

Canine mesenteric artery and vein convey no difference in the content of major contractile proteins

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Abstract

Background: Mesenteric arteries and veins are composed of tonic smooth muscles and serve distinct functions in the peripheral circulation. However, the basis for the functional disparity of the resistive and capacitative parts of the mesenteric circulation is poorly understood. We studied potential differences in the expression levels of six contractile proteins in secondary and tertiary branches of the inferior mesenteric artery and vein along with differences in the vessel wall morphology.

Results: Bright field and electron microscopy showed that both vessel walls had the same major structural elements. The arterial walls, however, had greater number, and more tightly assembled, smooth muscle cell layers compared to vein walls. The content of actin, myosin heavy chain, myosin light chain, and calponin was similar in the two blood vessels. The artery expressed higher amount of the actin-binding protein caldesmon than the vein (41.86 ± 2.33 and 30.13 ± 3.37 $\mu\text{g}/\text{mg}$ respectively, $n = 12$). Although the total tropomyosin content was almost identical in both blood vessels, the alpha isoform dominated in the artery, while the beta isoform prevailed in the vein.

Conclusions: Canine mesenteric artery and vein differ in vessel wall morphology but do not convey differences in the expression levels of actin, myosin light chain, myosin heavy chain and calponin. The two vascular networks express distinct amounts of caldesmon and tropomyosin, which might contribute to the fine tuning of the contractile machinery in a manner consistent with the physiological functions of the two vascular networks.

Background

Various smooth muscles differ in the content of contractile proteins and their isoforms, and these differences might contribute to manifestation of characteristic contractile phenotypes. For example, phasic and tonic behavior of gastrointestinal smooth muscles has been attributed to different expression levels of caldesmon and caldes-

mon-binding proteins, but not to differences in the relative proportions of myosin, actin, calponin, and tropomyosin [1,2]. Arteries and veins of different vascular beds are primarily composed of tonic smooth muscles. However these two types of blood vessels serve distinct functions in the circulation, i.e. resistance vs. capacitance. The basis for the functional dissimilarities between the re-

sistive and capacitative networks is poorly understood, although various possibilities have been considered. Arterial walls, for example, include a substantial layer of smooth muscle, which provides efficient adjustment of arteries to blood pressure changes. In contrast, the muscle layer of venous wall is thinner and veins better accommodate changes in blood volume. Differences in smooth muscle layer thickness, however, do not always satisfactorily explain why in some instances, including stimulation of postganglionic nerve terminals, veins are more responsive than corresponding arteries [3–6]. Although differences in the type and amount of neurotransmitters, receptor density and/or sensitivity for neurotransmitter action, or signal transduction mechanisms that couple membrane receptors to the contractile elements have been proposed to underlie the functional distinctions between capacitative and resistive regions of the mesenteric circulation, the possibility that mesenteric artery and vein

convey distinct expression levels of contractile proteins has not been ruled out. The present study was carried out to determine whether the artery and vein form the same circulatory bed (i.e. mesenteric circulation) demonstrate differences in the expression levels of six major contractile and thin filament-binding proteins, i.e. actin, myosin heavy chain (MHC), myosin light chain (MLC), tropomyosin (TM), calponin, and caldesmon. We also compared the anatomical structure of the vessel walls to obtain basic information about the morphology of the vessels under study, and to interpret the protein density measurements in relation to vessel wall morphology. Our experiments provide hints for understanding how the morphology and contractile protein content might contribute to distinct functions of the arterial and venous sites of the splanchnic circulation. In addition, this study contributes to the relatively rare comparative studies on capacitative and resistive blood vessels from the *same* vascular bed.

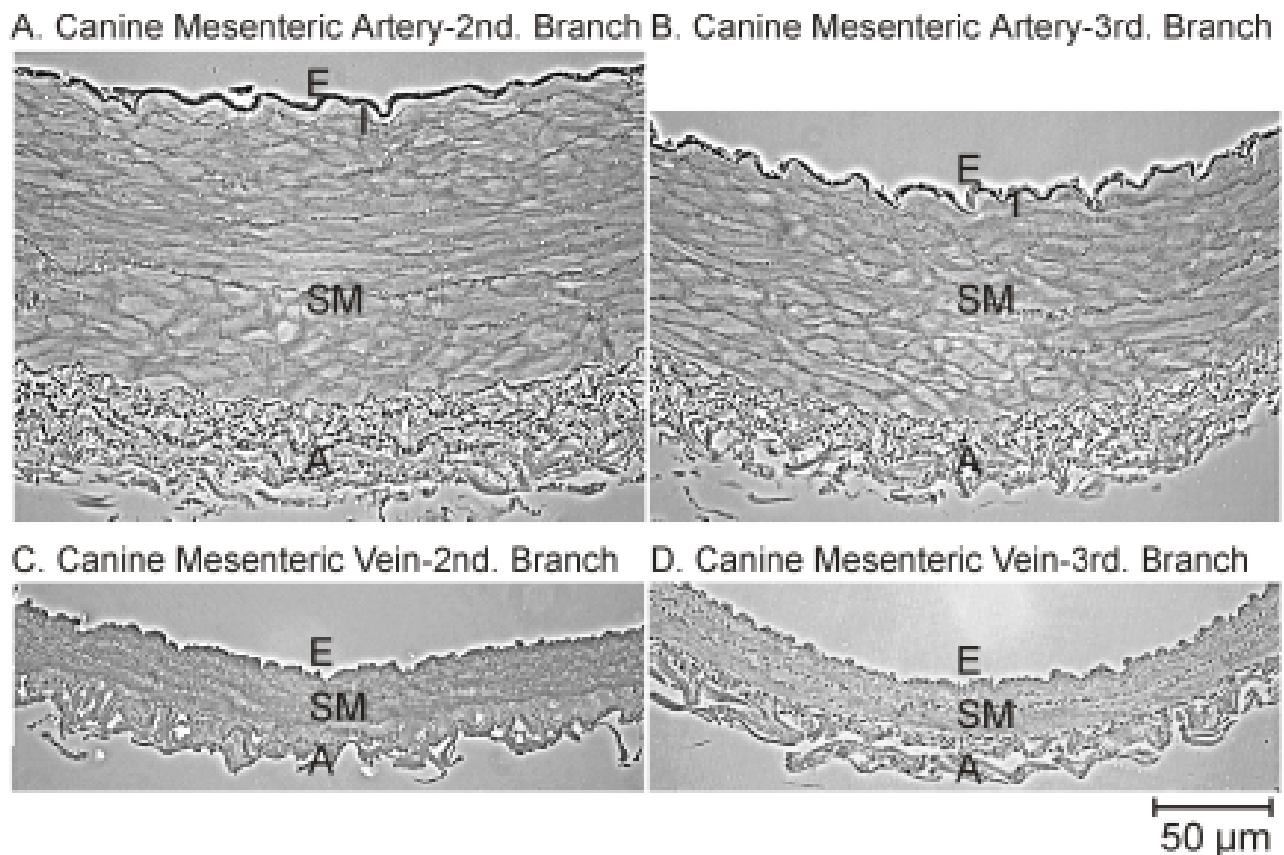


Figure 1

Phase contrast images of cross sections of canine isolated mesenteric artery and vein. Panels A and B represent secondary and tertiary branches of artery, Panels C and D refer to secondary and tertiary branches of vein. The panels depict endothelial layers (E), elastic lamina (l) smooth muscle cells (SM) and tunica adventitia (A). Scale bar applies to all panels.

Results

Morphology of mesenteric artery and vein

The gross organization of canine mesenteric artery and vein preparations was studied by conventional phase contrast microscopy. The walls of secondary branches of the artery were $180 \pm 5 \mu\text{m}$ in thickness and consisted of an inner endothelial layer (tunica intima), a tunica media and an outer tunica adventitia (Fig. 1, Panel A). Cross sections of tunica media revealed that it consisted primarily of smooth muscle cells, averaging 24 ± 0.5 cells in cross section ($n = 10$ fields of view, $N = 3$ animals). Although, the structure of secondary and tertiary branches of mesenteric artery was similar, the smooth muscle layers of tertiary artery consisted of 12.4 ± 0.4 smooth muscle cells in cross section (Fig. 1, Panel B), and were notably thinner ($130 \pm 4 \mu\text{m}$, $n = 10$, $N = 3$, $P < 0.001$).

Mesenteric veins and arteries exhibited similar gross organization, but expected differences were also observed in some individual structural elements. For example, secondary branches mesenteric vein consisted of 3.4 ± 0.2 smooth muscle cells and had an average wall thickness of $50 \pm 4 \mu\text{m}$ (Fig. 1, Panel C). Similarly, tertiary-branched mesenteric veins consisted of 3.3 ± 0.2 smooth muscle cells in cross section and were $40 \pm 2 \mu\text{m}$ thick (Fig 1, Panel D, $n = 10$ fields of view for each vessel). These observations point to another structural difference: unlike arteries, the average number of smooth muscle cells per cross section, and thus the difference of wall thickness, between secondary and tertiary veins, was insignificant ($P > 0.05$).

Ultrastructural details in vessel wall morphology were observed by electron microscopy of the canine mesenteric artery and vein. Fig. 2 shows the organization of endothelium, smooth muscle cells and nerves within the artery. Endothelial cells made close association with smooth muscle in regions of the inner aspect of the artery that was free of internal elastic lamina (Fig. 2, Panel A). Within the tunica media, smooth muscle fibers were organized so that the long axis of the cells ran at an angle to circular axis of the vessel wall (Fig. 2, Panel B). These cells were tightly packed with thick and thin filaments and had many of the ultrastructural characteristics of smooth muscle cells including dense bodies and bands, occasional mitochondria, sarcoplasmic reticulum, free ribosomes, caveolae and a basal lamina (Fig. 2, Panels C and D). Elastic connective tissue and an abundance of collagen fibrils occupied intercellular spaces between smooth muscle cells. In the adventitial layer there was an abundance of collagen fibrils. Occasional nerve trunks containing several axons were observed adjacent to the external elastic lamina and often in regions where the lamina was disrupted. Nerve axons contained several types of vesicles includ-

ing dense cored and small electron lucent vesicles (Fig. 2, Panel D).

The overall ultrastructural organization of the mesenteric vein (Fig. 3) was similar to that of the artery. The tunica media was primarily made up of smooth muscle cells and extracellular matrix proteins, the tunica adventitia was made up of collagen fibrils, an external elastic lamina, occasional nerve processes and fibroblasts. Since the same set of components were observed in sections of either blood vessel wall, the characteristic feature that distinguishes the artery from vein was wall thickness and number of smooth muscle cells that span through vessel wall.

Total protein content of mesenteric artery and vein

For these experiments we used mesenteric arteries and veins from 12 different dogs. The tissue homogenization conditions [1] favor extraction of the major smooth muscle cell contractile proteins actin and myosin, and the thin filament-binding components caldesmon, calponin, and tropomyosin (TM). The protein concentrations determined in the clear homogenates from the artery decreased after each extraction cycle from 6.26 mg/ml in the first, to 1.28 mg/ml in the second, and were lower than the detectable minimum (0.1 mg/ml) of the BCA micro assay after the third cycle. Likewise in the vein, the protein concentrations after the third cycle were lower than the detectable level. Therefore, the protein extraction after the third cycle was considered complete. The 3 supernatants from each artery and from each vein were pooled together, and the protein concentrations were assayed again. On an average, the total protein yield was significantly higher from the artery than from the vein: 0.312 ± 0.024 mg/mg dry tissue weight versus 0.225 ± 0.016 mg/mg, respectively ($P < 0.05$, Table 1). Thus, the calculated average ratio of total protein in the artery versus vein was 1.4:1.

Contractile protein content of mesenteric artery and vein

To test whether differences in the total protein content were associated with differences in the content of individual contractile proteins, we quantified proteins by using dilution series of purified protein standards, as illustrated with actin in Fig. 4. The band densities of increasing amounts of actin standards on Western immunoblots (Fig. 4, Panel A) were fitted to a linear relationship (Fig. 4, Panel B), which was then used to calculate the amounts of actin in extracts from the artery and vein (Fig. 4, Panel C). As shown in Table 1, densitometry of the immunoreactive actin bands and calculation of the actin amounts as a fraction of the separated total protein, revealed a lack of difference between the artery and vein ($P > 0.05$). Similar assays showed that the contents of MHC (200 kDa) and MLC (20 kDa) were also indistinguishable in the artery and vein (Table 1), and so were the actin/MHC ratios:

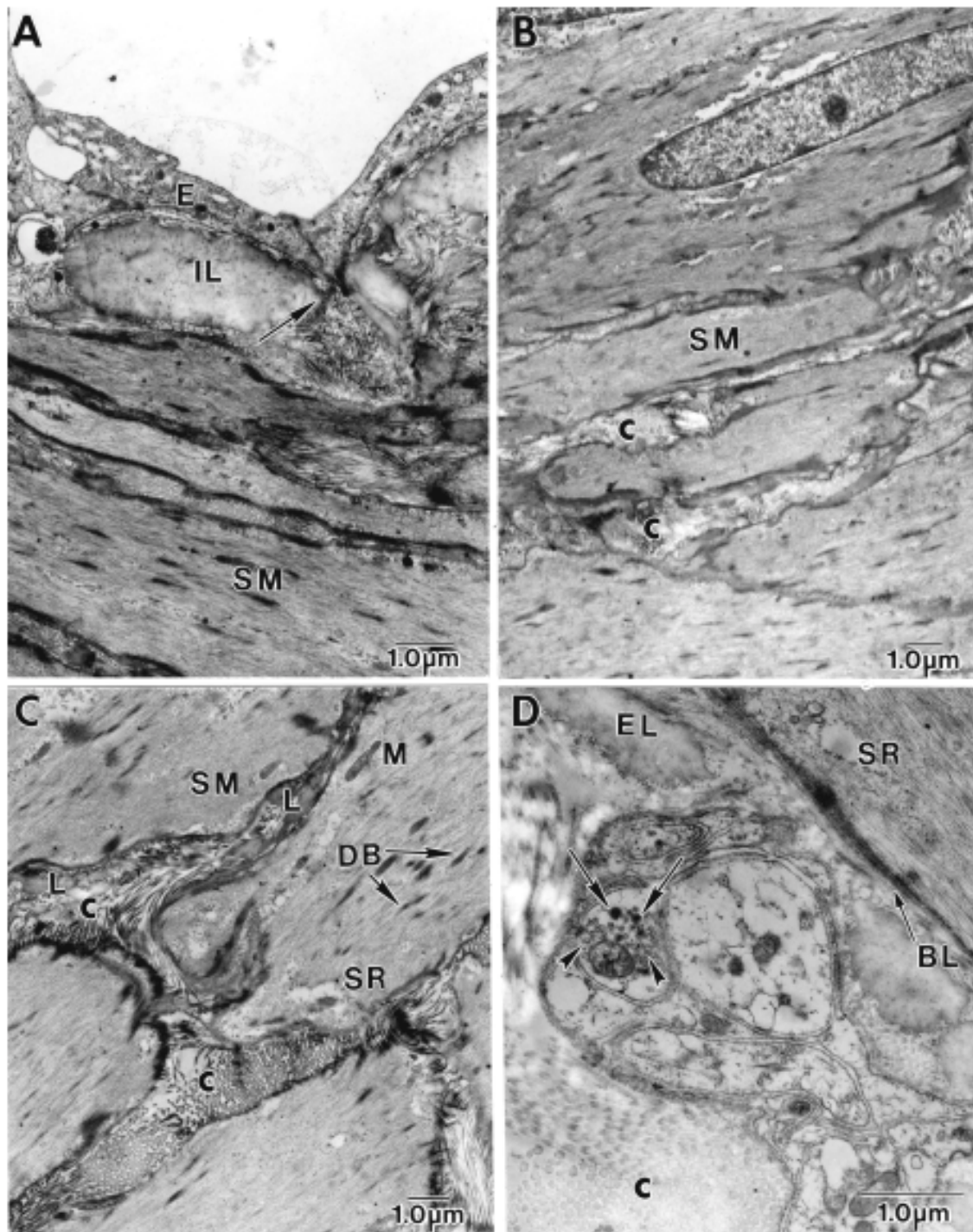


Figure 2
Electron micrographs of a tertiary mesenteric artery branch. Panel A: endothelial cell layer (E), internal elastic lamina (IL), and smooth muscle cells (SM). The long axis of the muscle fibers within tunica media runs at an angle to the circular axis of the vessel wall (Panel B). These cells have ultrastructural characteristics of smooth muscle cells including dense bodies (DB, Panel C) and bands, mitochondria (M, Panel C), sarcoplasmic reticulum (SR, Panels C and D), and a basal lamina (BL, Panel D). Elastin (L), collagen fibrils (C), elastic lamina (EL), dense cored vesicles (arrows), small electron lucent vesicles (arrowheads, Panel D). Scale bar is as indicated for each panel.

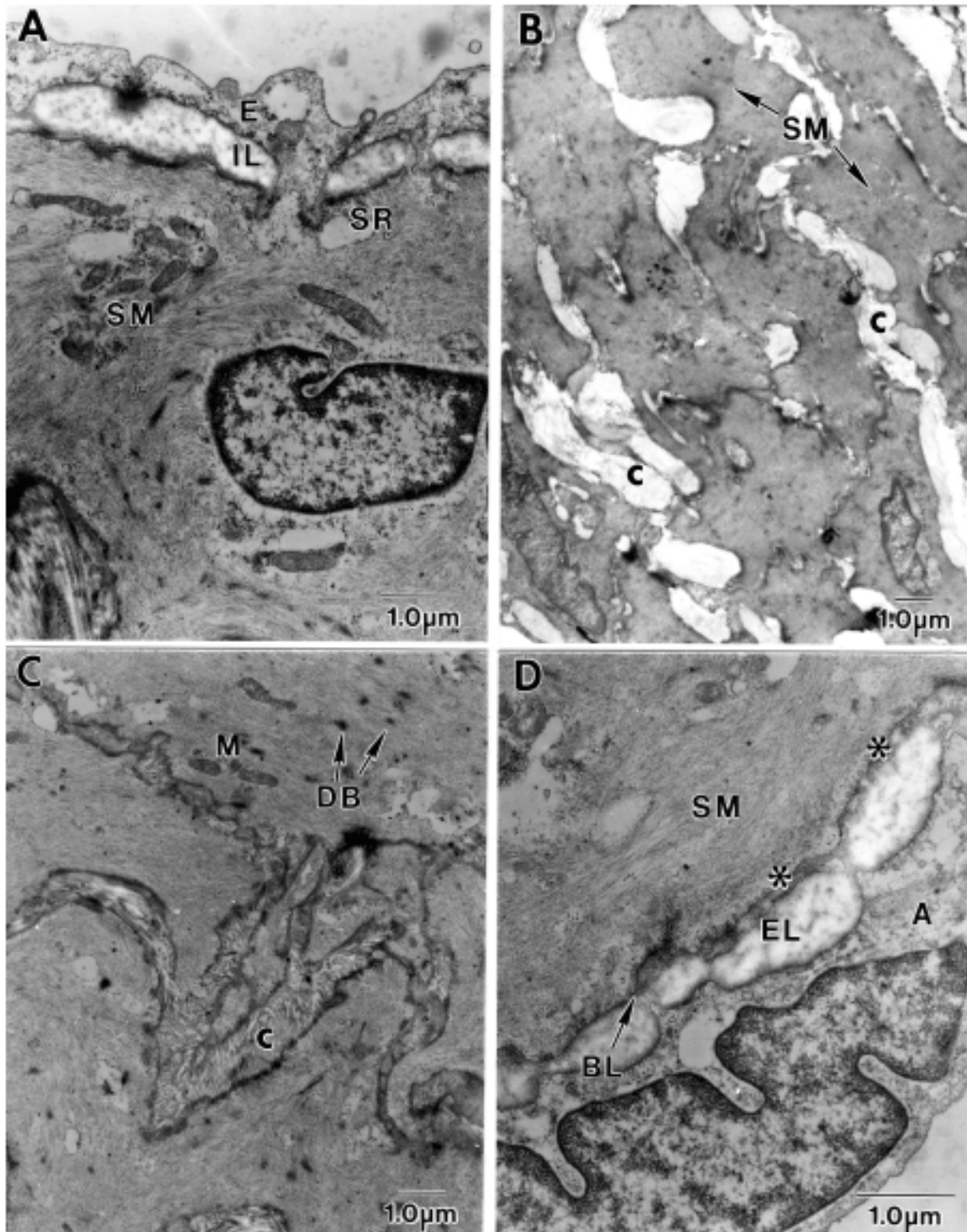


Figure 3
Electron micrographs of a tertiary mesenteric vein branch. Endothelial layer (E, Panel A), internal elastic lamina (IL), smooth muscle cells (SM, Panels A and B), collagen fibrils (C, Panels B and C), dense bodies (DB, Panel C), mitochondria (M, Panel C), sarcoplasmic reticulum (SR, Panel A), caveolae (*, Panel D) and a basal lamina (BL, Panel D), external elastic lamina (EL, Panel D), tunica advential layer (A, Panel D). Scale bar is as indicated for each panel.

Table 1: Content of major contractile proteins in mesenteric artery and vein (Mean \pm SEM, Student's t-test).

Protein	Measure	Artery	Vein	P
Total	($\mu\text{g}/\text{mg}$ dry tissue)	312.8 \pm 24.3	225.2 \pm 16.1	<0.05
α -Actin	($\mu\text{g}/\text{mg}$ total protein)	119.23 \pm 6.64	114.67 \pm 4.24	>0.05
MHC	($\mu\text{g}/\text{mg}$ total protein)	34.4 \pm 4.4	33.8 \pm 5.2	>0.05
MLC20	($\mu\text{g}/\text{mg}$ total protein)	22.0 \pm 3.35	23.8 \pm 4.26	>0.05
Calponin	($\mu\text{g}/\text{mg}$ total protein)	42.15 \pm 6.16	35.9 \pm 7.99	>0.05
Caldesmon	($\mu\text{g}/\text{mg}$ total protein)	42.15 \pm 3.12	29.89 \pm 1.98	<0.05
TM	($\mu\text{g}/\text{mg}$ total protein)	31.35 \pm 1.59	33.14 \pm 2.71	>0.05
TM- α	($\mu\text{g}/\text{mg}$ total protein)	24.24 \pm 2.03	18.0 \pm 1.55	<0.05
TM- β	($\mu\text{g}/\text{mg}$ total protein)	7.74 \pm 0.31	11.87 \pm 1.01	<0.05

3.72 \pm 0.38 in the artery and 3.59 \pm 0.26 in vein ($P > 0.05$). These data indicate that the fractions of the major motor proteins, actin and myosin, are similar in both blood vessels.

We assayed the contents of some thin filament binding proteins, proposed to play roles in modulation of the actin-myosin coupling, e.g. calponin, caldesmon, and TM. The assay pointed to a slightly bigger amount of calponin in the artery than vein, however the difference was not statistically significant ($P > 0.05$, Table 1). There were significant differences in the content of caldesmon. The high molecular weight caldesmon (h-caldesmon) is the major isoform of intact vascular smooth muscle [7]. On the Western blots h-caldesmon appeared as a single band with apparent molecular weight of 130 kDa, which comigrated with h-caldesmon standard, purified from porcine stomach [8]. Densitometry of immunoreactive bands revealed that the artery contained approximately 1.39 times more h-caldesmon than vein (Fig. 5 and Table 1). Tropomyosin is a dimer protein, composed of one heavy subunit (TM- α , 39 kDa) and one light subunit (TM- β , 36 kDa) (Fig. 6, Panel A). Analysis of the TM bands revealed a lack of significant difference of the total TM between the artery and vein (Table 1). However, differences were observed in the content of the TM isoforms (Fig. 6 and Table 1): (i) both, the artery and vein had significantly more TM- α than TM- β ; and (ii) the content of TM- α was bigger in the artery, while TM- β was more abundant in vein. These TM isoform levels determined significantly different TM- α /TM- β ratios: 3.26 \pm 0.48 in the artery versus 1.87 \pm 0.26 in vein ($P < 0.05$).

Discussion

The overall architecture and contents of individual contractile proteins are among the factors contributing to differences in the functional behavior of blood vessels [9]. However, comparative information about arteries and

veins from the same vascular bed is usually scarce, as it is with the arteries and veins of the mesenteric circulation. In the present study we focused on identifying differences in the structure and organization of mesenteric arterial and venous walls, which might help to better understand the physiology of these two vascular beds. The primary structural difference was that secondary arterial branches contain a greater number of smooth muscle cells within the tunica media and have 5 to 10 times thicker smooth muscle layer than that of veins. Moreover, arterial smooth muscle cells have smaller intercellular gaps. This tighter arrangement suggests closer intercellular coupling and more efficient production of force during muscle constriction; the arterial wall is thus better suited for vigorous mechanical resistance than the venous wall. From a structural point of view, mesenteric blood vessels comply with the general notion that arterial walls are thicker than venous walls from the same branches. Likewise, the walls of the arterial secondary branches are thicker compared to tertiary branches, consistent with the notion that a gradient of wall thickness is necessary for adjustments of the arterial network to rapid reduction of the blood pressure. Interestingly, however, secondary and tertiary mesenteric veins have almost identical thickness. It appears, therefore, that descending down the mesenteric tree, the contractile potential of arterial strips would decrease faster relative to that of veins. Thus, an awareness of the gradient of wall thickness might be useful in comprehending the relative potency of the artery and vein during comparative mechanical studies.

From the thicker smooth muscle layer of mesenteric artery we obtained a greater amount of total protein than from the thinner veins, but the fraction of the major motor proteins actin and myosin, as well as of thin filament-binding protein calponin, was indistinguishable. An intriguing finding in this study was that the actin/myosin ratio in the canine mesenteric circulation (~ 3.7 in the artery and ~ 3.6

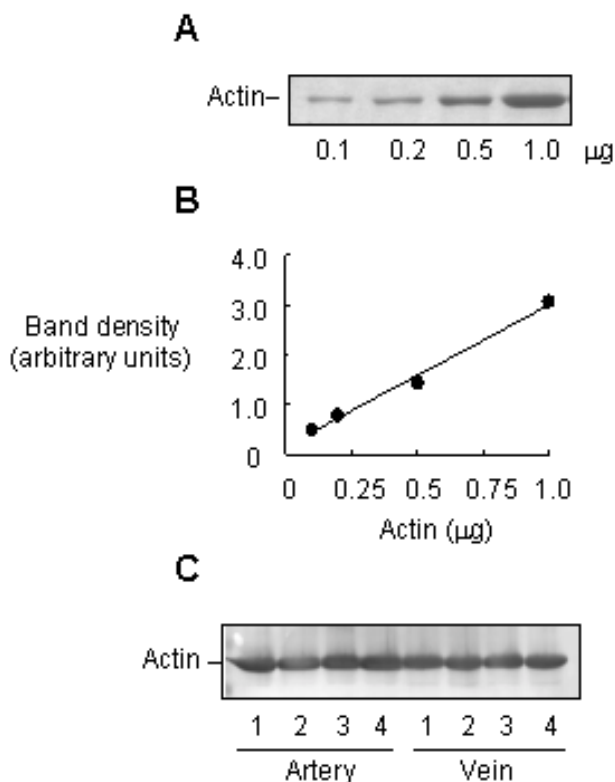


Figure 4
Quantification of contractile proteins in mesenteric artery and vein illustrated with actin. Panel A: Image of a Western blot depicting immunoreactive bands of actin standards. Band densities were assayed by scanning densitometry and were plotted against the respective amount of actin (Panel B). The linear relationship was used to calculate the amounts of actin in arterial and venous total tissue extracts. Tissue protein was separated by SDS-PAGE and actin was visualized by Western analysis, using a polyclonal anti- α -actin antibody (Panel C).

in the vein) is higher than in aorta, carotid and coronary arteries (~2.6), or in non-vascular smooth muscles (~1.5) of pig [10]. These observations suggest that the contractile protein composition might be inconsistent among vascular beds of one species, and might exhibit interspecies differences.

Caldesmon and TM were the two contractile proteins that displayed quantitative differences between artery and vein. Caldesmon is likely to play a modulatory role on the production of smooth muscle force via a tethering of actin to myosin [11] and/or of its effects on the actomyosin ATPase [12–14]. Similarly, TM does not seem to function as a major regulatory protein, but modulatory effects due to its ability to maintain the actin filamentous structure [15], to inhibit the Ca^{2+} -ATPase activity [16], alter the cytoskel-

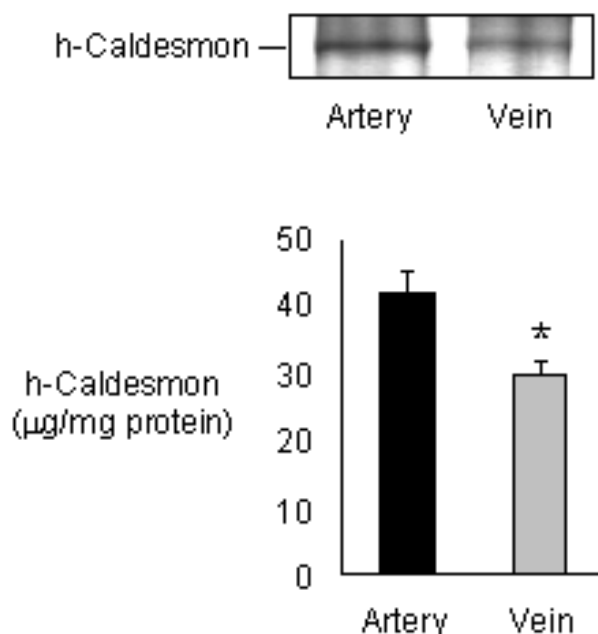


Figure 5
Mesenteric artery contains more h-caldesmon than vein. Total tissue protein was separated by SDS-PAGE and protein bands were visualized by densitometry of immunoreactive bands on Western blots (inset). The average contents of h-caldesmon in either blood vessel are plotted in the bar graph (Mean \pm SEM, n = 8, P < 0.05, Student's t-test). Asterisk denotes significant difference between artery and vein.

etal dynamics [17] and improve actin filament flexibility during the contraction/relaxation cycle [18,19] have been well documented. Differences in the content of caldesmon, or the TM isoforms, therefore, might be associated with modulatory effects on the time profile or magnitude of contraction. Moreover, it has been recognized that caldesmon and TM can modulate the actin-myosin interaction in a cooperative manner [20], suggesting that functional effects of these proteins on actin-myosin coupling and smooth muscle mechanics ought to be interpreted in parallel. It remains to be determined whether the relatively bigger amount of caldesmon and TM- α is advantageous for the artery in fulfilling resistive functions, and whether more TM- β is a prerequisite for veins to fulfill capacitative functions in the mesenteric circulation.

Conclusions

The results of this study confirm previous studies that thicker and more tightly assembled smooth muscle layer, rather than the profile of the major contractile proteins, is the likely cause for a higher mechanical potential of the mesenteric artery compared to vein. While in various circumstances the mesenteric veins display a greater responsiveness to contractile stimuli than the corresponding

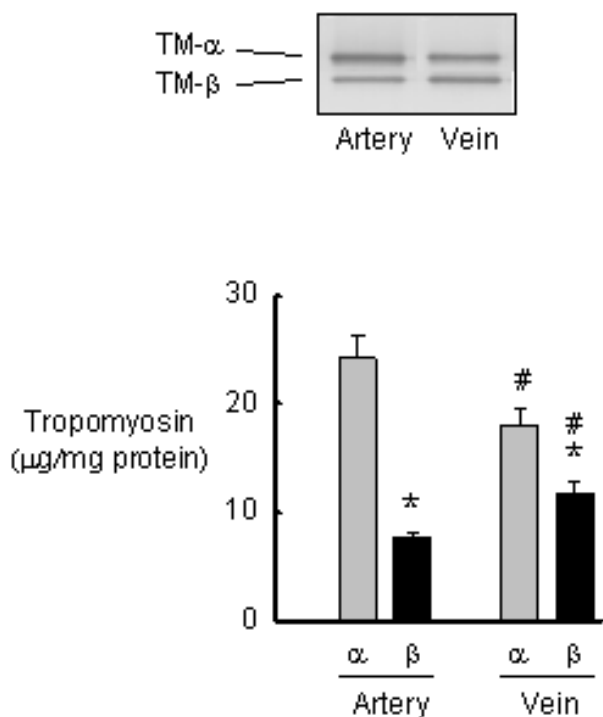


Figure 6
Mesenteric artery and vein contain more TM- α (39 kDa) than TM- β (36 kDa). Vein contains more TM- β than artery. Total tissue protein was separated by SDS-PAGE and TM bands were quantified by densitometry of immunoreactive bands on Western blots (inset). The average contents of TM in either blood vessel are plotted in the bar graph (Mean \pm SEM, $n = 10$, $P < 0.05$, two-way ANOVA). Asterisks denote significant differences between TM- α and TM- β ; pound signs denote significant differences between artery and vein.

arteries [5,21], this difference should be attributed to the specificity of the neuroeffector coupling, or to signal transduction mechanisms underlying the functional distinctions between capacitative and resistive regions of the mesenteric circulation.

Methods

Tissue preparation

Twelve mongrel dogs of either sex (averaging 15 kg) were obtained from vendors licensed by the United States Department of Agriculture. The use of dogs for these experiments was approved by the Institutional Animal Care and Use Committee at the University of Nevada. The animals were euthanized with an overdose of pentobarbitone sodium (100 mg/kg intraperitoneally). The abdomen was opened and segments of second and third order branches of the inferior mesenteric artery (0.7–1 mm in diameter) and vein (0.8–1.2 mm in diameter) were dissected out

and bathed in cold (10°C) Krebs solution of the following composition (mM): 118.5 NaCl; 4.2 KCl; 1.2 MgCl₂; 23.8 NaHCO₃; 1.2 KH₂PO₄; 11.0 dextrose; 1.8 CaCl₂. The tissues were continuously aerated with a mix of 95% O₂ and 5% CO₂. The vessel segments used for morphological examination were cleaned of connective tissues and the endothelial cell layer was left intact. The arteries and veins used for protein biochemistry experiments were perfused with distilled water for 30 min to remove endothelium. In previous experiments we have shown that this procedure successfully removes the endothelial cell layer without affecting the smooth muscle contractility [5,6].

Conventional phase contrast and electron microscopy

Mesenteric artery and vein segments (15 mm in length) were ligated with suture thread and fixative solution containing 4% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was injected into the lumen to partially distend the vessel. The whole preparations were then immersed and incubated in fixative solution for 4 hours at 4°C. Vessels were then rinsed with fresh fixative buffer and post-fixed in 1% osmium tetroxide for 2 hours at 4°C. Tissues were subsequently rinsed with distilled water, block-stained with saturated aqueous uranyl acetate solution for 1 hour, dehydrated through a graded series of ethanol solutions and embedded in Epon epoxy resin (Ted Pella, Inc. Redding CA, USA). Semi-thin sections (2 μ m) were cut with a Reichert microtome and visualized using phase contrast microscopy with a Leitz Diaplan microscope. Images were collected using a Leica LEI-750 digital camera and Metamorph 3.0 software (Universal Imaging Corp. West Chester, PA, USA). Final images were constructed using Adobe Photoshop (4.0) and Corel Draw (7.0). For electron microscopy imaging, ultra-thin sections were stained with uranyl acetate and lead citrate (5 minutes each) and viewed under a Philips CM10 transmission electron microscope.

Extraction and assay of contractile proteins

A previously described general protocol for extraction of contractile proteins [1] was applied to the canine mesenteric arteries and veins. Smooth muscle preparations were frozen by immersion in ice-cold acetone containing 5 mM NaF (-80°C). The acetone was then evaporated in a speed-vac centrifuge. Smooth muscle strips were weighed and total protein was extracted by glass-glass homogenization using 50 μ l per milligram dry tissue sodium dodecyl sulfate (SDS)-based extraction buffer: 25 mM Tris-HCl, pH 7.4, 2% SDS, 10% glycerol, 1 mM dithiothreitol (DTT), 1 μ M leupeptin, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium orthovanadate, 5 mM NaF and 1 mM phenylmethylsulfonyl fluoride (PMSF). Tissue extracts were boiled for 5 min, sonicated for 3 min and incubated at room temperature for another 30 min to enhance the protein yield. Homogenates were then

centrifuged at 10,000 rpm for 20 min, supernatants were transferred into clean tubes and stored at 4°C. Pellets were resuspended in extraction buffer and protein was extracted twice as described above. Total protein content of all supernatants was assayed colorimetrically by the Micro bicinchoninic acid (BCA) Protein Assay kit (Pierce, Rockford, IL, USA).

Protein separation and quantification by SDS-PAGE and immunoblotting

Equal amounts of total sample protein (usually 15 µg) were resolved by SDS-PAGE. Purified standard proteins were resolved along with the total tissue extract to identify the position of each protein in the gel. Proteins were transferred onto nitrocellulose membranes (Genie blotter, Idea Scientific Company, Minneapolis, MN, USA) for 1 hour, at 24 V and 4°C, using transfer buffer composed of 25 mM Tris-HCl, 192 mM glycine and 10% methanol. Membranes were then blocked with 0.5% solution of gelatin in TNT buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 2 hours at room temperature. Labeling with the primary antibodies took place in 0.1% gelatin-TNT buffer for 1 hour at room temperature, with the following dilutions: 1:500 of the anti-MHC antibody (Sigma, Saint Louis, MO, USA); 1:2,000 of the anti-TM antibody (Sigma, Saint Louis, MO, USA); and 1:10,000 of the anti-caldesmon (gift from Dr. L. Adam, Bristol-Myers-Squibb, USA), anti-calponin (Sigma, Saint Louis, MO, USA), anti- α -actin (Biomeda Corporation, Foster city, CA, USA) and anti-MLC20 (gift from Dr. S. Gunst, Indiana State University, USA). The unbound primary antibodies were removed by three 5-min washes with TNT buffer. The membranes were then incubated for 1 hour with goat-anti-rabbit or goat-anti-mouse alkaline-phosphatase-conjugated secondary antibodies (Promega Corp., Madison, WI, USA) diluted 10,000 times with 0.1% gelatin-TNT. Excess secondary antibody was removed by three 5-min washes with TNT buffer and color was developed using the 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate. Stained gels and blots were scanned with a UMAX Powerlook flatbed scanner (BioRad, Hercules, CA, USA) to obtain images. Protein bands on these images were analyzed by scanning densitometry, using Molecular Analyst program (BioRad, Hercules, CA, USA).

Statistics

The protein amounts in each artery or vein were averaged and presented as mean \pm SEM. Student's t-test for paired and unpaired data was applied as appropriate. Two-way ANOVA was applied for multiple group comparisons. Values of $P < 0.05$ were considered statistically significant.

Authors' contributions

Author IAY participated in the Western blot analysis and quantification of the studied contractile proteins. Author SMW was involved in preparation of the microscope specimens and analysis of vascular wall structure. Author VNM-Y designed and coordinated the study. All authors participated in drafting the manuscript.

All authors read and approved the final manuscript.

Abbreviations

MHC, myosin heavy chain; MLC, myosin light chain; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TM, tropomyosin; WB, Western immunoblot.

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