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Preferential Myosin Heavy Chain Isoform B Expression May Contribute to the Faster Velocity of Contraction in Veins versus Arteries

By Catherine M. Rondelli, Irna T. Szasz, Anas Kayal, Keshari Thakali, Ralph E. Watson, Arthur S. Rovner, Thomas J. Eddinger, Gregory D. Fink, and Stephanie W. Watts

Smooth muscle myosin heavy chains occur in 2 isoforms, SMA (slow) and SMB (fast). We hypothesized that the SMB isoform is predominant in the faster-contracting rat vena cava compared to thoracic aorta. We compared the time to half maximal contraction in response to a maximal concentration of endothelin-1 (ET-1; 100 nM), potassium chloride (KCl; 100 mM) and norepinephrine (NE; 10 μ M). The time to half maximal contraction was shorter in the vena cava compared to aorta (aorta: ET-1 = 235.8 ± 13.8 s, KCl = 140.0 ± 33.3 s, NE = 19.8 ± 2.7 s; vena cava: ET-1 = 121.8 ± 15.6 s, KCl = 49.5 ± 6.7 s, NE = 9.0 ± 3.3 s). Reverse-transcription polymerase chain reaction supported the greater expression of SMB in the vena cava compared to aorta. SMB was expressed to a greater extent than SMA in the vessel wall of the vena cava. Western analysis determined that expression of SMB, relative to total smooth muscle myosin heavy chains, was 12.5 ± 4.9 -fold higher in the vena cava compared to aorta, while SMA was 4.9 ± 1.2 -fold higher in the aorta than vena cava. Thus, the SMB isoform is the predominant form expressed in rat veins, providing one possible mechanism for the faster response of veins to vasoconstrictors.

Introduction

The myosin proteins are a large superfamily of proteins that share a common motor domain which, through interacting with actin and adenosine triphosphate (ATP) hydrolysis, produces movement. There are at least 15 classes of myosin, of which smooth muscle myosin is in class II [1]. Members of myosin class II are hexameric enzymes that are composed of 2 heavy chains and 2 pairs of light chains. Myosin has 3 functional subdomains: (1) the motor domain which interacts with actin and binds ATP, (2) the neck domain which binds light chains or calmodulin and (3) the tail which anchors myosin to filaments in the muscle wall [1, 2].

Alternative splicing of the heavy chain of class II myosin (MHC) produces 4 MHC isoforms (1A, 1B, 2A, 2B) [for head isoforms, see 3–5; for tail isoforms, see 6–8]. MHC 1 and MHC 2 differ only at the carboxy terminus of the myosin tail, while SMA lacks a 7 amino acid insert in the ATP binding pocket of the motor domain head which is present in SMB [2, 9]. SMB

has higher ATPase activity, faster actin translocation velocity [10] and faster unloaded shortening velocity than SMA [11], allowing for faster stretch activation and tension unloading (activity via electrical stimulation) [12].

Much of the work regarding tissue-specific myosin isoform expression relates to striated tissue (skeletal and cardiac) or isolated cardiac and vascular smooth muscle cells. Little has been reported about intact smooth muscle vascular tissue at the protein level, although mRNA analysis has shown increased SMB expression in fetal tissue [13], isolated cardiac smooth muscle cells [14], lymphatic tissue [15] and specific organs such as the stomach [11] and kidney [16]. Meer and Eddinger [17], Eddinger et al. [18] and Eddinger and Meer [19] have shown that while the head isoforms (SMA and SMB) correlate with unloaded shortening in single cells, this is not the case for the tail isoforms (SM1 and SM2) for MLC17 (A and B). We and others have shown that veins contract faster than arteries to agonists [20, 21], despite proportionally fewer smooth muscle cells present in veins compared to arteries. Therefore, we hypothesized that increased SMB expression in veins compared to arteries allows veins to contract faster than arteries.

Methods

Animals

Male Sprague-Dawley rats (0.225–0.250 kg; Charles River, Portage, Mich., USA) were used. Experiments were approved by the Michigan State University Institutional Animal Use and Care Committee.

Smooth Muscle Isometric Contractile Measurement

Rats were deeply anesthetized with pentobarbital intraperitoneally (50 mg kg^{-1}) to the point of a loss of corneal reflex and lack of withdrawal from painful stimuli. Thoracic aorta, vena cava and tail artery were placed in physiologic salt solution consisting of (in mM): NaCl, 130; KCl, 4.7; KH_2PO_4 , 1.18; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.17; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.6; NaHCO_3 , 14.9; dextrose, 5.5; CaNa_2EDTA , 0.03. Tissues were cleaned of fat and connective tissue, left with an intact endothelium, mounted as rings (3–4 mm long) on stainless steel hooks and placed on stainless steel holders in tissue baths (30 ml) for isometric tension recordings using Grass polygraphs and transducers as previously described [22]. Functional integrity of the endothelial cells was evaluated by testing relaxation caused by acetylcholine ($1 \mu\text{M}$) in tissues contracted with 10–100 nM α -adrenergic agonist norepinephrine (vein) or phenylephrine (artery). Individual tissues were challenged with a maximal concentration of endothelin (ET-1; 100 nM), potassium chloride (KCl; 100 mM) and norepinephrine (NE; 10 μM), and the time to half maximal contraction was

measured in seconds. Each tissue was exposed to 2 agonists. NE or KCl was added first, and additions were randomized throughout the experiments. Tissues were then washed for 1 h, with washes every 5 min. ET-1 was then added, as contraction stimulated by ET-1 is virtually irreversible and difficult to wash out.

Reverse Transcription and Polymerase Chain Reaction

The MELTTM total RNA isolation system (Ambion, Austin, Tex., USA) was used according to the manufacturer's instructions. Briefly, 10-mg sections of rat tail artery, thoracic aorta and vena cava were cleaned in cold nuclease-free water and enzymatically digested while being vortexed. The homogenate was bound to magnetic beads for RNA purification, DNase digestion and washing. A magnetic stand adapted for 96-well plates was used to recapture the RNA binding beads throughout the experiment. RNA was finally eluted from the magnetic beads in 10–20 μ l elution solution. Total RNA was quantitated on a Nanodrop spectrophotometer. The A260/280 and A260/230 ratios were considered acceptable if they were situated around 2 (80.15). The integrity of RNA was assessed by MOPS formaldehyde gel electrophoresis when the RNA isolation method was initially established, however, vena cava RNA yield (usually around 1 μ g) did not allow for repeating this verification with each sample.

Primers for rat SMA/SMB were based on those reported by Shiraishi et al. [16]. SMB mRNA contains a 21-nucleotide insert in the 5'-end coding region that is lacking in SMA mRNA. The primers were 5'-TACAGAAGCATGCTACAAGATCGT-3' and 5'-GCGGGAGGAGTTGTCATTCTTGAC-3'. Primers for β 2-microglobulin, as a housekeeping gene, were purchased as a kit from Super Array (Frederick, Md. USA).

One microgram of total RNA, DNase treated, was used as starting material for all samples. First-strand cDNA synthesis was performed using oligo(dT)12–18, dNTP, 5x first-strand buffer, 0.1 M DTT and the Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif., USA) in a 1-hour incubation at 42°C, followed by a 15-min 70°C incubation for reverse transcriptase denaturation. Conventional polymerase chain reaction using the Platinum Taq High Fidelity DNA polymerase (Invitrogen) was performed, and final products separated on a 3% agarose gel in Tris-acetate-EDTA buffer using DNA ladders separated by 100 bp (Invitrogen).

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded thoracic aorta and vena cava (8 μ m) were cut, air dried overnight, deparaffinized, and taken through standard protocol using a Vector kit. Slides for smooth muscle α -actin were unmasked through the Vector Laboratory Antigen Unmasking protocol, modified to use a microwave instead of a pressure cooker. After blocking with 1.5% serum in phosphate-buffered saline (PBS), sections incubated overnight at 4°C with

an antibody for total (SMA + SMB) MHC [1 µg/ml, mouse antibody from Santa Cruz Technologies (Santa Cruz, Calif., USA), with 1.5% blocking serum in PBS], SMB antibody (1:20, with 1.5% blocking serum in PBS), SMA antibody (1:100, with 1.5% blocking serum in PBS), smooth muscle α -actin (0.5 fg/ml, mouse antibody from Calbiochem, with 1.5% blocking serum in PBS) or no antibody (1.5% blocking serum in PBS). SMB antibody was generated in the laboratory against the short peptide sequence QGPSFAYGELEC in rabbit [23]. SMA antibody was generated in the laboratory against the short peptide sequence KKDTSITGELEC in rabbit [11]. Sections were washed thrice with PBS, incubated for 30 min with the appropriate secondary antibody, washed again and incubated for 30 min with Vector ABC Elite reagent. Antibody binding was detected by incubating sections for 1 min with a DAB developing solution (Vector Laboratories, Burlingame, Calif., USA). Primary antibody binding was observed by a dark brown/black precipitate. Sections were photographed using an inverted Nikon T2000 microscope connected to a SPOT Insight color camera using MetaMorph® software. Images were processed using Adobe® Photoshop. Masson's trichrome staining was performed by the Investigative Histopathology Laboratory at Michigan State University

Western Analysis

Tissues were isolated directly from the animal, cleaned and snap-frozen in liquid nitrogen. Tissue lysates were prepared as previously described [24]. Fifty micrograms of total protein was boiled for 5 min with standard 4:1 sample buffer. Proteins were separated on 1-mm-thick, 7% polyacrylamide SDS gels using a Mini Bio-Rad III apparatus with a positive control for SMB of rat tail artery homogenate [25], and transferred to Immobilon PVDF membranes. Membranes were blocked overnight in 5% milk (4°C, PBS + 0.025% NaN₃) and blots were incubated with primary antibodies [1:500 total smooth muscle MHC (SM-MHC), 1: 1,000 SMB, 1:1,000 SMA] for 24 h at 4°C. Blots were then rinsed thrice in Tris-buffered saline + Tween (0.1%) with a final rinse in Tris-buffered saline and incubated with a horseradish peroxidase-linked antirabbit secondary antibody (1:2,000; Cell Signaling Technology, Beverly, Mass., USA) for 1 h at 4°C (rocking). ECL® reagents (Amersham Life Sciences, Arlington Heights, Ill., USA) were used to visualize bands. Gels were stained with Gel Code Blue® (Pierce, Rockford, Ill., USA) to verify protein loading and blots were reprobbed with smooth muscle α -actin primary antibody (1:1,000; Oncogene Research Products, Boston, Mass., USA).

Data Analysis

Data are presented as means \pm SEM for the number of animals in parentheses. Time to half maximal contraction is reported as seconds. Band density was quantified using the public domain program NIH Image (version 1.62). When comparing 2 groups, the appropriate Student t

test was used. ANOVA followed by the Student Newman Keuls post hoc test was performed when comparing 3 or more groups. In all cases, $p \leq 0.05$ was considered statistically significant.

Results

Vena Cava Contract Faster than Aorta to Exogenous ET-1, KCl and NE

Rat aorta and vena cava from the same animal were stimulated with a maximal concentration of ET-1 (100 nM), KCl (100 mM) and NE (10 fM). In separate experiments, the speed of response of the tail artery to these same agonists was investigated. Figure 1a depicts a raw tracing of the tail artery, aorta and vena cava response to a maximal concentration of ET-1 (100 nM). In general, vena cava generated significantly less tension than aorta due to the lower muscle mass present per milligram of tissue. In the contractile experiments, tissues responded to the 3 agonists in the following manner: aorta NE: $3,325 \pm 480$ mg, vena cava NE: 96 ± 16 mg; aorta KCl: $2,470 \pm 344$ mg, vena cava KCl: 222 ± 31 mg; aorta ET-1: $1,855 \pm 242$ mg, vena cava ET-1: 553 ± 57 mg. The time to half maximal contraction was significantly shorter in veins compared to arteries, regardless of agonist. Time to half maximal contraction ranged from 1.94- to 2.83-fold lower in the vein compared to the artery (aorta ET-1: 236.8 ± 14 s, vena cava ET-1: 122 ± 16 s; aorta KCl: 140 ± 33 s, vena cava KCl: 50 ± 7 s; aorta NE: 20 ± 3 s, vena cava NE: 9 ± 3 s; fig. 1b). By comparison, the tail artery – a thermoregulatory artery – was used as a control for an SMB-positive tissue and contracted with significant speed to these 3 agonists (ET-1 = 29.3 ± 2.0 s, KCl = 6.1 ± 0.5 s, NE = 3.2 ± 0.2 s).

SMB mRNA Is Observed at a Higher Magnitude in Vena Cava Compared to Aorta

Primers designed to detect the mRNA variants for SMA and SMB were used in equivalent amounts of cDNA generated from tail artery, aorta and vena cava. As expected, 2 products were observed at 261 bp (SMB) and 240 bp (SMA) (fig. 2). While aorta weakly expressed SMB, all vena cava examined expressed a significantly higher amount of SMB message as densitometry revealed an increase of over 200% of that observed in the aorta. By comparison, the tail artery provided a robust signal for SMB and a weaker signal for SMA. Thus, these data validate our use of the tail artery as a positive control for SMB and demonstrate the potential for preferential expression of SMB protein in the vena cava compared to the aorta.

Stronger Immunohistochemical Staining for SMB Is Observed in Vena Cava Compared to Aorta

Paraffin-embedded sections from normal rats were used in the immunohistochemical analysis of SM-MHC isoforms. Masson's trichrome staining of the aorta (fig. 3a) and vena cava (fig. 4a) demonstrates significantly different structures of arteries and veins. The aorta has

several distinct and regular layers of smooth muscle cells that run parallel to the intimal endothelium, as confirmed by α -actin staining. The arterial adventitia was primarily a collagenous layer surrounding the highly organized muscle. In contrast, trichrome staining of the vena cava revealed a collagenous and relatively disorganized tissue with a thin layer of smooth muscle underneath the endothelial layer, as confirmed by α -actin staining (fig. 4a). Total SM-MHC and SMB expressions were spread relatively uniformly throughout the arterial wall from the endothelium to the adventitia with similar patterns weakly seen with SMA (fig. 3b). Total SM-MHC expression in the vena cava appears to be limited to the endothelial/ subendothelial cell layer while SMB and to a significantly lesser extent SMA expression was observed throughout the collagenous vein wall (fig. 4b).

Western Analysis Demonstrates Higher SMB Expression in Vena Cava than Aorta

Figure 5a shows Western blots of total SM-MHC, SMB and SMA expression in aorta and vena cava. Rat tail artery was used as a positive control for SMB [16]. Densitometry of total SM-MHC, SMB and SMA expression was normalized to the expression of smooth muscle α -actin present in a given sample (fig. 5b). There was notably less α -actin present per microgram of tissue in the vein compared to the artery (fig. 5 c). While total SMMHC and SMA expression was significantly less in vena cava compared to aorta, SMB isoform expression tended to be higher in the vena cava than aorta (fig. 5b). The relative expression of SMA to the total SM-MHC expression was significantly greater (4.9 ± 1.2 -fold) in the aorta compared to vena cava. The relative expression of SMB to the total MHC expression was significantly greater (12.5 ± 4.9 -fold) in the vena cava compared to aorta.

Discussion

The ability of vasculature to respond to contractile stimuli from neuronal innervation and blood is crucial for stable maintenance of blood pressure. The measurably different speed of contraction between arteries and veins suggests an important role of venous contractility in the moment-to-moment control of blood pressure, allowing for a quick response to natural stimuli. For example, blood must quickly be redirected from the lower body towards the head upon movement from a reclining to a vertical position to avoid loss of consciousness. The present work begins to define one possible mechanism by which the fast response of veins to stimuli is facilitated.

Time to Half Maximal Contraction Is Less in Veins versus Arteries

Interestingly, each of the vasoconstrictor stimuli tested demonstrated a significant difference in response time between tissues, and vena cava consistently contracted faster than

aorta. The tail artery, which possesses predominantly SMB protein, had the fastest time to half maximal contraction for all agonists examined. The agonists used elicit contraction via different mechanisms. ET-1 binding to ET_A and ET_B receptors activates phospholipase C-β to increase intracellular Ca²⁺ [26]. KCl causes membrane depolarization and extracellular Ca²⁺ influx through L-type calcium channels [27] while Ca²⁺ intercellular release minimally contributes to contraction [28]. NE binds α-adrenergic receptors which leads to G protein-coupled increase in intracellular Ca²⁺ [29, 30]. Though membrane depolarization is a common factor between each of the agonists tested [2, 24, 25, 31], the mechanisms by which these agonists cause contraction are varied enough to consider the difference in relative speed of contraction in artery and vein to be an inherent property of these tissues. We proposed that differences in SM-MHC isoform expression in arteries and veins may account for differences in speed of contraction.

Structural Characteristics of Arteries and Veins

The architecture of the aorta and vena cava were strikingly different. The qualitative difference between the smooth muscle tissue of aorta and the predominantly more structural collagen fibers of vena cava can be seen in figures 3 and 4. We have assumed that vascular smooth muscle cells are wholly responsible for contraction of aorta and vena cava. The question has been raised as to whether this is a fair assumption given the relative paucity of smooth muscle in veins. The pronounced SMB staining in figure 4 compared to total MHC staining is likely due to the sensitivity of the respective antibodies in this particular protocol, as is the comparably lighter SMA staining in all tissues. It is possible that the relative penetrance of antibodies in arterial versus venous tissues is different, thereby explaining why there is less staining for total MHC than for SMB.

One potential concern is that the basic passive elements of the blood vessels contribute to the speed of contraction. This is possible, but it is not an avenue we have yet explored. Veins and arteries possess different complements of framework proteins. Arteries are more highly elastic than veins, while veins are compliant vessels that can allow for the pooling of blood. One potential way to determine the contribution of the passive framework to contraction would be to investigate the kinetics of contraction in the freshly dissociated, isolated vascular smooth muscle cell from the vein and the artery. This will be an interesting question to examine.

Role of SMB in Contraction

A recent provocative study performed in renal blood vessels was the stimulus for investigating the expression of faster SMB in arteries and veins. Shiraishi et al. [16] performed immunohistochemical and Western analysis of MHC in renal afferent and efferent arterioles. The

connection between similar kinetic features and physiological roles led us to quantitate SMB, which has been suggested by others to be an important intrinsic difference in these tissues [16, 23]. Shiraishi et al. [16] noted that there was little to none of the fast isoform (SMB) in the slower-contracting efferent arterioles, although total SMMHC measurements did show the presence of some form of SM-MHC. In contrast, faster-contracting afferent arterioles expressed significantly more SMB. Afferent arterioles were comparable to veins in their ability to rapidly contract to chemical depolarization of the membrane. Efferent arterioles were similar to arteries in depolarization response, which is significantly slower than in afferent arterioles. With Western analysis, we observed a similar difference in the relative expression of SMB to total SMMHC protein. The vena cava has a significantly greater expression of SMB than the aorta, though total SM-MHC content is less. As expected, the reverse was true for SMA, as relative expression of SMA to total SM-MHC was higher in the aorta than the vena cava. We used a colorimetric detection system for our immunohistochemistry instead of the fluorescence system used in the paper by Shiraishi et al. [16] due to the intense amount of autofluorescence observed in the whole tissue.

The tail artery was used throughout this study as a positive control for SMB expression; this was originally reported by Shiraishi et al. [16]. We were able to validate the preferential expression of SMB mRNA and protein in the tail artery. The tail artery does express some SMA mRNA and protein (fig. 2, 5). Moreover, it is an artery, and we are here trying to argue that the general mechanics of contraction, as based on SM-MHC function, are different between veins and arteries. However, the rat tail artery is a unique artery in its dedication to thermal regulation of the rat. It is the vasodilation of this artery that enables a rat to shed heat, and it is thus governed by stimuli that are significantly different from those which would govern a systemic artery. Thus, while there are limitations to using the tail artery, it provides one of the best controls available when examining SMB expression and in connecting our work to that of others.

Limitations and Questions

There are no specific pharmacologic inhibitors of SMA or SMB to further examine our hypothesis. An ideal experiment would be to knock down native SMB to compare the speed of vessel contraction in the presence and absence of SMB. Periasamy's group has generated an SMB knockout mouse and other laboratories have used this model with conflicting results [32, 33]. While Karagiannis and Brozovich [32] demonstrated differences in shortening velocities of bladder smooth muscle tissue between normal and the slower knockout mice, no direct visualization methods were used to confirm the total loss of SMB in the target tissue. However, Patzak et al. [33] did not observe differences in constriction velocity between efferent and

afferent arterioles of normal and SMB knockout mice. While the loss of SMB was confirmed through immunohistochemistry, the possibility of compensatory mechanisms masking changes in physiology could not be ruled out. We have also not addressed the potential differences between arteries and veins in the SM1 and SM2 class of MHC proteins, and thus, we cannot comment upon how these particular proteins may contribute to the kinetics of agonist-induced contraction.

One potential concern is the finding that while the vein possessed a greater amount of protein and mRNA for SMB as opposed to SMA, the relative amount of mRNA did not correspond exactly with the protein expressed. This is somewhat different than what was found by Shiraishi et al. [16] in the afferent/efferent arterioles. We did confirm the nearly pure expression of the SMB isoform in the tail artery and thus have faith in the primer sets used. One speculation for the discrepancy in these findings is that the SMB mRNA may be more efficiently translated than the SMA mRNA, clearly the dominant mRNA form in the vena cava but not the dominant protein.

Finally, other factors which influence arterial versus venous contraction may exist in smaller arteries and veins that we do not see in the large vessels. What are the differences in the rates of signal transduction pathways for each agonist in each tissue? Are there cellular composition differences not detected in the trichrome staining that may contribute to a contractile response, such as the presence of myofibroblasts? These remain unanswered questions.

Conclusion

In conclusion, we have quantitatively demonstrated that the proportion of SMB expression is greater in veins compared to SMA. The vena cava had little to no measurable SMA expression. The possibility that SMB plays a significant role in the kinetic contractile response indicates the need for further experimentation, such as additional kinetic studies in the presence of an SMB-specific inhibitor or SMB knockout. Nonetheless, the faster phenotype of veins versus arteries is one argument for the important role played by veins in moment-to-moment control of blood pressure.

Acknowledgment

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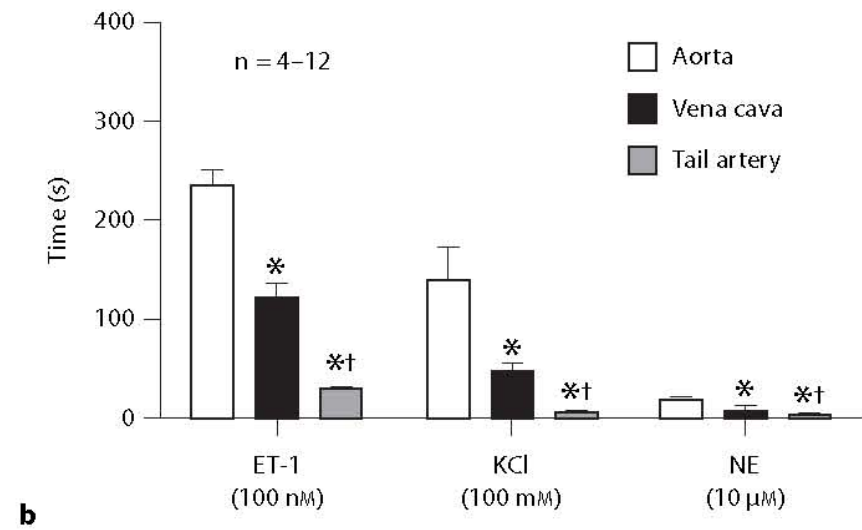
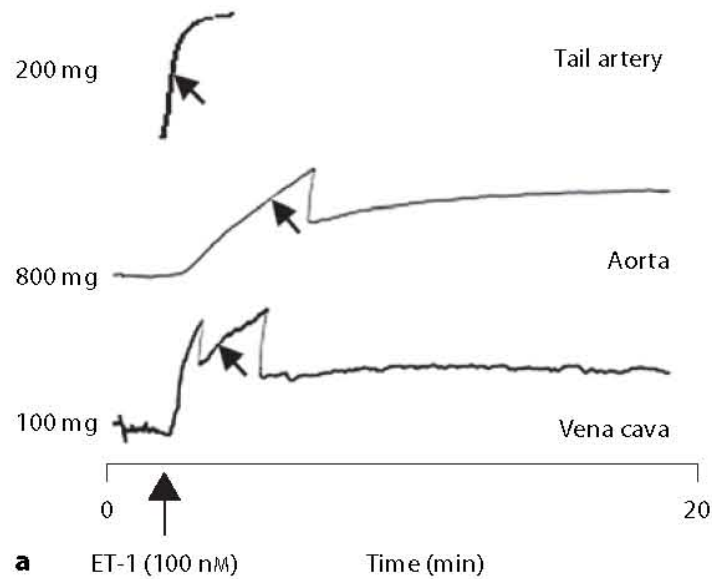
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Appendix

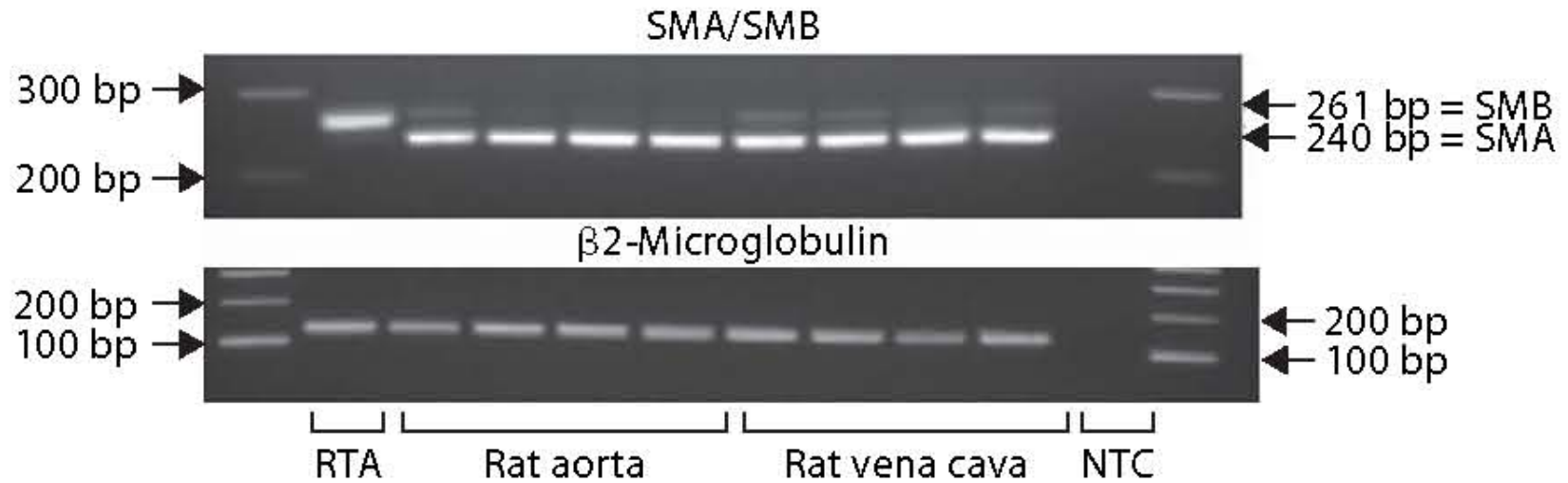
Figure 1



a Tracing of the contraction to a maximal concentration of ET-1 in the rat tail artery, aorta and vena cava. The arrows on the tracings indicate the time to half maximal contraction.

b Time to half maximal isometric contraction of rat aorta and vena cava in the presence of ET-1 (100 nM), KCl (100 mM) and NE (10 μM). Bars represent means \pm 8 SEM. * $p < 0.05$ vs. rat aorta; † $p < 0.05$ vs. vena cava.

Figure 2



Polymerase chain reaction products for SMA (240 bp)/SMB (261 bp) and β 2- microglobulin amplification of cDNA from rat tail artery, rat aorta and rat vena cava. DNA ladders were run on both the left and right side of the gel. RTA = Rat tail artery; NTC = no template control.

Figure 3

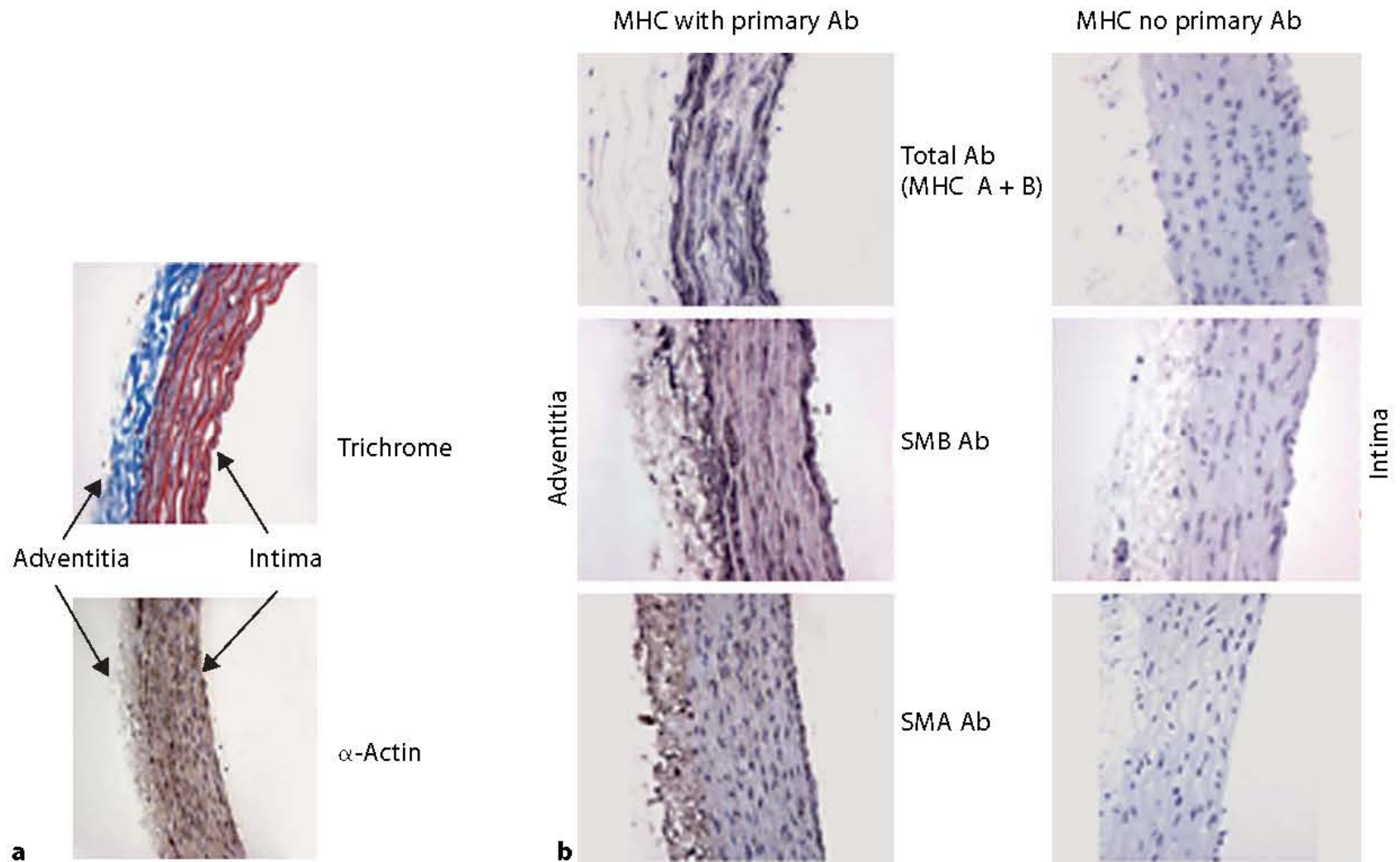


Figure 4

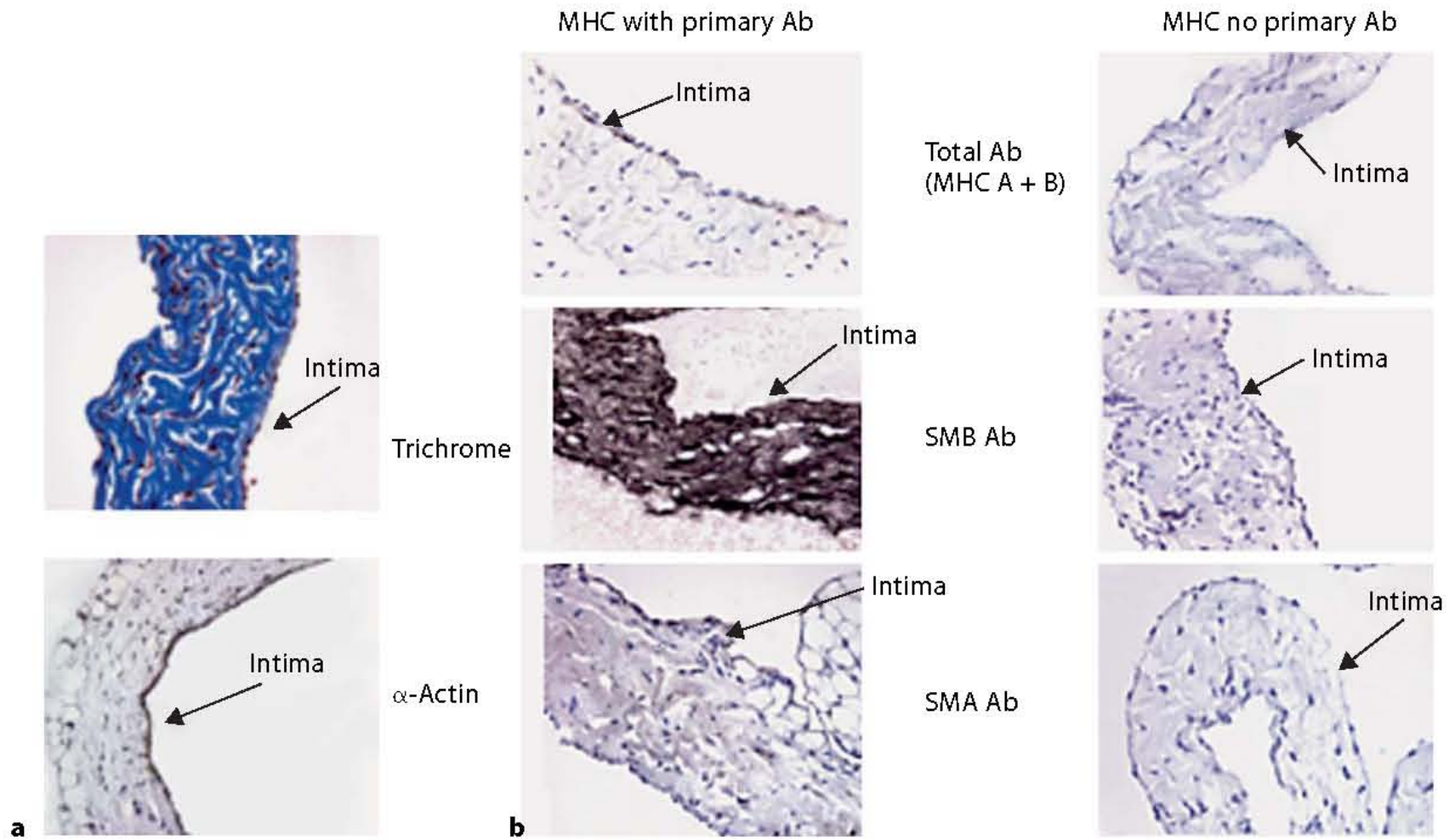
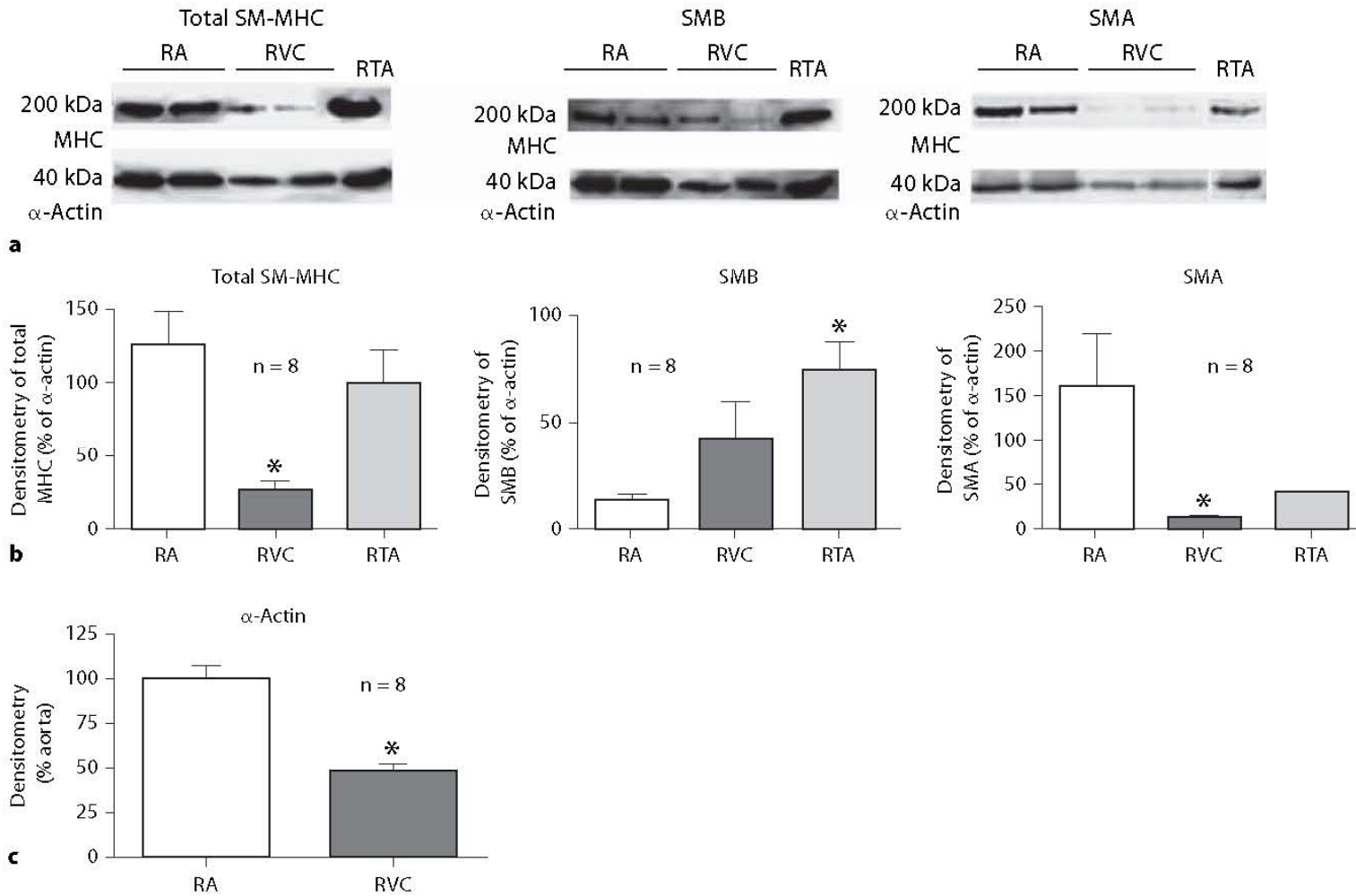


Figure 5



a Western blot of total SM-MHC, SMB and SMA. The upper bands are SM-MHC, while the lower bands are the respective smooth muscle α -actins. Each lane contains 50 μ g total protein.
b Densitometry analysis of total SM-MHC, SMB and SMA expression. Each value was normalized to the relative density of α -actin.
c Relative difference in α -actin in aorta and vena cava per microgram protein. RA = Rat aorta; RVC = rat vena cava; RTA = rat tail artery. * $p < 0.05$.