the abdominal contents.

1.3.3. Collect the mesenteric arcade into a silicon-coated Petri dish.

1.3.4. Spread and pin down the mesenteric network in the Petri dish to reveal the branching of mesentery and connective tissue meshwork.

1.3.5. Under a microscope (10x), and with fine-scissors and forceps, carefully dissect out surrounding connective tissues. Avoid damaging the adventitial layer. Alternatively, surrounding adipose tissue can be retained around the blood vessel for experiment (if needed).

1.3.6. Using the fine scissors and forceps, excise the secondary branches of mesenteric arteries in ice-cold Krebs buffer.

NOTE: Each researcher should have his/her own set of dissection kit, strings and stirrups. These tools should be kept properly and cleaned-up every time after the experiment as some drugs are hard to wash away and residue can stick to them.

1.3.6.1. Keep the blood vessels in cold Krebs buffer while separating the surrounding connective tissues, including PVAT. During handling of the blood vessel, be gentle to prevent unnecessary damage to the endothelium.

1.3.6.2. If the experiment involves the study of PVAT, retain a 1.5 to 2 mm-diameter sphere of PVAT around the blood vessel. Alternatively, same quantities of adipose tissues can be added in each chamber for experiment.

1.3.7. (Optional) Remove the endothelium from the dissected blood vessel as a control to evaluate the endothelium-dependency of the responses. For mesenteric arteries, remove the endothelium by gently rolling it over a wire stirrup or a hair.

1.3.8. Cut the blood vessel prepared as above into small rings (~2 mm length) and put them in a plastic dish full of aerated (95% O_2 and 5% CO_2) Krebs buffer for subsequent mounting into the chambers of a wire myograph[27].

1.3.9. Transfer the vessel rings into a myograph chamber placed under the microscope. Rings should be placed evenly, with the upper and lower stirrups parallel. The attaching wire (40 µm) should be newly prepared as drugs may bind to the strings.

1.3.10. Thread the blood vessel ring onto a suitable length (2 cm) of the wire and secure to one jaw of the mounting chamber by screwing to fix the position.

1.3.11. Pass a second wire through the ring and anchor to the opposite jaw.

1.3.12. With rings threaded and secured to chamber jaws, mount the chamber onto the myograph setup and turn the micrometer screw clockwise to move the wires close to each other until the force reading on the user interface corresponding to the chamber mounted is zero or just below.

NOTE: The wire attached to the upper jaw should be of minimal length to ensure that tension can be fully transduced to the detector.

1.3.13. Equilibrate the preparations at 37 °C for at least 30 min prior to the first application of force using the adjustable micrometer.

1.3.14. Assess the tissue viability in 115 mM high potassium (NaCl replaced by KCl on a molar basis) Krebs containing 4.6 mM NaCl, 115 mM KCl, 2.5 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.17 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.1 mM D-glucose at pH 7.4.

NOTE: Isolated vessels are considered viable if the contractile force transduced and recorded as deflection above the baseline in the data recording software of the myograph system is more than 40% of their resting tone, in response to a contractile agent. If the artery does not contract appropriately, then either the optimal basal tension/wall pressure has not been properly adjusted or the artery may have been damaged during isolation or mounting of the vessel.

1.3.15. (Optional) Assess the integrity of endothelial cells by applying phenylephrine to induce contraction of the vessel to 50% of the initial response to KCl (as recorded by the force transducer in the data recording software), followed by adding 1 µM acetylcholine.

NOTE: A good blood vessel preparation is crucial for obtaining consistent and accurate results. A preparation should not be used for the experiment either if the endothelial integrity test is unsatisfactory or it does not respond to KCl, indicating that endothelial function or vascular smooth muscle contractility, respectively, are not satisfactory. In this case, the preparation should be replaced with a new ring from the same blood vessel or a new blood vessel.

2. Normalization to determine the optimal initial tension

NOTE: The normalization procedure allows the determination of the optimal internal diameter (IC) of arteries at which the blood vessel experiences a suitable resting transmural pressure (100 mmHg or 13.3 kPa for mesenteric arteries) and produces maximal active forces in response to vasoactive agents.

2.1. Switch on the computer and open the data recording software (see the **Table of Materials**).

2.2. Save the experiment as a "LabChart data file" with a new name to avoid overwriting the original setting file.

2.3. Open the **normalization settings** window and set the *k* factor as 1. Accept the default values for eyepiece calibration (0.3, if vessel length is unknown, or 1 if vessel length is known), target pressure (13.3), online averaging time (2) and delay time (60). Click **OK** to save the settings.

2.4. Select channels of interest and input the wire diameter (40 µm), tissue endpoints (a1: 0; a2: tissue length as measured), initial micrometer reading in the normalization window.

2.5. Start the normalization procedure by applying the first passive stretch to the blood vessel (turn the micrometer screw counter-clockwise).

2.6. Wait for the vessel to stabilize (3 min) and input the new micrometer reading in the normalization window. The wall tension is automatically calculated and shown as a point on the graph.

NOTE: The micrometer "steps" used during the passive stretch do not need to be the same. The first few stretches could be 20 µm each. As the stretches get closer to the isobar line, the steps can be reduced to 10 µm, 5 µm, 2 µm or even smaller. Have the main chart window open while adjusting the micrometer settings—if a large spike exceeding the isobar line on a length/tension graph (which indicates points of pressure corresponding to a pre-determined value) appears, reduce the tension.

2.7. After each passive stretch, replace the control Krebs with an iso-osmotic high potassium Krebs containing 115 mM KCl. When the contraction reaches a plateau (about 3 min), record the active force (F) by subtracting the passive force at each stretch from the potassium-activated force. Calculate the wall tension as well as the internal circumference (IC) values.

2.7.1. Measure active tension as the deflection above the baseline. The active tension (T) is calculated based on the equation F (mN) = T (mN/mm) x 2 x vessel length (mm). Internal circumference (IC) values are calculated from the micrometer data (IC = 205.6 µm + 2 x "gap").

2.8. Remove the high potassium condition by replacing with fresh Krebs. Repeat washing for three times over 5 min.

2.9. Repeat steps 2.5 to 2.8 (by inducing passive stretches followed by active contraction in alternate turns) until the active tension starts to decrease (**Figure 2**).

2.10. After multiple rounds of alternate stretches, the passive length/tension curves give the value of IC100, the internal circumference of the vessel at a transmural pressure of 100 mmHg, as the crossing point with the isobar line.

NOTE: Each micrometer value during the passive stretches is manually introduced in the software **Normalization Module**. The program automatically records the corresponding force measurement to generate the passive length/tension curve, which gives the value of IC100 as the crossing point with the isobar line (**Figure 2**, right panels). The closer the last point is to the isobar line but just above the better normalization is without damaging the vessels. A point too far above the isobar line may physically damage the mounted vessel, causing unreliable results during the experiment.

2.11. Create the active length/tension curves to determine IC1 values and calculate the normalization *k* factor as the ratio of IC1/IC100, which will be used for this type of blood vessel in subsequent myography experiments.

NOTE: The active length/tension curves are created by plotting the IC values calculated from the micrometer data on the x-axis and active tensions on the y-axis. The IC1 is the value lying within the peak plateau region (red traces in **Figure 2**, right panels). After plotting the active length/tension curves and determining IC1, the normalization *k* factor is calculated as the ratio of IC1/IC100. Based on the normalization *k* factor, the optimal IC for baseline, denoted as IC1, will show on the passive length/tension curve. The micrometer setting for this IC appears under the curve and should be used to set the micropositioner for subsequent myography experiments. The initial tension (T) equals to target pressure (Pi) x IC/2π and the optimal force (F) applied to the vessel equals to T x 2 x vessel length.

2.12. Thoroughly wash out the high potassium Krebs and equilibrate the preparations for another 30 to 45 min. Reset the basal tensions to "zero" so that only active contractile responses will be recorded during the subsequent experiment.

3. Phenylephrine-induced contractions

NOTE: Drugs that can be selected for inducing the vasoconstrictive responses include the unspecific adrenoceptor agonist norepinephrine, the selective α-1 adrenoceptor agonist phenylephrine, the peptide hormone angiotensin II, and the monoamine neurotransmitter 5-hydroxytryptamine. Phenylephrine is used in the present protocol for examination (**Table of Materials**).

3.1. Prepare and mount paired arterial rings as described in section 1.3, one with PVAT intact and the other with PVAT removed, from the adjacent sections of each artery for the experiment.

3.2. After normalization (described in section 2), pre-contract the arterial segments with high potassium Krebs buffer by adding 120 mM KCl solution to the chamber containing Krebs.

3.3. Wait for contraction to plateau (3 min), wash out the high potassium and replace with fresh aerated Krebs buffer. Repeat washing three times over 5 min.

3.4. Repeat the KCl stimulation and washing three times and record the maximal contractile response/tension to KCl by subtracting the baseline tension from the tension due to KCl stimulation.

3.5. After the last contraction and washing, refill the chamber with warm, aerated Krebs buffer and allow the artery to recover for about 30 min before performing the next task.

3.6. To each chamber, add cumulative amounts of phenylephrine (half-log increments from 10^{-10} to 10^{-4} M) to induce the concentration-dependent increases in isometric tension of the quiescent preparations.

3.6.1. Start by adding a low concentration of the agonist to the chamber. After allowing enough time for a stable contraction (3–5 min), add the next concentration. Repeat the steps with increasing concentrations of phenylephrine.

3.7. After adding the last dose of agonist (phenylephrine), wash out the drug thoroughly and refill the chamber with fresh Krebs buffer. Plot the concentration-dependent responses as increasing percentages of the KCl-induced maximal contractions (**Figure 3**).

3.8. (Optional) To assess the contribution of NO, incubate the preparations with the NO synthase inhibitor, L-NAME (10^{-4} M), for 30 min prior to the addition of phenylephrine. L-NAME enhances phenylephrine-induced contractions in the quiescent preparations of mesenteric arteries (**Figure 4**).

NOTE: The inhibitors or antagonists must have sufficient time to achieve equilibration, usually 30–45 min (be consistent for any set of experiments).

3.9. For performing a second concentration-response curve sequentially, wash the chamber completely and repeatedly to remove all of the previous agonists, until no further changes in tone are observed.

NOTE: Parallel experimentation exposes at least two rings obtained from the same blood vessel to the agonist, one under control conditions and one in the presence of the inhibitor(s); in each ring the concentration-response curve will be performed only once. It is preferred to perform parallel experiments, since this provides a better control for the drug's action and blood vessel sensitivity. Serial experiments obtain a concentration-response curve to an agonist in a single ring; washing it out, changing the experimental conditions (e.g., adding an inhibitor), and then repeating the concentration response curve on the same ring. In this case, time controls are needed to show that the drug responses are not due to changes of the tissue over time. One can never be certain that the tissue is in exactly the same state after exposure to a concentration-response experiment. Sufficient time (at least 30–60 min) must be given to allow the vessel segments to return to their resting (basal) tension although in some case, this may not occur instantly after dissociation of the high affinity agonist from the receptors. In addition, high potassium Krebs can be applied between the cumulative concentration-response curves to

reduce desensitization[28]. Remember that most antagonists cannot be washed out completely, thus keep adding it for the rest of the experiment.

4. Endothelium-dependent relaxations/contractions

4.1. Pre-contract a freshly mounted arterial segment (as described in steps 3.2 to 3.5). Again, record the maximal contractile response/tension to KCl by subtracting the baseline tension from the tension due to KCl stimulation.

4.2. (Optional) Incubate the preparations with the NO synthase inhibitor, L-NAME (10^{-4} M), for 30 min prior to the addition of U46619.

4.3. Add the pre-calculated concentrations of U46619 to the chamber and allow a stable, sustained contraction of the artery segments.

NOTE: Vasodilatory responses are induced in mesenteric arteries pre-contracted to about 80% of the maximal responses to 115 mM KCl. Different agonists can be used to induce contraction by activation of their specific receptors. Here, the blood vessel segments with or without PVAT are pre-contracted with U46619 (1–3 x 10^{-8} M; **Table of Materials**), a thromboxane A2 receptor agonist, to induce stable and sustained smooth muscle contractions.

4.4. Add cumulative concentrations of acetylcholine (10^{-10} to 10^{-4} M) to the organ chamber. Concentration-dependent vasodilatory responses of artery segments are presented as percentage of U46619-induced contractile responses (**Figure 5**).

NOTE: For most of the experiment, the next concentration of the relaxing agonist should be added immediately when a plateau is observed to prevent rebound in tension. Concentration-dependent vasodilatory responses of artery segments are normalized as percentage of U46619-induced contractile responses to adjust for minor differences in innervation and diameter between the artery segments (**Figure 5**). Small variabilities in responsiveness between individual rings obtained from the same blood vessel become minimal when a group of six or more experiments are analyzed statistically. When expressing responses as a percentage of the individual tissue's own maximal contractions, it is as appropriate to use a paired analysis (e.g., paired Student's t-test) to compare responses of the same type of tissues from different animals as to compare responses of a single tissue before and after an intervention. When analyzing the effects of PVAT, two-way ANOVA is used followed by multiple-comparison test.

4.5. After adding the final dose of the relaxing agonist, remove the drug from each chamber and refill with fresh Krebs buffer. Wash the chamber thoroughly with Krebs buffer and let the artery stabilize for at least 45 min before performing any additional experiments.

REPRESENTATIVE RESULTS:

Examination of the length/tension relationships to obtain the normalization factor *k*

The amount of stretch applied to a vessel segment influences the extent of the actin-myosin interaction and hence the maximal active force developed. Thus, for every type of blood vessel, determining the amount of stretch needed for maximal active force is required for proper myography studies. Here, normalization of the length/tension relationship is performed for mesenteric arteries isolated from mouse models (**Figure 2**). The arterial segments were suspended in a four-chamber wire myograph system (see **Table of Materials**) on stainless steel pins (40 µm diameter). Isometric tension was recorded using an analog-to-digital converter connected to a computer with a recording program. Chambers contained 5 mL of Krebs buffer, kept at 37 °C and aerated with 95% O_2 and 5% CO_2 to maintain pH at 7.4 throughout the experiment. A passive length/tension relationship was established by incremental stretching of the artery segments until the internal circumference corresponding to 100 mmHg transmural pressure (IC100) was obtained. After each stretch (blue arrows), 115 mM KCl was applied to stimulate contractions (green arrows). The active length/tension curves (red) were plotted by extracting the active force data (subtracting the passive force at each stretch from the KCl-activated force) on the Y-axis and then a graph was created manually with the IC values calculated from the micrometer data on the X-axis. An IC value lying within the peak plateau is IC1 (dashed red lines). The normalization factor *k* was calculated as IC1/IC100 ratios, which could then be applied to samples of the same vessel type in the subsequent experiment.

Presentation and calculation of concentration-response curves

Most concentration-response curves are performed in a cumulative manner. A low concentration of the agonist is added to the bath (preferably starting with a concentration below the threshold for response). After allowing enough time for a possible response (3–5 min), the next concentration is added. When a response is observed, it is allowed to reach a plateau before the next maximal response is obtained. The half-log (average 3.16-fold) increments in concentration of phenylephrine are applied here to study agonist-induced contractions (**Figure 3** and **Figure 4**).

In most cases, contraction-response curves are not expressed as the raw values of tension/force, but as a percentage of the reference response to KCl obtained at the optimal point of the length/tension curve of the individual blood vessel segment. This adjusts for variability in the size or smooth muscle content of the blood vessel as well as corrects for remodeling changes due to aging or pathology. Here, the maximal contractions induced by 115 mM KCl are obtained at the beginning of the experiment and used for calculating phenylephrine-stimulated contractions of mesenteric arteries with or without PVAT, in the absence or presence of L-NAME (**Figure 3** and **Figure 4**).

To study agonists producing relaxation, the vessels are usually contracted to a uniform level—around 50–80% of the maximal contractile response of that tissue. Since the responses to phenylephrine-induced contractions are different between the experimental groups, the present protocol uses U46619 to stimulate stable contractions before applying the cumulative concentrations of acetylcholine. The smooth muscle relaxation is expressed as a percentage of the initial contraction induced by U46619 (**Figure 5**).

The concentration-response curves can be compared as the presence of a leftward or rightward shift (e.g., between a control curve and one obtained in the presence of an antagonist) by determining the concentrations producing equal responses, e.g., 30% or 50% of the maximum. These are termed EC_{30} and EC_{50}, respectively (**Figure 3B**). Statistical comparison of the mean EC_{50} values should be performed on the logarithm of their values. Depression of curves is examined by comparing their respective maximal responses (Emax) (**Figure 3B**). In the examples shown, the phenylephrine-induced contractions in mesenteric arteries were enhanced by L-NAME and the concentration-response curves showed a leftward shift as well as an elevation in the maximal contractions (**Figure 4B**). The acetylcholine-induced relaxations in mesenteric arteries were inhibited by L-NAME, and the concentration-response curve showed a rightward shift as well as reduction in the maximal relaxations (**Figure 5B**).

Vascular responses to various pharmacological agents can be computed as the area-under-the-contraction-curve (AUCC) for contractions and area-above-the-relaxation-curve (AARC) for relaxations, respectively, by using the nonlinear logistic regression analysis for comparison[10] (**Figure 3C**, **Figure 4C** and **Figure 5C**). The effect of L-NAME can be compared by the values of the AUCC/AARC to determine the NO bioavailability (**Figure 6**). The basal and stimulated release of NO in mesenteric arteries with or without PVAT can be expressed as the differences in the concentration-response curves of phenylephrine-stimulated contraction (ΔAUCC) and acetylcholine-induced relaxation (ΔAARC), respectively, in the presence or absence of L-NAME (**Figure 6A** and **Figure 6B**). In the example shown, the presence of PVAT reduced the NO bioavailability in mesenteric arteries collected from mice fed with high fat diet (**Figure 6C**).

FIGURE AND TABLE LEGENDS:

Figure 1: A schematic diagram of the wall structure of arteries. Endothelial cells in the tunica intima mediate endothelium-dependent relaxation/contraction of the vascular smooth muscle, whereas signals released from the perivascular adipose tissue modulates the cross-talks between different layers of the arterial wall.

Figure 2: Representative traces illustrating an experiment to determine the optimal initial tensions for mouse mesenteric arteries. The blood vessel segments were prepared from mesenteric arteries collected from the 16-week-old mice fed with standard chow (**A**) or high fat diet (**B**). After mounting, the passive and active length/tension curves were obtained by step-wise stretching and sequential stimulation with 115 mM KCl (**left panels**). The active contraction generated with each stimulation should increase as the vessel is progressively stretched, until it reaches a plateau at the optimal length. Further stretch will lead to a decrease in the active contraction. The IC100 and IC1 were determined by plotting the passive and active length/tensions curves, respectively (**right panels**). Note that the passive length/tension curve was generated by the Normalization Module after manually inputting the micrometer and force values, whereas the active length/tensions curves plotted manually after calculating the tension and IC values at each step of KCl stimulation. The IC1/IC100 ratios were calculated as normalization *k* factor (**right panels**).

Figure 3: Output recordings of the vasoconstrictor responses to phenylephrine in mesenteric arteries with or without surrounding PVAT. Contractility studies are performed on preparations of mesenteric arteries from 16-week-old Adipo-SIRT1 transgenic mice, in which the human SIRT1 is overexpressed selectively in adipose tissues[29]. Cumulative concentrations of phenylephrine were applied to stimulate the contractions of mesenteric arteries collected from 16-weeks old Adipo-SIRT1 mice (**A**). The contractile responses were recorded and calculated as percentage of 115 mM KCl-induced maximal contraction (**B**). The area-under-the contraction curves (AUCC) were plotted for comparison (**C**). Note that PVAT from Adipo-SIRT1 mice elicited an anti-contractile effect on the response to phenylephrine. -PVAT, arterial rings prepared without PVAT; +PVAT, arterial rings prepared with the surrounding PVAT.

Figure 4. Output recordings of the vasoconstrictor responses to phenylephrine in mesenteric arteries with or without the surrounding PVAT, and in the absence or presence of L-NAME. Mesenteric arteries were collected from 16-week-old wild type mice fed with high fat diet. Thirty min after adding 10^{-4} M L-NAME or vehicle control, cumulative concentrations of phenylephrine were applied to stimulate the contractions of mesenteric arteries (**A**). The contractile responses were recorded and calculated as percentage of 115 mM KCl-induced maximal contraction (**B**). The area-under-the contraction curves (AUCC) were plotted for comparison (**C**). Note that PVAT from dietary obese mice did not elicit anti-contractile effects on the response to phenylephrine. -PVAT = arterial rings prepared without PVAT; +PVAT = arterial rings prepared with the surrounding PVAT; -L-NAME = in the absence of L-NAME; +L-NAME = in the presence of L-NAME.

Figure 5. Output recordings of the vasodilator responses to acetylcholine in mesenteric arteries with or without the surrounding PVAT, and in the absence or presence of L-NAME. Mesenteric arteries were collected from 16-week-old mice fed with high fat diet. At 30 min after adding 10^{-4} M L-NAME or vehicle control, the blood vessel segments with or without PVAT are pre-contracted with U46619 (1–3 x 10^{-8} M; **Table of Materials**), a thromboxane A2 receptor agonist, to induce stable and sustained smooth muscle contractions. Cumulative concentrations of acetylcholine were then applied to stimulate the relaxations of mesenteric arteries with or without the surrounding PVAT (**A**). The relaxation responses were recorded and calculated as percentage of U46619-induced contraction (**B**). The area-above-the relaxation curves (AARC) were plotted for comparison (**C**). -PVAT = arterial rings prepared without PVAT; +PVAT = arterial rings prepared with the surrounding PVAT; -L-NAME = in the absence of L-NAME; +L-NAME = in the presence of L-NAME.

Figure 6. Illustration of the procedure to calculate NO bioavailability. The area-under-the-contraction-curves (AUCC) and the area-above-the-relaxation-curves (AARC) were calculated based on the responses to cumulative concentrations of phenylephrine (**A**) and acetylcholine (**B**), respectively. The differences between preparations pre-treated without and with L-NAME were defined as ΔAUCC (**A**) and Δ AARC (**B**) to represent basal NO contribution and stimulated NO release, respectively. Accordingly, the ΔAUCC (calculated from **Figure 4C**), ΔAARC (calculated from **Figure 5C**), and the sum of both (total NO bioavailability) were presented for comparing the NO bioavailability in mesenteric arteries without and with the surrounding PVAT (**C**). -PVAT =

arterial rings prepared without PVAT; +PVAT = arterial rings prepared with the surrounding PVAT; -L-NAME = in the absence of L-NAME; +L-NAME = in the presence of L-NAME.

DISCUSSION:

Apart from the endothelial cells, signals derived from PVAT play an important role in the regulation of smooth muscle tone reactivity[30]. Healthy PVAT releases NO and anti-inflammatory adiponectin to exert an anti-contractile effect on arteries, which is lost under pathological conditions such as obesity and metabolic syndrome[31,32]. In disease states, PVAT contributes to the development of endothelial dysfunction and other cardiovascular abnormalities[33,34]. Abnormal eNOS expression and function have been reported in PVAT of arteries from obese animals[35,36]. Since both endothelial and PVAT dysfunctions contribute to the development of cardiovascular and metabolic abnormalities[23,37], when performing ex vivo vascular experiment, their role should be considered by including in or removing them from the preparations.

The wire myography system provides a convenient platform to dissect the vasoactive signals released from PVAT using different pharmacological probes[10,38]. However, the compositions in PVAT of different types of arteries, or the same arteries from animals of different genetic background, are not same[39]. Therefore, the wire myography results involving PVAT should not be compared across different types of arteries or the same type of arteries from mice of different strains. Age and the underlying disease states also affect the cellular compositions in PVAT. Here, mice from the same genetic background but with different genetic modifications in their adipose tissue were used for comparing the vasomodulating activity of PVAT.

As a main source of resistance to blood flow, mesenteric arteries are chosen for the present study. Resting tension determines the amount of vasomotor responsiveness[40]. The optimal initial tension of the blood vessel is affected by the type of artery, age, diet, treatment and genetic background of the animals, thus should be determined individually before examining relaxation/contraction-response curves. For the present demonstration, the superior mesenteric arteries were collected from 16-week-old mice fed with standard chow or high fat diet starting from the age of four weeks. The present protocol emphasizes the establishment of optimal settings for maximal active force production of the arterial segments before assessing pharmacological responses. Both passive and active length/tension relationships are studied for mesenteric arteries collected from in-house mouse models. A normalization *k* factor of 1 has been established for preparations from the 16-week-old animals, which is different from the default value of 0.9 or those used by previous publications[41]. Caution is needed when comparing the normalization ratios in the literature due to possible differences in the technique, buffer composition and instrument models, etc. In particular, age, diet and other pathophysiological conditions affect the passive and active tension as well as the pharmacodynamics characteristics of arteries[42].

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DISCLOSURES:

Authors have nothing to disclose.

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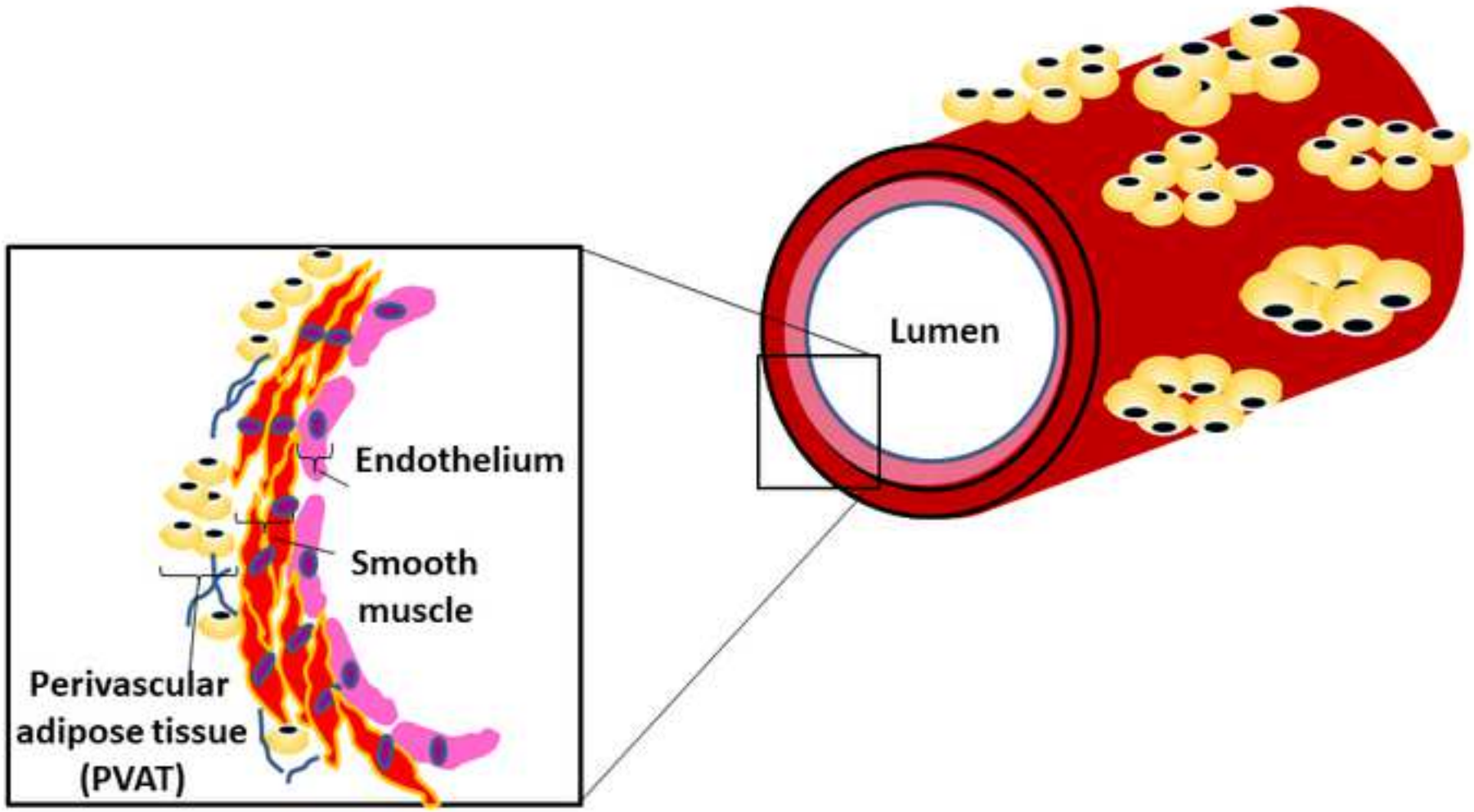

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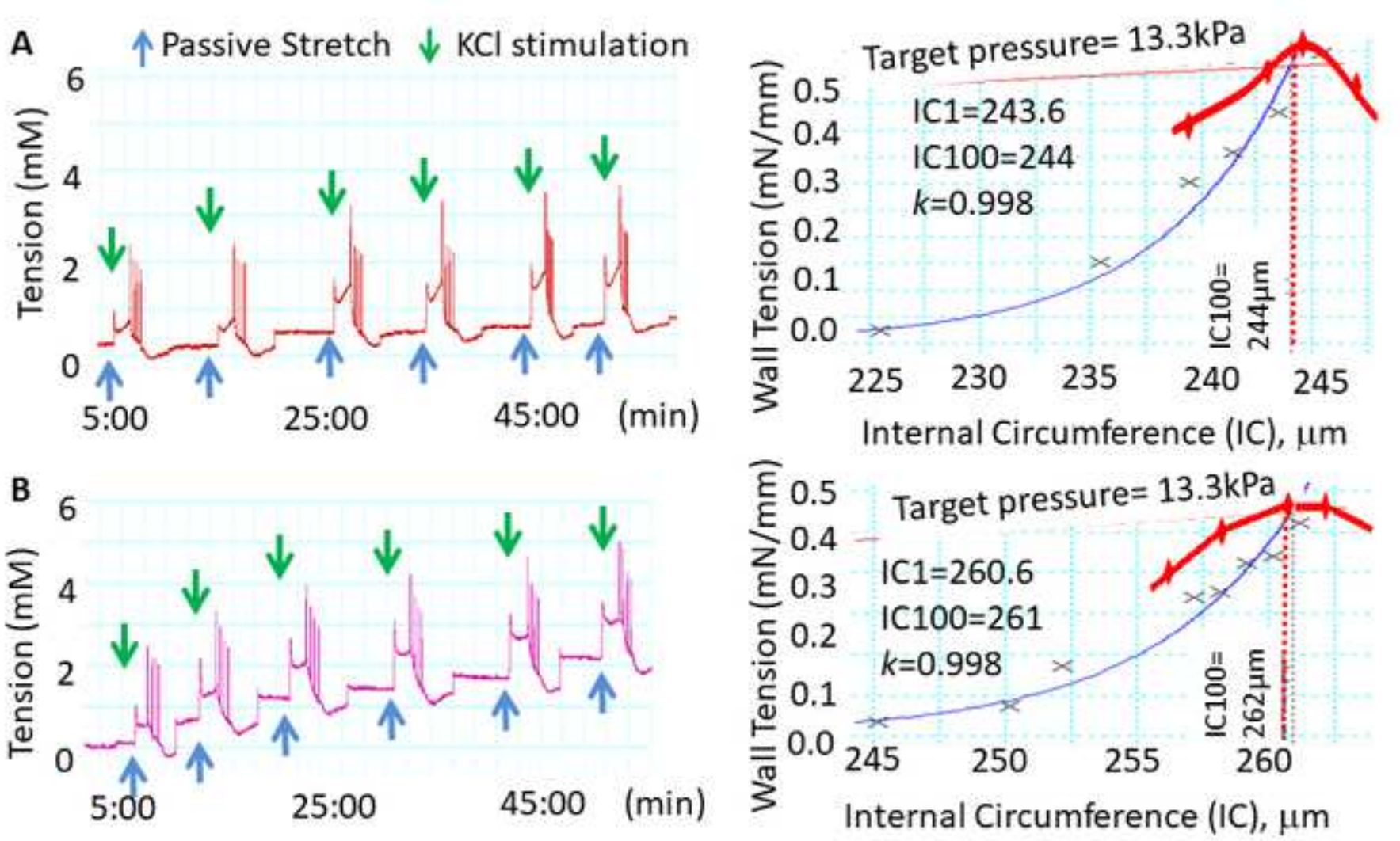

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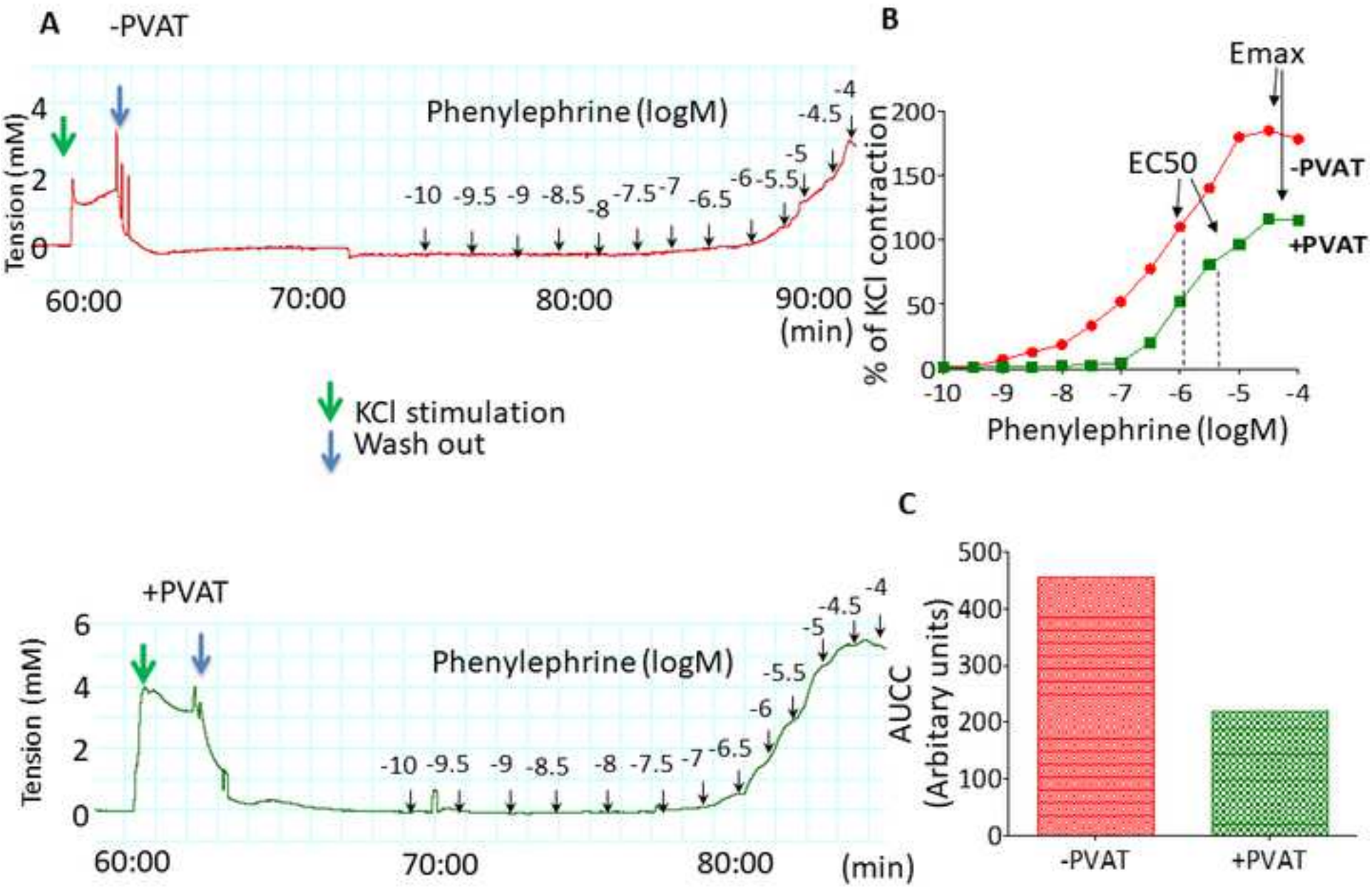

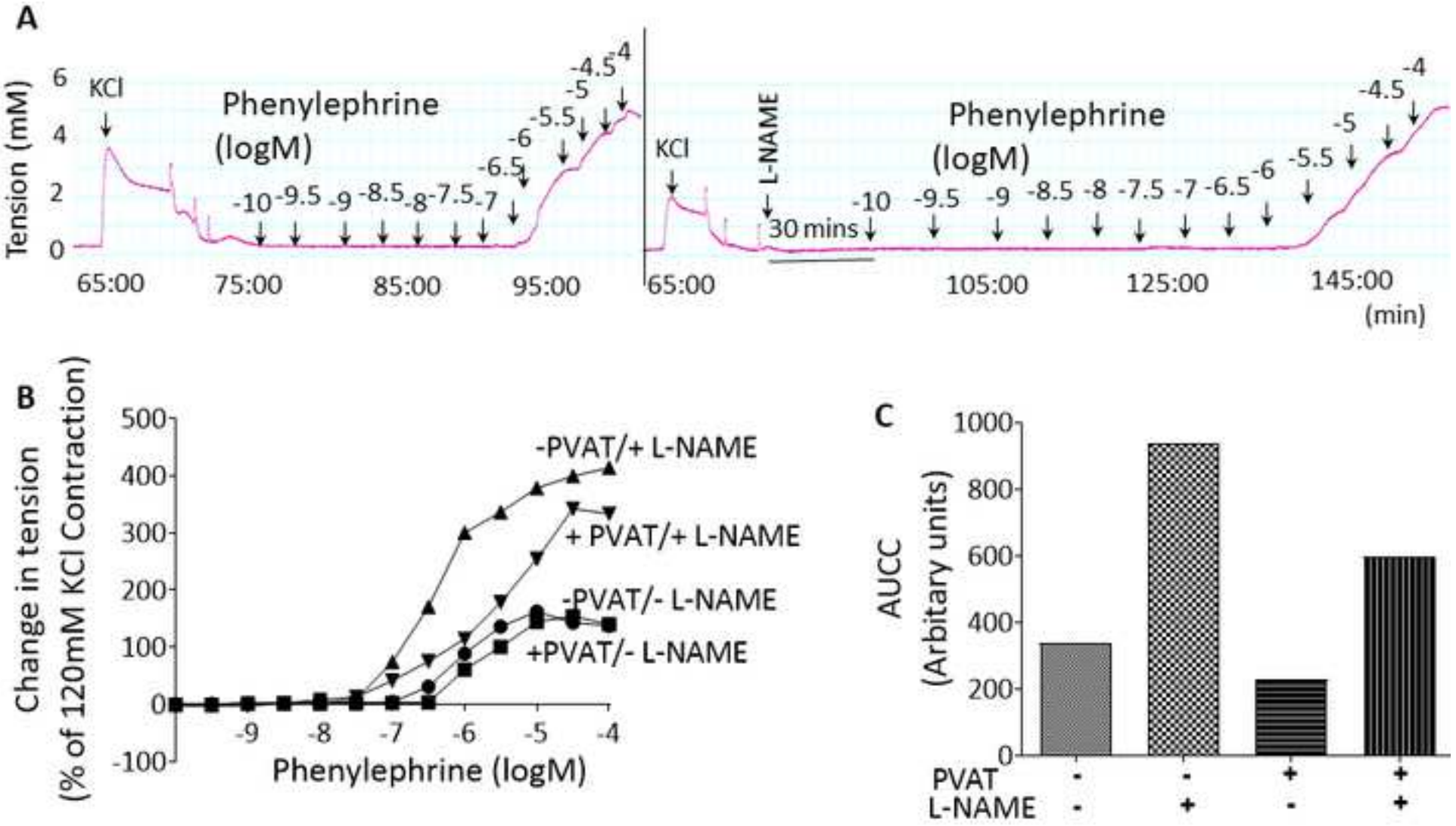

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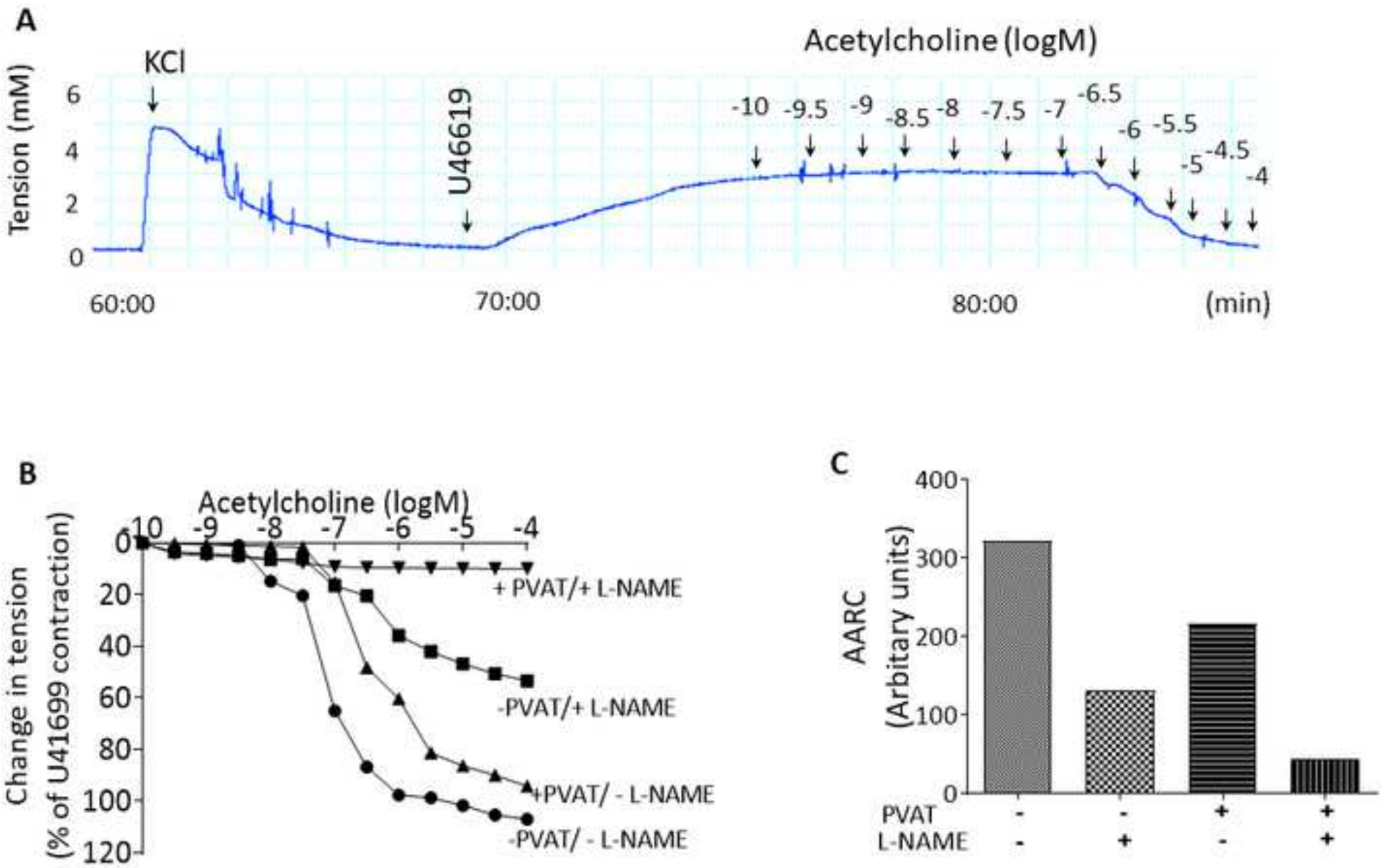

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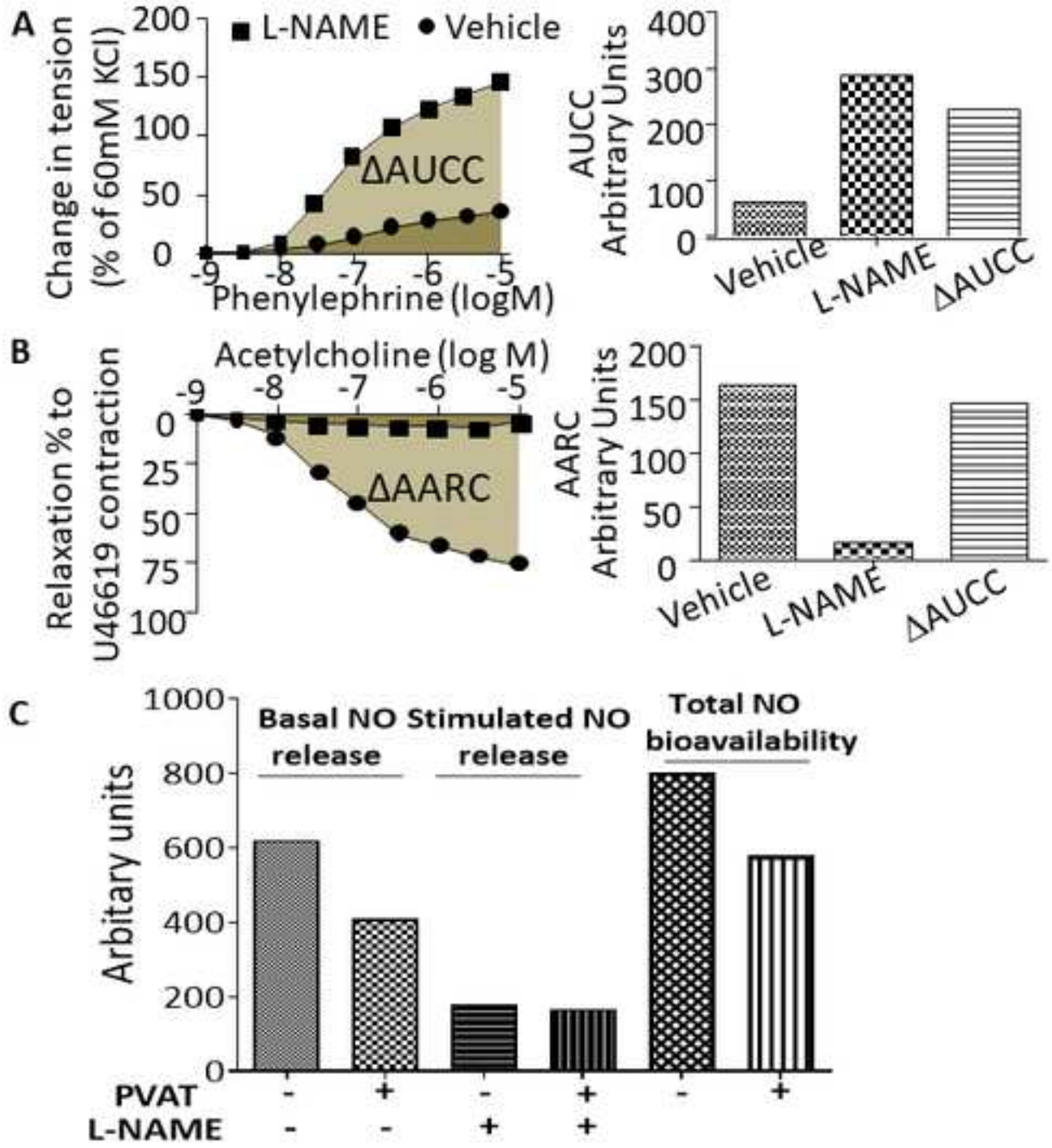

Table 1

Acetylcholine	Stocks	Concentrations (M)	Working solutions
Prepared and stored in small aliquots at -20°C	A	10^{-1} M (146.2 mg in 10 ml distilled H2O)	Prepared ir
Prepared and stored at -20°C for experiment within one week	B	10^{-2} M (50 µL A in 450 µL Krebs)	H
	C	10^{-3} M (100 µL B in 900 µL Krebs)	I
	D	10^{-4} M (100 µL C in 900 µL Krebs)	J
	E	10^{-5} M (100 µL D in 900 µL Krebs)	K
	F	10^{-6} M (100 µL E in 900 µL Krebs)	L
	G	10^{-7} M (100 µL F in 900 µL Krebs)	M
			N
			O
			P
			Q
			R
			S
			T

Cumulative concentrations (M)
n the chamber during experiment
10^{-10} M (5 µL G in 5 mL Krebs)
$10^{-9.5}$ M (10 µL G in 5 mL Krebs)
10^{-9} M (35 µL G in 5 mL Krebs)
$10^{-8.5}$ M (10 µL F in 5 mL Krebs)
10^{-8} M (35 µL F in 5 mL Krebs)
$10^{-7.5}$ M (10 µL E in 5 mL Krebs)
10^{-7} M (35 µl E in 5 ml Krebs)
$10^{-6.5}$ M (10 µl D in 5 ml Krebs)
10^{-6} M (35 µL D in 5 mL Krebs)
$10^{-5.5}$ M (10 µL C in 5 mL Krebs)
10^{-5} M (35 µL C in 5 mL Krebs)
$10^{-4.5}$ M (10 µL B in 5 mL Krebs)
10^{-4} M (35 µL B in 5 mL Krebs)

Table of Materials

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Name of the reagent/Equipment	Company	Catalog /Model number	Stock concentration
Acetylcholine	Sigma-Aldrich	A6625	10^{-1} M
L-NAME (Nω-nitro-L-arginine methyl ester)	Sigma-Aldrich	N5751	3 x 10^{-2} M
Phenylephrine	Sigma-Aldrich	P6126	10^{-2} M
U46619 (9,11-dideoxy-9α,11αmethanoepoxy prostaglandin F2α)	Enzo	BML-PG023-0001	10^{-5} M
Multiwire myograph	Danish MyoTechnology (DMT)	620M	-
PowerLab 4/26	ADInstruments	ML848	-
Labchart7	ADInstruments	-	-
Adipo-SIRT1 wild type mice	Laboratory Animal Unit, The University of Hong Kong	CULATR NO.: 4085-16	
Silicon-coated Petri dishes	Danish MyoTechnology (DMT)		
Tungsten wires	Danish MyoTechnology (DMT)	300331	
Surgical tools			

Working concentration
10^{-10} to 10^{-5} M
10^{-4} M
10^{-10} to 10^{-5} M
1-3 x 10^{-8} M
-
-
-

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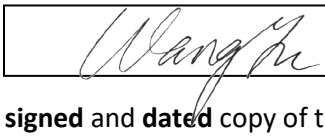
CORRESPONDING AUTHOR

Name:	Yu Wang
Department:	Department of Pharmacology and Pharmacy
Institution:	The University of Hong Kong
Title:	Professor
Signature:	
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Dear Dr. DSouza,

RE: Your manuscript, JoVE59688 "Assessment of vascular tone responsiveness using isolated mesenteric arteries: modulation by perivascular adipose tissues," has been editorially and peer reviewed, and the following comments need to be addressed.

We are grateful to you for sending us the valuable comments and suggestions from the editors. Please find our detailed point-by-point responses to the **editorial comments**.

Comment A1:

Please note that the protocol was edited in terms of structure to match JoVE's style requirements. E.g., I merged all the notes from section 5 to follow the relevant step.

Response: The authors note your valuable comments and formatting, and are grateful.

Comment A2:

Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution

Response: Ethical approval statement included.

Comment A3:

Please a

Response: Statement in Comment A3 is not complete, so we are unable to provide response.

Comment A4:

How? Mention surgical tools used.

Response: surgical method and tools included as sub-section 1.3.3.

Comment A5:

Mention animal strain, age, sex, weight. Add animal strain to the table of materials.

Response: animal information included as sub-section 1.3.1. Information also included in table of materials.

Comment A6:

Mention euthanasia method.

Response: Euthanasia method included as sub-section 1.3.2

Comment A7:

Add to the table of materials. If coated in-house mention silicon concentration.

Response: Revised as sub-section 1.3.4; also included in the table of materials.

Comment A8:

How is this done? Mention tools used.

Response: Surgical procedure and tools included as sub-sections 1.3.5 and 1.3.6.

Comment A9:

Magnification?

Response: Magnification included in sub-section 1.3.6

Comment A10:

Define

Response: PVAT explained in sub-section 1.3.6

Comment A11:

This note is in reality a description of steps to be followed and must be re-written in the imperative voice. Please edit and make this into substeps of 1.3.4. Please ensure correct ordering of events.

Response: Note revised as sub-section 1.3.11 to 1.3.14

Comment A12:

Remove the product name from the manuscript and add this to the table of materials.

Response: Product name removed from manuscript and added to table of materials.

Comment A13:

Assessed by naked eye? Is the contraction measured by the force transducer? Please clarify

Response: Clarified as ‘note’ under sub-section 1.3.16

Comment A14:

Is the contraction measured by the force transducer? Please clarify

Response: Clarified in sub-section 1.3.17

Comment A15:

One of JoVE's policies is that the narrative in the video should sound objective and not biased towards the "features and benefits" of a product featured in the video, i.e the video should not feel like a commercial. To this end, we ask that you please reduce the number of instances of “LabChart” within your text.

Response: Revised.

Comment A16:

Define

Response: Defined in sub-section 2.7.1

Comment A17:

mention step numbers to be repeated.

Response: Revised as sub-section 2.9

Comment A18:

Unclear. Is Krebs added back?

Response: Revised as sub-section 2.8

Comment A19:

I have unhighlighted 2.8-2.9 as they lack filmable content

Response: we note your correction with thanks.

Comment A20:

Define

Response: Corrected - IC100 defined in sub-section 2.10.

Comment A21:

unclear what exactly is done here and it appears that several steps have not been described. How are the segments mounted? What is the K+ concentration in Krebs buffer. What is the KCl concentration? How is the measurement performed? Mention button clicks

Response: Corrected and rephrased as sub-sections 3.1 to 3.6.

Comment A22:

Without any K+?

Response: Corrected - included in sub-section 3.2

Comment A23:

How? What is done exactly? Mention button clicks

Response: Revised as sub-section 3.5

Comment A24:

Unclear. Please describe in detail or reference the steps where this was described previously.

Response: Corrected – referenced in sub-section 4.1.

Comment A25:

Unclear. Please describe in detail or reference the steps where this was described previously.

Response: Corrected – explained in sub-section 4.1.

Comment A26:

Please avoid commercial names. Ensure that this item is listed in the table of materials.

Response: Commercial names removed from text, and items included in table of materials.

Comment A27:

Please avoid commercial names. Ensure that this item is listed in the table of materials.

Response: Commercial names removed from text, and items included in table of materials.

Comment A28:

Please avoid commercial names. Ensure that this item is listed in the table of materials.

Response: Commercial names removed from text, and items included in table of materials.