Video Article

Isolating and Using Sections of Bovine Mesenteric Artery and Vein as a Bioassay to Test for Vasoactivity in the Small Intestine

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Abstract

Mammalian gastrointestinal systems are constantly exposed to compounds (desirable and undesirable) that can have an effect on blood flow to and from that system. Changes in blood flow to the small intestine can result in effects on the absorptive functions of the organ. Particular interest in toxins liberated from feedstuffs through fermentative and digestive processes has developed in ruminants as an area where productive efficiencies could be improved. The video associated with this article describes an *in vitro* bioassay developed to screen compounds for vasoactivity in isolated cross-sections of bovine mesenteric artery and vein using a multimyograph. Once the blood vessels are mounted and equilibrated in the myograph, the bioassay itself can be used: as a screening tool to evaluate the contractile response or vasoactivity of compounds of interest; determine the presence of receptor types by pharmacologically targeting receptors with specific agonists; determine the role of a receptor with the presence of one or more antagonists; or determine potential interactions of compounds of interest with antagonists. Through all of this, data are collected real-time, tissue collected from a single animal can be exposed to a large number of different experimental treatments (an *in vitro* advantage), and represents vasculature on either side of the capillary bed to provide an accurate picture of what could be happening in the afferent and efferent blood supply supporting the small intestine.

Video Link

The video component of this article can be found at http://www.jove.com/video/52020/

Introduction

Alterations in blood flow to a tissue bed can have a large impact on organ function. A primary function of the small intestine is nutrient absorption. Arterial blood flow to the absorptive surface of the gut is required for nutrient absorption and blood flow increases to aid in nutrient absorption as digesta moves along the surface[1]. A decrease in blood flow can cause a reduction in nutrient absorption due to a decrease in the transepithelial gradient[2]. In addition to nutrients, the small intestine can also be exposed to secondary metabolites, drugs, or toxins that exert an effect on localized blood flow in the mesentery. In the case of the ruminant animal, compounds can be liberated from a feedstuff (*e.g.*, nutrients such as amino acids, or toxins such as ergot alkaloids) through fermentative processes of the foregut. If these compounds survive the microbial metabolism of ruminal fermentation, they are now available for absorption or interaction as they travel through the gastrointestinal tract of the animal.

There are a number of different methods available to measure blood flow *in vivo* (*e.g.*, Doppler ultrasound, indwelling blood flowmeters, radiolabeled microspheres, and indicator-dilution techniques) that permit evaluation of various experimental scenarios or treatments. However, to obtain information regarding the mechanical or pharmacological properties of vascular smooth muscle, methods remained limited to large vessels until Mulvany and Halpern[3] published an article describing a technique using wire mounted vascular ring preparations in a myograph. Since the development of this technique, modifications continue to be made to the associated myograph systems that permit a variety of different applications for evaluation of tubular structures. The system has also been adapted to utilize fixed rods for mounting larger vessels[4] where perfusion techniques are not desired.

Because of dissimilarities in vessels from different anatomical origins and distinctions in the same vessels from different species of animal, data from vessel and animal type cannot easily be extrapolated across different vessels or the same vessel in different animal types[5]. Consequently, separate bioassays must be developed and validated anytime these aspects are changed. Recently several bioassays have been developed with these technologies for use in cattle lateral saphenous vein and right ruminal artery and vein[6,7].

This bioassay was developed to specifically investigate the effects that ergot alkaloids have on vasculature supporting the small intestine. It was reported that 50-60% of fed alkaloids appear in abomasal contents, but only 5% are recovered in feces[8]. Strickland *et al.*[9] stated in a review of ergot alkaloids, that available data suggest that the small intestine may be the most important site for ergopeptine absorption. Eckert *et al.*[10] reviewed biopharmaceutical aspects of ergot alkaloids and stated that once they cross the epithelial barrier, ergot alkaloids are transported either by lymphatic system to the subclavian vein or via mesenteric vein and into portal blood. Rhodes *et al.*[11] reported a decrease in blood flow to duodenum and colon in steers consuming a high endophyte-infected (high ergot alkaloid) diet. Using the right ruminal artery and vein

bioassay, Foote *et al.*[12] demonstrated that ergot alkaloids are vasoactive in ruminal vasculature. Foote *et al.*[13] subsequently demonstrated in vivo that ruminal exposure to ergot alkaloids results in a decreased rumen epithelial blood flow. This decrease in blood flow to the absorptive surface of the rumen concomitantly caused a reduction in nutrient (volatile fatty acid) flux. Given the quantity of ergot alkaloids passing on to the small intestine from the foregut; it was hypothesized that a similar effect on small intestinal vasculature and nutrient absorption would occur. This necessitated the development of the bovine proximal ileal mesenteric artery and vein bioassay.

Protocol

Procedures used in this study did not require approval from the University of Kentucky Animal Care and Use Committee because no live animals were used. Prior to collection of any sample used herein, all animals were stunned with a captive bolt and exsanguinated. This was conducted at a federally inspected abattoir facility at the University of Kentucky. An official representative of the USDA Food Safety and Inspection Service observed all activities that dealt with the live animal and handling of the carcass.

1. Preparation of Instrumentation

1. To minimize the amount of time between collections of tissue samples to the start of an experiment, set up equipment and prepare buffers prior to collecting experimental tissue (or if additional personnel are available, these tasks can occur simultaneously).
   NOTE: This not only provides the greatest amount of time that the tissue remains viable to conduct experiments, but some experiments can run for long periods of time (or there is a desire to complete multiple experiments in a day), therefore it is usually desirable to get started as early as feasible.
2. Power up the associated computer, data acquisitions equipment, water bath (for buffer(s)) and myograph unit(s). Place the calibration equipment on the myograph unit(s) and turn on myograph heat (preset to 37.5 °C) and allow units and calibration equipment to warm to preset temperatures.
   1. Open a previously created Chart software settings file on the computer and start a run (but not acquiring data).
3. Warm up the equipment for 10 min.
   1. Start acquiring data.
   2. Zero all channels.
   3. Check, and conduct calibration (if necessary) on each channel (4 channels per multimyograph) to standardize the electric signal supplied from the respective force transducer associated with that channel to a 2-g force supplied by a certified weight.
   4. After calibration and a units conversion are completed, store all subsequent data as grams. Change accordingly based on desires of each separate laboratory; the equipment and software permit the use of other units, such as milliNewtons and volts.
4. Make sure that the gas lines are clear of blockages and turn on gas supply (95% O_2/5% CO_2) to myograph(s) units. Continuously gas all buffers (in the myograph chambers) during the entire procedure.
5. Fill all myograph chambers with 70% ethanol and soak for 10 min, remove ethanol, and repeat for a second 10 min soak.
   1. After removing the ethanol, rinse three times with deionized water, followed by three rinses with prepared buffer (see Section 2 for buffer preparation), leaving the third addition of buffer in the chambers.
      NOTE: At this point the equipment can remain idle in this state until buffer and tissue are prepared and staff is ready to proceed with the experiment.

2. Preparation of Buffers

1. Prepare a 1 L Krebs-Henseleit buffer solution for use in transportation and processing of tissue samples to achieve final concentrations of 11.1 mM D-glucose; 1.2 mM $MgSO_4$; 1.2 mM KH_2PO_4; 4.7 mM KCl; 118.1 mM NaCl; 3.4 mM $CaCl_2$; 24.9 mM $NaHCO_3$.
   1. Mix 9.6 g of Krebs salts into approximately 900 ml of deionized water on a stir plate.
   2. Mix in 0.373 g calcium chloride dehydrate followed by 2.1 g sodium bicarbonate per L of buffer desired.
   3. Gas buffer solution for 20 min with 95% O_2/5% CO_2.
   4. After gassing, adjust pH to 7.05 (filtration of buffer increases pH; final target pH should be 7.4) and adjust volume to 1 L.
   5. Filter sterilize buffer into a clean autoclaved 1 L media bottle and store buffer at 4 °C until ready for use.
2. Prepare separate Krebs-Henseleit buffer solution for use in myograph experiments as described in step 2.1 for the transportation Krebs-Henseleit buffer with additional compounds that pertain to the contractility experiments.
   NOTE: Typically 2 L will suffice for experiments described in this article, but this volume may need to be increased or reduced based on the length of the experiment, number of myograph chambers, and number buffer replacements (relative to treatment additions).
   1. Prior to the gassing step (step 2.1.3), add 9.1 mg (per L of buffer being prepared) of desipramine-HCl to buffer.
   2. Prepare a 1.0 µM solution of propranolol-HCl and add 1 ml (per L of buffer being prepared) of this solution to the Krebs-Henseleit buffer solution. Make this buffer on the day of use and keep at myograph operating temperatures (a temperature that yields a 37.5 °C temperature in the myograph).
      NOTE: The desipramine is added to inhibit the biogenic amine reuptake mechanisms and allow clearing of experimental additions from the buffer bathing the blood vessels to occur more rapidly. The addition of propranolol prevents the non-specific binding of treatment compounds to β-adrenergic receptors.

3. Collection and Preparation of Vasculature

1. As soon as possible, remove the gastrointestinal tract from the carcass. Once the animal no longer exhibits any involuntary reflex, remove the head and hide and then raise the carcass vertically. Removal of the gastrointestinal viscera (esophagus to anus) from the carcass should be completed by approved abattoir personnel (<20 min from time of stunning).
   NOTE: How soon, is generally limited by the location/facility where the intestinal tract is being collected from and standard operating procedures or federally mandated procedures followed by the facility personnel.
   1. In most facilities, conduct manipulations of the gastrointestinal tract at a separate location from the processing of the carcass that is destined to enter the food supply. Use a nearby convenient location where the gastrointestinal tract can be taken and spread out for examination.

2. Once the gastrointestinal tract is spread out, identify the small intestine, the ileocecal fold connecting the small intestine to cecum, and the ileal flange extending proximal to the ileocecal fold (**Figure 1A**).
   1. Using a scalpel or knife, very lightly make an incision in the mesenteric membrane in the center of the ileal flange. Using two index fingers, bluntly dissect away the fat and connective tissue exposing the mesenteric vasculature (**Figure 1B**).

3. From this flange or bulge in the intestinal mesentery, dissect out multiple branches (~2 cm in length) of the exposed mesenteric artery and vein bundles that support this portion of the ileum (**Figure 1C**).
   1. If using forceps to grasp tissue, take care to not grasp directly or pull on the blood vessels at all while removing them as any sort of stretching can damage and negatively effect tissue performance[14]. Blood vessel removal is best done, by cutting across each end of the section to be removed and then cutting alongside or parallel to the now isolated section. For ease, remove some of the surrounding tissue along with vessels to provide some tissue to grasp with a pair of forceps.
   2. With a pair of forceps, submerge tissue sample in a tube containing ice cold Krebs-Henseleit buffer and store on ice until processing can occur in the laboratory.

4. Place tissue sample on a cutting surface or in a petri dish and partially submerge in ice cold Krebs-Henseleit.
   1. Using #5 jewelers forceps, Noyes iris scissors, and magnifying lamp or dissecting scope (2.5 to 5.0X magnification is sufficient), carefully dissect away the surrounding fat and connective tissues and separate the artery and vein. Identifying the vessel opening at one end of the section and carefully grasp the fascia surrounding the vessel with the forceps.
   2. Make a cut with the scissors parallel to the vessel by sliding the tip of the scissor underneath the raised fascia. After the initial incision, the fat and connective tissues can be further detached from the vessel by cutting down either side with the scissors. Ensure that the vessels are as clean as possible while minimizing the amount of time the vessels spend on the bench.
   3. Return blood vessels to tubes of fresh Krebs-Henseleit buffer and store at 4 °C (samples can remain stored for up to 24 hr after collection and still produce valid contractility data).
   4. Using a razor blade, slice vessel to be used in myograph into desired number of 2-mm sections (it is helpful to use a tissue slicer to obtain consistent cross-sections).
   5. Examine each section under a dissecting scope (12.5X magnification) to ensure that each section has no abnormalities, branches, valves, or superficial damage inadvertently done during dissection and cleaning. Replace the vessel section if there is any abnormality, branch, valve, or superficial damage.

5. Store acceptable sliced vessel sections (submerged in Krebs-Henseleit buffer) on ice or at 4 °C until ready for mounting in myograph chambers (usually <30 min).
6. Gently mount the vessel on the myograph by inserting the supports through the lumen and increase the tension (using the micropositioner on the myograph) being careful to not stretch the vessels above a 2 to 3 g reading.
7. Once all chambers are covered, vacuum the buffer from all chambers and refill with 5.0 ml of buffer and start a 15 min timer to begin the equilibration period and the experiment.

4. Experiment

1. Equilibrate all vessel sections for 1.5 hr to achieve a stable resting tension of 1.0 g.
   1. Replace the Krebs-Henseleit incubation buffer every 15 min.
   2. During the equilibration, constantly adjust the tension on the blood vessel sections up to 2 g and then allow to relax down to approximately 0.80 g. Try not to allow vessels to relax too much (they may slip off the mounts). Try to achieve a steady baseline tension, where the vessel holds 1 g tension without requiring adjustment.

2. During the equilibration, prepare an aqueous 1.32 M solution of KCl to be used as a reference.
3. Following the completion of a satisfactory equilibration, add 500 µl of the 1.32 M KCl solution to result in a 0.12 M solution in the 5.5 ml solution bathing the vessel.
   1. Following the addition of the 1.32 M KCl, do not adjust the tension manually as the maximal response from this addition is used to evaluate tissue viability and can be used to normalize treatment data (*e.g.* concentration responses to an agonist).
   2. Replace the buffer in 15-min intervals until tension has returned to the baseline value of 1.0 g.

4. Once baseline is reached, change the buffer and simultaneously begin a 1 min timer. Do this for all chambers simultaneously to avoid a staggering of start times.
   1. Vortex the standard to be added and prepare a comment for chamber 1 during the 1 min countdown.
   2. Add the standards in 25 µl aliquots to each chamber as the experiment dictates (this keeps the treatment below 0.5% of the total volume).
   3. When the last standard has been added, start a 9 min timer.

4. At the end of the 9 min standard incubation, remove the treatment-containing buffer from the chambers and add 5.0 ml of fresh buffer, and start the 2.5 min timer.
5. Repeat step 4.4.4.
6. At the end of the second 2.5 min rinse, vacuum the chambers and add fresh buffer, and start a 1 min timer to countdown the commencement of the second standard addition.

5. Continue this cycle for all of the day's standard additions.
6. During the final standard addition and subsequent 9 min incubation prepare the 1.32 M KCl reference compound for an end of run reference dose addition of 500 µl.
7. Following the 1 min interval after the final treatment addition, add the KCl and start a 9 min timer.
   NOTE: The KCl addition is to confirm tissue viability at the conclusion of the experiment and is helpful if administering a treatment that results in negligible response.
8. At the conclusion of the final 9 min reference compound incubation, vacuum or remove buffer from all of the chambers. Do not add fresh buffer. Conclude the experiment.
9. Save Chart file, remove blood vessel sections and dispose of properly, and follow the clean up protocol (provided by manufacturer and can be lab specific) for myograph equipment.

Representative Results

The blood vessels used to generate the included results were collected from 6 Holstein steers (425 ± 8 kg) within a 3 week interval. An example of a typical mesenteric vein contractile response to KCl and treatment additions increasing in concentration is presented in **Figure 2**. The magnitude of response will vary some with the size of the vessel (correlated with the size of the donor animal), but can also be influenced (negatively) by improper handling (stretching) of the vessels during collection and cleaning. Therefore, it is important to observe a significant contractile response in the vessels exposure to KCl. If a negligible response is observed at this point, it is not advisable to proceed any further into the procedure with that vessel. As seen in the 9 min incubation periods in **Figure 2**, there are incremental increases in the response that correspond with increasing concentrations. Although desirable for this particular experimental design, it may not always occur or be sought after with other treatments. The KCl addition at the conclusion of the experiment, although not used in the processing of data collected, is very important as it confirms the viability of the tissue at the conclusion of the experimental period (becomes more important if the vessel is not responding to any of the treatment additions).

The data presented in **Figure 2** are recorded in grams of tension. It is important to normalize these data to minimize any animal-to-animal and vessel-to-vessel variation. Thus, in addition to being used as an indicator of viability, the maximum KCl response is used to normalize all of the treatment additions. This generates contractile response data as a percentage of the KCl maximum. This is how mesenteric artery and vein response curves to increasing concentration of 5-hydroxytryptamine (serotonin; **Figure 3**) and norepinephrine (**Figure 4**) are presented. The data in these figures have been fit sigmoidal concentration response curve using a non-linear regression.

The mesenteric vein response to both 5-hydroxytryptamine (**Figure 3**) and norepinephrine (**Figure 4**) started out as negative values. This is a result of correcting the maximum tension recorded during each 9 min incubation period by the baseline tension recorded prior to the initial KCl addition. This baseline correction ensures that any slight differences in tension are not included in the analyses and allow the data presented to reflect only a change in tension. The negative value results from the blood vessel relaxing and dropping below pre-KCl baseline levels before the subsequent treatment addition. This is especially common in venous samples where the initial concentrations of the treatment or agonist are too low to cause a contractile response. This was not the case for the mesenteric artery response to either biogenic amine, which held tension above baseline until the vessel began to respond to the treatment additions. This difference exemplifies the differences in vessels on either side of the capillary bed and why they were both considered as part of this bioassay.

This bioassay can be used to evaluate vasoactivity of compounds on blood vessels supplying nutrients to the small intestine as well as blood vessels carrying absorbed nutrients away from the small intestine. Data in **Figures 3 and 4** are simple examples of how this may be accomplished. In the case of 5-hydroxytryptamine (**Figure 3**), there was not a difference in the arterial or venous contractile response. Conversely, the contractile response by the mesenteric vein to increasing concentrations of norepinephrine was greater than that of the mesenteric artery.

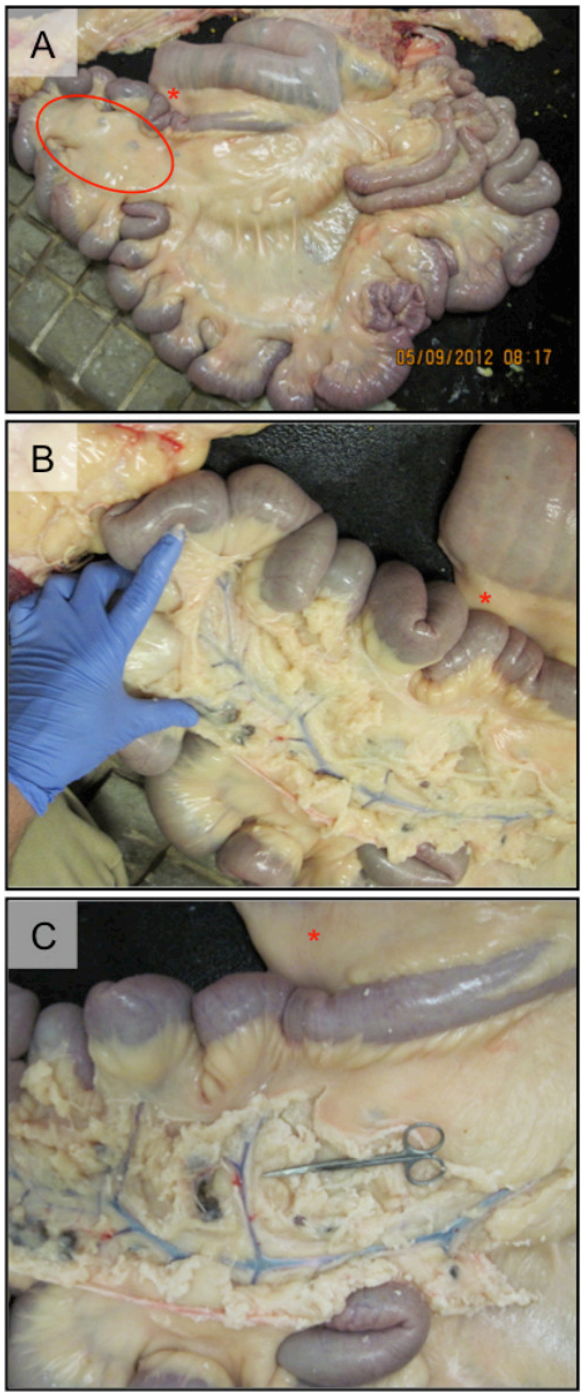

Figure 1: An example of bovine intestinal tract used as source of mesenteric blood vessels. The red asterisk indicates the visible portion of the ileocecal fold in all three panes as a point of reference. (**A**) The whole tract with the red oval indicating the ileal flange where sampling of vasculature occurred. (**B**) Exposure of mesenteric vascular bed. (**C**) Iris scissors are pointing towards an exposed branch of mesenteric vein (visible) and artery (not visible) just prior to collection.

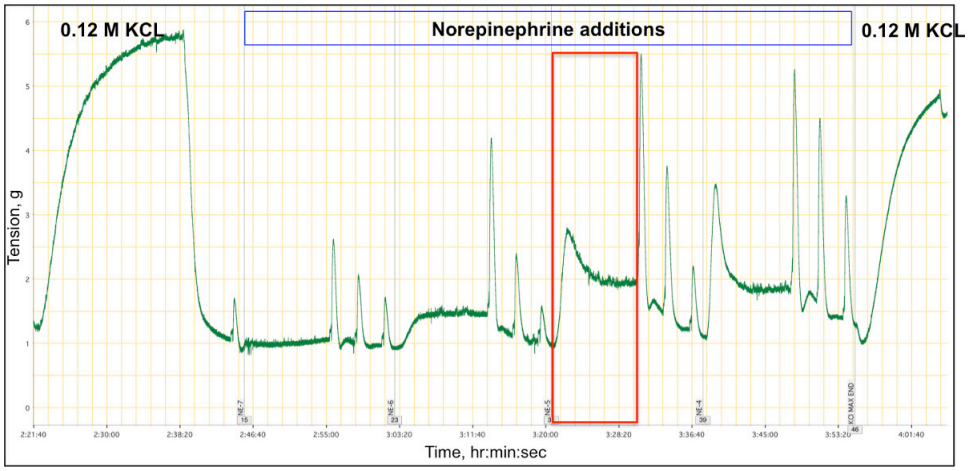

Figure 2: Example of a concentration response of a mesenteric vein to increasing concentrations of norepinephrine (1×10^{-7} to 1×10^{-4} M). The large events at the beginning and end of the trace are responses to the 0.12 M KCl additions. The red rectangle signifies the data collection region corresponding with the 9 min incubation period for each treatment addition. The 3 slender spikes that follow this are artifacts generated by the buffer replacements and are not included in the analysis. Please click here to view a larger version of this figure.

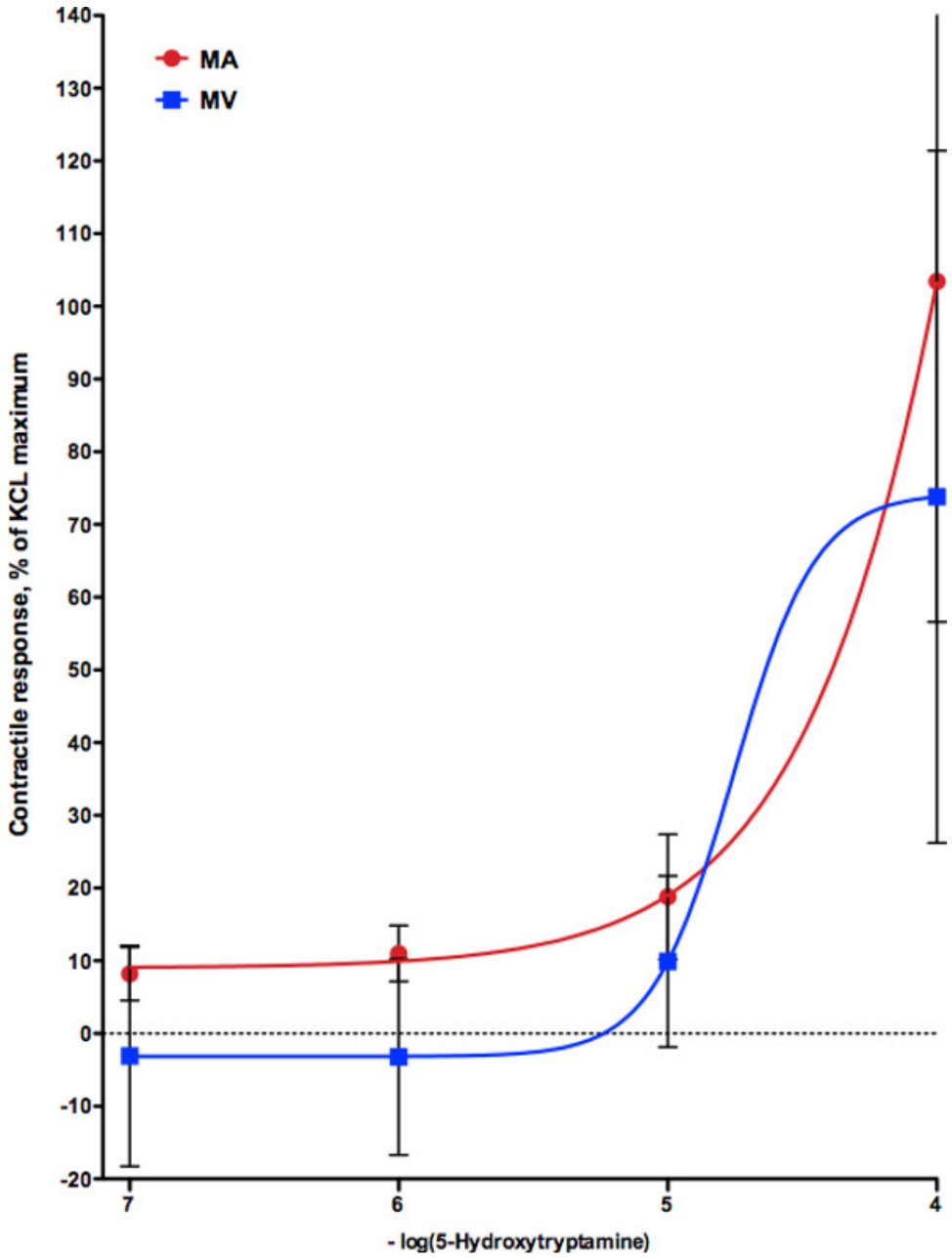

Figure 3: Mean contractile responses of mesenteric artery (MA) and vein (MV; n = 6 steers) to increasing concentrations of 5-hydroxytryptamine (serotonin). Lines shown were generated using non-linear regression analysis to fit the data to a sigmoidal concentration response curve which utilized the following equation: y = bottom + [(top – bottom) / (1 + $10^{(\log EC_{50} - x)}$)], where top and bottom are the percentage of 120 mM KCl maximum contractile response at the plateaus, and the EC_{50} is the molar concentration of alkaloid producing 50% of the KCl maximum response.

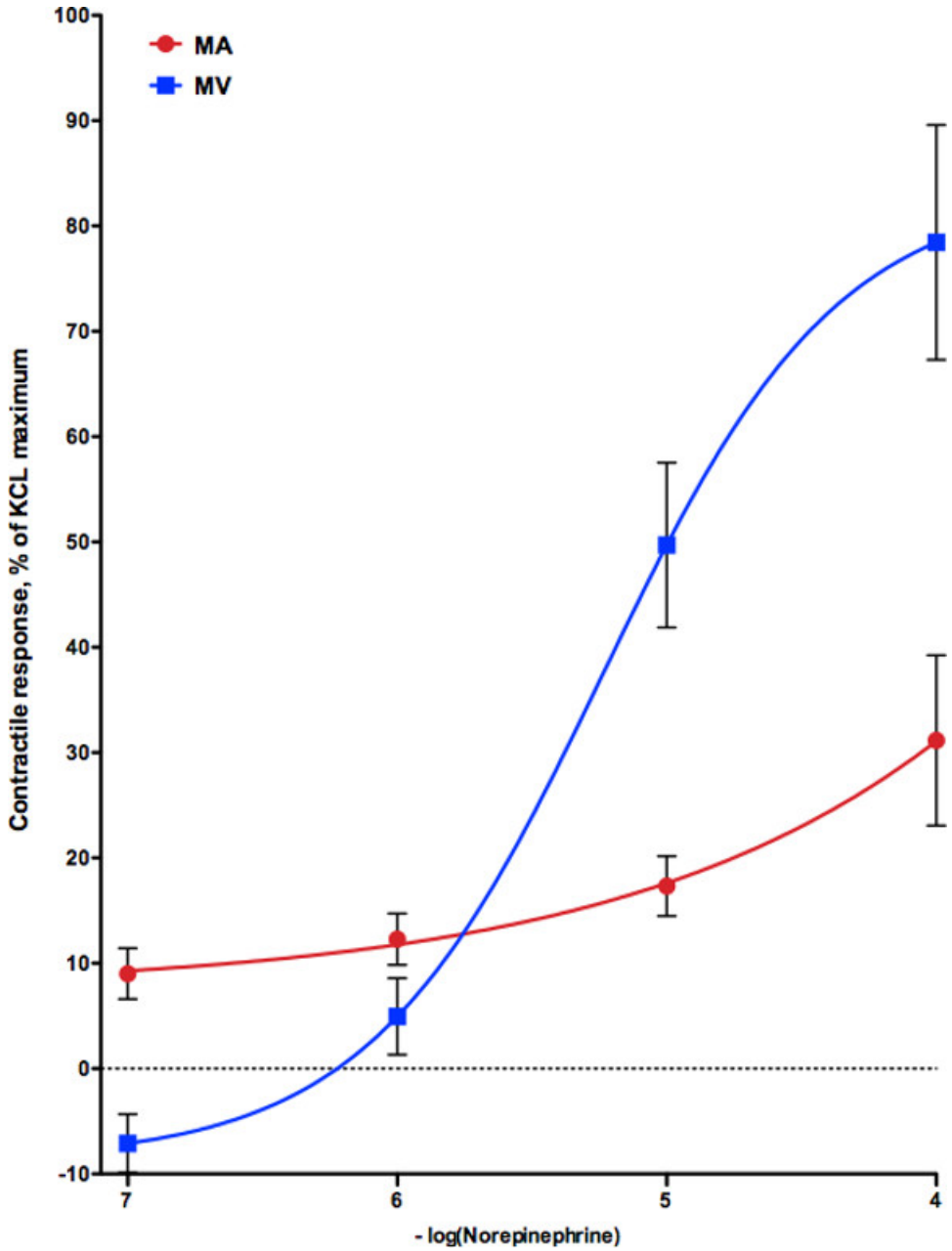

Figure 4: Mean contractile responses of mesenteric artery (MA) and vein (MV; n = 6 steers) to increasing concentrations of norepinephrine. Lines shown were generated using non-linear regression analysis to fit the data to a sigmoidal concentration response curve which utilized the following equation: y = bottom + [(top – bottom) / (1 + $10^{(\log EC_{50} - x)}$)], where top and bottom are the percentage of 120 mM KCl maximum contractile response at the plateaus, and the EC_{50} is the molar concentration of alkaloid producing 50% of the KCl maximum response.

Discussion

The initial challenge in the development of this bioassay was the establishment of a repeatable collection site for mesenteric vasculature. Sample site consistency is critical, as some of the functions of the small intestine change during the progression from the jejunum through the ileum and consequently the mesentery vary in a similar pattern. The ileal branches of mesenteric artery and vein were the most easily identified through anatomic landmarks. By locating the cecum and following the ileocecal fold to its terminus on the left side of the mesentery, this is near the termination of the cranial mesenteric artery[15]. The area of longer mesentery (termed the flange) and the extension of the ileocecal fold (unique to the bovine animal)[16] made sampling across numerous animals very repeatable. One of the larger pitfalls that can be made is at this phase of the bioassay. Mechanical stretching has a large negative impact on subsequent vessel performance[14] and the researcher will notice that vessels that are excessively manipulated or stretched during removal and subsequent cleaning will not respond to the reference compound being used. This unfortunately is the only way to truly evaluate the quality of the vascular preparation at a functional level and vessels that do not respond to KCl should not be considered further for generation of reliable data.

Two other method validation steps (not shown) that led to the final method presented herein were the use of other chemicals as reference compounds and testing the viability of blood vessels following 24 hr storage. Norepinephrine and serotonin were evaluated as reference compounds, but responses by the mesenteric artery and vein were too variable across vessel type and across animals for them to be successfully used. Conversely, the addition of 120 mM KCl resulted in a very reproducible response across both artery and vein preparations. Also, artery and vein responses were evaluated the day of collection and reevaluated 24 hr post collection and there were no differences in agonist response by either the artery or the vein. This was an important aspect of the bioassay, as there are often a large number of treatments (or various treatment combinations) that can be applied, but researchers are limited by space on the myograph and time. By extending the period of viability out 24 hr from collection, the time for additional experimentation is increased greatly.

It is important for the researcher to understand the flexibility of the treatment structure applied to this bioassay. A researcher can add any compound to the incubation buffer that he or she is interested in chronically exposing the blood vessels to in order to control something of biologic relevance or as a treatment (in the case of Klotz *et al.*[17], this was done with lateral saphenous veins continuously exposed to ketanserin, which antagonized a serotonin receptor of interest). The mesenteric blood vessels could be pretreated with a compound of interest prior to conducting a contractile response experiment. The bioassay, as presented, was designed to conduct a cumulative concentration response experiment looking at ergot alkaloids as agonists and whether prior dietary exposure to ergot alkaloids affected the vascular response[18]. The representative results demonstrate that this method can produce a measurable vascular response and only four concentrations were used to achieve this. In the case of Egert *et al.*[18], there were up to 10 different concentrations used to construct a response curve. This method is a way to evaluate a large number of different compounds or receptors on blood vessels from a single animal in a small window of time.

Disclosures

Mention of trade name, proprietary product, or specified equipment does not constitute a guarantee or warranty by the USDA and does not imply approval to the exclusion of other products that may be available.

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