Expression of Smooth Muscle Myosin Heavy Chain B in Cardiac Vessels of Normotensive and Hypertensive Rats

Ulrike Wetzel, Gudrun Lutsch, Hannelore Haase, Ursula Ganten, Ingo Morano

Abstract—We investigated expression of the 5′-spliced isoform of smooth muscle myosin heavy chain (SM-MHC-B) in smooth muscle cells of cardiac vessels of the left ventricle of normotensive (Wistar-Kyoto) and spontaneously hypertensive rats of the stroke-prone strain by immunofluorescence microscopy. In parallel, liver and bladder were studied for characterization of the nature of vessels expressing SM-MHC-B and for semiquantitative evaluation of its abundance. Smooth muscle cells were detected by staining with a monoclonal antibody specific for α-smooth muscle actin. Abundance of the SM-MHC-B isoform in these cells was evaluated by using an antibody raised against the seven–amino acid insert at the 25K/50K junction of the myosin head (a25K/50K) that specifically recognized SM-MHC-B. In the ventricle, a25K/50K immunoreactivity was observed in smooth muscle cells of precapillary arterioles but not in larger vessels or aorta. The a25K/50K immunoresponse of those vessels with the highest expression level of SM-MHC-B closely resembled the signal observed in the smooth muscle layer of urinary bladder known to preferentially express SM-MHC-B. Interestingly, in left ventricles of stroke-prone spontaneously hypertensive rats, there was a significantly reduced fraction of a25K/50K-positive precapillary arterioles compared with normotensive control rats. **(*Circ Res*. 1998;83:204-209.)**

Key Words: smooth muscle ■ myosin heavy chain ■ hypertrophy ■ hypertension ■ cardiac vessel

Perfusion of the heart with blood is a main determinant of the contractile state of the myocardium and depends largely on the tone of SMCs of cardiac vessels. This tone is generated by the molecular motor type II myosin, which is composed of two heavy chains (MHCs), each associated with two types of light chains (MLCs).[1–3] MHC and MLC isoforms of smooth muscle (SM) are produced by both expression of different genes and differential splicing of single genes.[4] The SM-MHC gene transcript is alternatively spliced at both the 3′ and 5′ ends of the gene. Alternative splicing of a 39-nucleotide exon of SM2 (200 kDa) at the most 3′ terminus generates SM1 (204 kDa).[5–7] 5′ splicing is accomplished by excision of 21 nucleotides (seven amino acids) near the ATP-binding 25K/50K junction.[8–10] SM-MHCs without 5′ insertion were designated as the "A" forms (SM1A and SM2A); those with the insert were designated as the "B" forms (SM1B and SM2B).

Little is known about regulation of alternative splicing of MHC and MLC isoforms in SMCs as well as functional consequences of alternative splicing. During pregnancy, the B forms become downregulated in the rat myometrium.[11] Downregulation of the B forms could also be observed in the megacolon in Hirschsprung's disease[12] as well as in hypertrophied urinary bladder.[13] We recently demonstrated that steroid hormones are involved in the regulation of myosin subunit expression: testosterone but not estrogen favored the expression of SM1 and 5′-inserted MHC, whereas estrogen reduced it.[14] MHCs containing the 25K/50K insert revealed a higher ATPase activity and moved actin filaments faster in in vitro motility assays than did MHCs without the 5′ insert.[10,15] In physiological experiments, however, downregulation of the SM-MHC-B isoforms in the megacolon and pregnant myometrium is associated with an increased shortening velocity.[12,16] There is a tissue-specific regulation of SM-MHC splicing. As demonstrated by mRNA analysis, ≈85% of rat bladder MHCs contained the 25K/50K insertion, whereas MHCs of rat aorta and myometrium contained very low levels of SM-MHCs with 25K/50K insertion.[8] The B form has therefore been designated as the "intestinal" form, and the A form has been designated as the "vascular" form.

In the present study, we investigated expression of the SM-MHC-B isoform in cardiac vessels of normotensive rats (WKY) and SHRSP using immunofluorescence microscopy. We found for the first time that SMCs of precapillary arterioles in the heart contain considerable amounts of the intestinal B form. In contrast, larger arteries contain predominantly the A form. Furthermore, SHRSP reveal a smaller fraction of precapillary arterioles in which detectable levels of the B form are found.

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Selected Abbreviations and Acronyms

a25K/50K = anti–25K/50K insertion
aSM-actin = anti–smooth muscle actin
MHC = myosin heavy chain
MLC = myosin light chain
SHRSP = stroke-prone spontaneously hypertensive rat(s)
SM = smooth muscle
SMC = smooth muscle cell
WKY = Wistar-Kyoto rat(s)

Materials and Methods

Animals and Tissue

We investigated 20-week-old male normotensive rats (WKY) and SHRSP held in accordance with institutional guidelines. Blood pressure was measured by the tail-cuff method performed in light and short ether anesthesia (<2 minutes). SHRSP revealed a significantly higher blood pressure ($P<0.001$) and higher ratio of heart weight to body weight than did WKY ($P<0.001$) (Table 1). The animals were weighed and killed by cervical dislocation, and the heart, aorta, liver, and bladder were excised. After the heart was blotted and weighed, the atria and right ventricles were removed, and the left ventricles were immediately frozen in liquid nitrogen. The aorta, liver, and bladder were also immediately frozen in liquid nitrogen.

Antibody Preparation

A peptide antibody specific for the 25K/50K insertion (a25K/50K) of rat SM-MHC (5′ insert) was generated based on the amino acid sequence QGPSFAY.[8] At the amino terminus of the peptide, *N*-chloroacetylglycine was added to facilitate cross-linking to carriers. The peptide was synthesized by the solid-phase method, purified, coupled to keyhole limpet hemocyanine, and used for immunization of rabbits as described previously.[17] The resulting antibody fractions were purified on a peptide-affinity column.[14] Specificity of the antibody has been characterized previously.[11,14]

Immunofluorescence Microscopy

Cryosections of 5-μm thickness were prepared with a Jung Frigocut 2800N cryostat (Leica). Sections were fixed with 4% formaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for 15 minutes at room temperature. Immunolabeling was performed with a25K/50K (see above) and aSM-actin antibodies (clone asm-1, Boehringer). In double-labeling experiments, primary antibodies were visualized by staining with DTAF- and Cy3-conjugated anti-rabbit and anti-mouse secondary antibodies (Dianova). To suppress unspecific labeling, cryosections were preincubated with a solution containing 20 mmol/L Tris-HCl, pH 8.4, 630 mmol/L NaCl, 0.05% Tween 20, 0.02% NaN_3, and 1% BSA (1% BSA-Tris) for 30 minutes at room temperature. Primary and secondary antibodies were diluted with the same solution to a protein concentration of 0.2 to 5 μg/mL and 5 to 20 μg/mL, respectively. Incubation with primary antibodies was performed for 1 hour at 37°C, and incubation with secondary antibodies was performed for 30 minutes at 37°C. During the immunolabeling procedure, nuclei were stained simultaneously by DAPI (Sigma). Washing steps were performed with 1% BSA-Tris. To prove specificity of immunoreaction, a25K/50K was preincubated with a 1600-fold molar excess of the peptide corresponding to the 25K/50K-insert sequence at 4°C overnight. This caused a complete suppression of the fluorescence signal (not shown). Tissue sections were evaluated with an Axioplan fluorescence microscope (Carl Zeiss) with appropriate filter systems. Micrographs were taken with an MC100 automatic camera (Carl Zeiss) with Kodak TMax 400 film.

To evaluate the fraction of cardiac vessels containing the SM-MHC-B isoform, all aSM-actin–positive and a25K/50K-positive vessels in a section were counted (two sections per ventricle, six animals per group). The fraction of a25K/50K-positive vessels was expressed in percentage of vessels detected by aSM-actin staining.

TABLE 1. Blood Pressure, Body Weight, Heart Weight, and Ratio of Heart Weight to Body Weight Multiplied by 100 of Normotensive WKY and SHRSP

	Blood Pressure, mm Hg	Body Weight, g	Heart Weight, g	Ratio ×100*
WKY	114±9	413.5±10.1	1.51±0.003	0.365±0.007
SHRSP	225±19†	309±9.9	1.44±0.04	0.466±0.006†

*Ratio of heart weight to body weight multiplied by 100.
†$P<0.001$, SHRSP vs WKY.

Statistical Evaluation

Results are expressed as mean±SD. Significance analysis was performed with the Student′s *t* test. In case of significant differences between SDs, the nonparametric Mann-Whitney test was used.

Results

Localization of SM-MHC-B in Left Ventricles of Normotensive and Hypertensive Rats

Cryostat sections of rat left ventricles were investigated with respect to the presence of a splice variant of SM-MHC (SM-MHC-B) by immunofluorescence microscopy with the use of a double-labeling method. An aSM-actin antibody that specifically reacts with the actin expressed in SMCs was used to identify cardiac vessels, and the a25K/50K peptide antibody was used for simultaneous detection of the SM-MHC-B isoform in the same sections. As seen in Figure 1, both antibodies stain specifically cardiac vessels, whereas cardiomyocytes are not stained. The antibodies differ, however, in their labeling pattern in vessels of different size. Most small vessels of ventricular tissue were labeled with both antibodies, whereas large vessels were only labeled with the aSM-actin antibody (Figure 1). The a25K/50K-positive vessels are characterized by a wall thickness $\leq$10 μm and a lumen diameter $\leq$25 μm, with the majority ($\approx$85%) in the range of $\leq$5 μm for wall thickness and lumen diameter. From wall thickness and simultaneous staining of nuclei with DAPI (Figure 2), it can be concluded that most of the a25K/50K-positive vessels are surrounded by a single layer of SMCs. In these vessels staining intensity is evenly distributed in the vessel wall (Figures 1 and 2). Irregular distribution of staining intensity was found in only a small fraction of a25K/50K-positive vessels with a wall thickness >5 μm, which indicated no preferred location toward the lumen or the outer surface of the vessel (not shown). No a25K/50K-positive vessels with a wall thickness >10 μm have been observed.

The staining pattern of the a25K/50K antibody is virtually identical to that of the aSM-actin antibody, as demonstrated at higher resolution in Figure 2. A cross-sectioned part (Figure 2A through 2D) and a tangentially sectioned part (Figure 2E through 2H) of a small vessel show unambiguously that SMCs stained specifically with the aSM-actin antibody (Figure 2B and 2F) are also labeled with the a25K/50K antibody (Figure 2A and 2E). Endothelial cells located in the lumen of the vessel are not stained. The location of endothelial cells is demonstrated by staining of their nuclei with DAPI (Figure

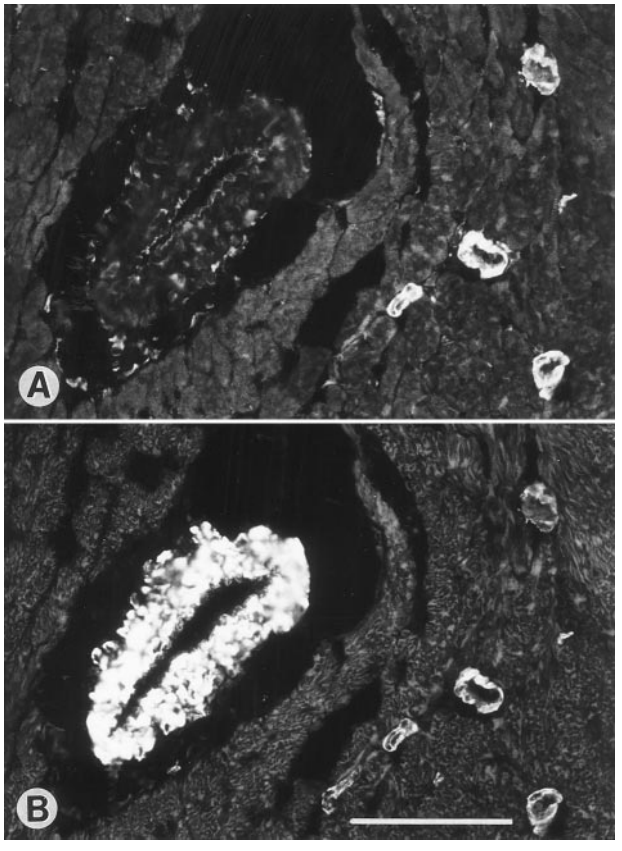

Figure 1. Immunofluorescence micrographs of left ventricle from WKY double-labeled with a25K/50K (A) and aSM-actin (B) antibodies. Primary antibodies were visualized with anti-rabbit and anti-mouse secondary antibodies, respectively, labeled with DTAF (A) and Cy3 (B). Bar=100 μm.

2C and 2G) and by the structures visible in the corresponding differential interference contrast images (Figure 2D and 2H). Thus, it can be concluded that SMCs of small ventricular vessels express the B form of SM myosin.

To characterize the nature of vessels labeled with the a25K/50K antibody in ventricular tissue, immunolabeling was performed with cryostat sections of rat liver, where arterioles and veins can be differentiated histologically according to their specific location in liver lobules. As can be seen in Figure 3, small vessels were intensively stained by the a25K/50K antibody, whereas the larger vein of the portal triangle did not show any immunoreaction. We therefore suppose that a25K/50K-positive vessels in liver and heart tissue are arterioles, whereas a25K/50K-negative vessels are venules. In summary, the immunofluorescence data characterize precapillary arterioles of rat ventricular tissue as sites of SM-MHC-B expression.

In another set of experiments we compared the proportion of a25K/50K-labeled vessels in left ventricles of WKY and SHRSP. As shown in Table 2, the proportion of a25K/50K-positive vessels was significantly higher in WKY than in SHRSP, the difference amounting to ≈17% related to the total number of counted vessels. In both rat strains the percentage of vessels with a wall thickness >10 μm was ≈5%, and no a25K/50K-positive vessels were found in this size range. Therefore, we conclude that the difference in the percentage of a25K/50K-positive vessels resulted from differences in SM-MHC-B abundance in ventricular vessels of WKY and SHRSP.

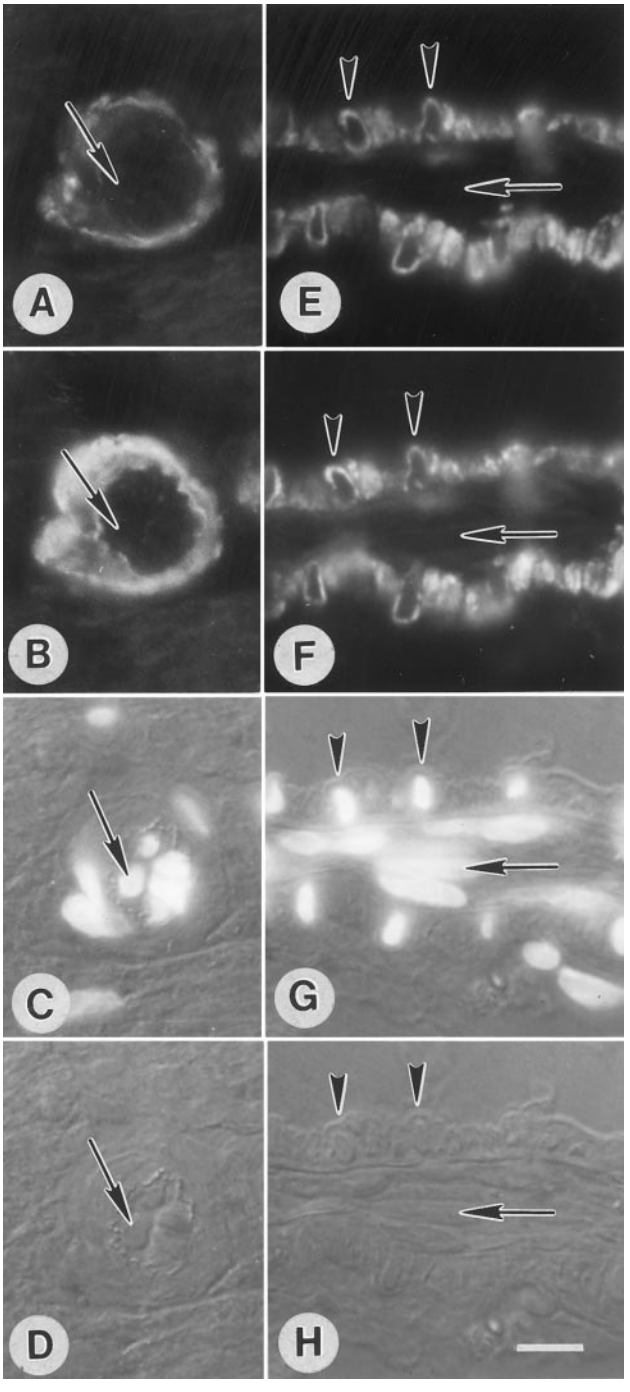

Figure 2. Immunofluorescence (A, B, E, and F) and differential interference contrast (C, D, G, and H) micrographs of small cardiac vessels double-labeled with a25K/50K (A and E) and aSM-actin (B and F) antibodies. Nuclei were stained with DAPI (C and G). Primary antibodies were visualized with anti-rabbit and anti-mouse secondary antibodies, respectively, labeled with Cy3 (A and E) and DTAF (B and F). Dark regions in SMCs indicate unstained nuclei; arrowheads, SMCs; and arrows, endothelial cells. Bar=10 μm.

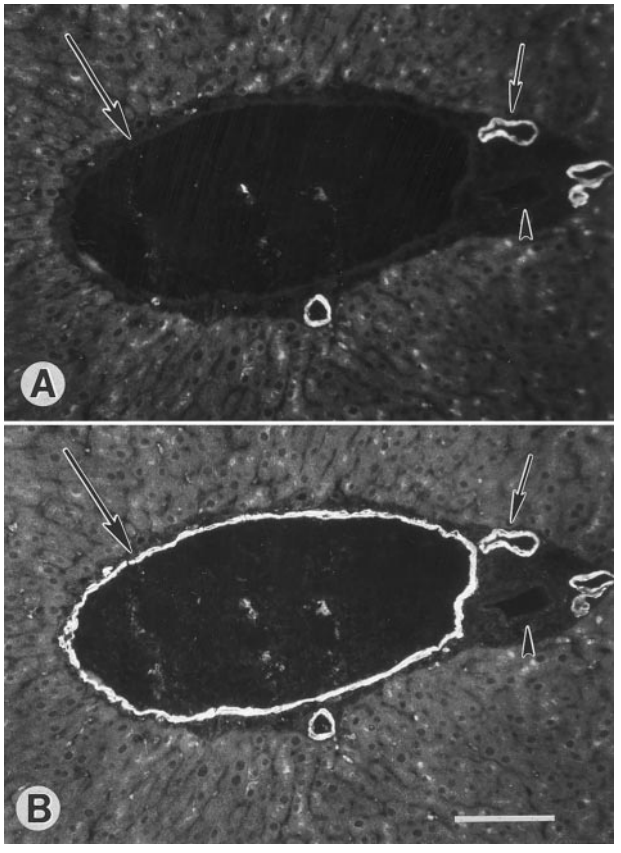

Figure 3. Immunofluorescence micrographs of rat liver double-labeled with a25K/50K (A) and aSM-actin (B) antibodies. Primary antibodies were visualized with anti-rabbit and anti-mouse secondary antibodies, respectively, labeled with Cy3 (A) and DTAF (B). Large arrows indicate veins; small arrows, arterioles; and arrowheads, bile ducts. Bar=100 μm.

Semiquantitative Evaluation of SM-MHC-B Level by Immunoblotting and Immunofluorescence Microscopy

To compare the level of SM-MHC-B in cardiac vessels with that of other tissues with known expression level of SM-MHC-B, Western blot analyses were performed with the a25K/50K antibody and SDS-extracted proteins from the aorta, bladder, and left ventricle of WKY. The antibody reacted strongly and specifically with the 200-kDa MHC band in bladder (Figure 4, lanes 4 to 6), whereas no

TABLE 2. Relation of Vessel Wall Thickness and Reaction With a25K/50K and aSM-actin Antibodies in Ventricular Vessels of WKY and SHRSP

Reaction With a25K/50K Antibody	Reaction With aSM-actin Antibody	Vessel Wall Thickness, μm	Proportion* of Vessels in WKY, %	Proportion* of Vessels in SHRSP, %
+	+	<10	69.7±5.7	53.0±11.1†
+	+	>10	0	0
−	+	<10	25.4±4.9	41.3±12.0†
−	+	>10	4.9±2.6	5.6±4.0

*Values are mean±SD calculated from investigations of 6 animals per group and 2 sections per ventricle (n=12). Total numbers of evaluated vessels were 673 and 438 for WKY and SHRSP, respectively.

†$P<0.001$, SHRSP vs WKY.

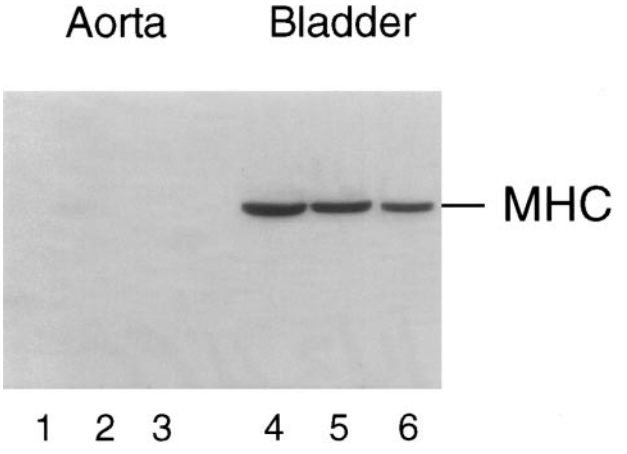

Figure 4. Western blot analysis of rat tissues with the aSM-MHC-B antibody. Transfers of SDS-extracted proteins from aorta (lanes 1 to 3) and urinary bladder (lanes 4 to 6) were probed with the antibody directed against the seven–amino acid insert in the SM-MHC-B isoform at 1 μg IgG per milliliter. The protein concentration applied per lane was 60, 40, and 20 μg for lanes 1, 2, and 3, respectively, and 10, 5, and 2.5 μg for lanes 4, 5, and 6, respectively.

immunoreaction was obtained with SM-MHC of aorta (Figure 4, lanes 1 to 3) and left ventricle (not shown). No cross-reactivity with other proteins was observed. These results confirm previous data demonstrating predominant expression of SM-MHC-B in urinary bladder[8] and are in accordance with the immunofluorescence studies described above with respect to a very low level of SM-MHC-B in ventricular tissue. Obviously, in protein extracts of the ventricle, the concentration of SM-MHC originating from cardiac vessels did not reach the detection limit in the Western blot analysis.

Additional immunofluorescence studies were performed with cryostat sections of urinary bladder and aorta to compare the immunoreaction of the a25K/50K antibody in two tissues with very different expression levels of SM-MHC-B. In accordance with the results of immunoblotting, no a25K/50K staining was seen in aorta (not shown), whereas double-labeling experiments with aSM-actin and a25K/50K antibodies performed on cryostat sections of urinary bladder revealed that the SM layer of bladder wall as well as SMCs of small vessels were strongly labeled by both antibodies (Figure 5). Similar to cardiac tissue, we observed vessels that were labeled solely with the aSM-actin antibody but not with the a25K/50K antibody (large arrows in Figure 5). To evaluate the SM-MHC-B level, double-labeling experiments were performed with decreasing concentrations of the a25K/50K antibody (4 to 0.2 μg/mL) and constant concentrations of the aSM-actin antibody on cryostat sections of left ventricles and urinary bladder of WKY. As expected, staining intensity in urinary bladder gradually decreased with antibody dilution, suggesting very similar levels of SM-MHC-B throughout the SMC layer of urinary bladder wall. In ventricular tissue, however, the number of positively reacting precapillary arterioles declined from ≈75% at an antibody concentration of 4 μg/mL to ≈5% at an antibody concentration of 0.2 μg/mL (related to aSM-actin–positive vessels). Below an

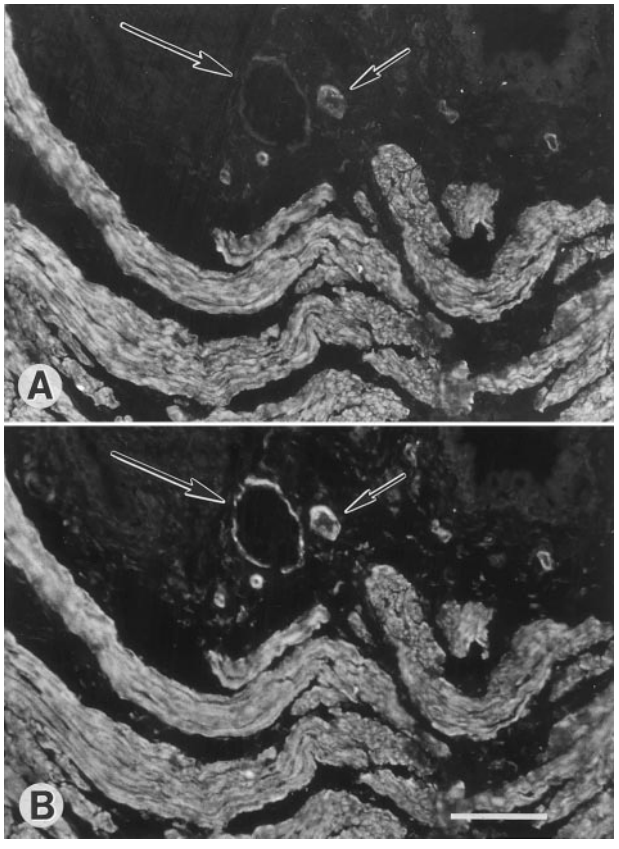

Figure 5. Immunofluorescence micrographs of rat urinary bladder double-labeled with a25K/50K (A) and aSM-actin (B) antibodies. Primary antibodies were visualized with anti-rabbit and anti-mouse secondary antibodies, respectively, labeled with DTAF (A) and Cy3 (B). Large arrows indicate venules; small arrows, arterioles. Bar=100 μm.

a25K/50K antibody concentration of 0.2 μg/mL, no fluorescence signal was observed in either bladder or ventricular tissue. This finding indicates the existence of different levels of SM-MHC-B in ventricular precapillary arterioles. Furthermore, the data show that the highest level of SM-MHC-B in SMCs of ventricular precapillary arterioles, ie, in positively reacting vessels at an antibody concentration of 0.2 μg/mL, closely resembles that of SMCs of urinary bladder.

Discussion

One main finding in the present study is that cardiac vessels in rat left ventricle contain the SM-MHC-B isoform; ie, a major fraction of cardiac vessels expresses the intestinal MHC isoform on the protein level. The SM-MHC-B–containing cardiac vessels have been interpreted as terminal arterioles. Expression of 5′-inserted SM-MHC in arterioles of the heart is in agreement with a previous report in which SM-MHC mRNA with the 5′ insert was described as being preferentially expressed in distributing femoral and saphenous arteries.[18] As demonstrated by our antibody dilution experiments, the level of the SM-MHC-B isoform, at least in a small fraction of precapillary arterioles of rat left ventricles, was comparable to that in urinary bladder, which is known to preferentially express SM-MHC-B.[8]

The physiological importance of the observation of considerable amounts of SM-MHC-B in cardiac blood vessels is still not clear because of the conflicting results of experiments performed in vitro and in physiological models. In vitro, SM-MHCs with the 25K/50K insertion revealed a higher actin-activated ATPase activity as well as a higher velocity of actin filament sliding in the in vitro motility assay.[10,15] Results obtained by in vitro motility assays predict an increased shortening velocity. However, this could not be verified in physiological experiments. Although ≈85% of SM-MHC mRNA of the rat bladder contained the 5′-insertion,[8] it has a maximal shortening velocity that is very similar to that of the myometrium,[11] where only minor amounts of 5′-inserted SM-MHCs are found.[8] Furthermore, although rat myometrium and aorta contain comparable low levels of the 5′ insertion,[8] they revealed very different shortening velocities (compare Reference 16 with Reference 19). In the hypertrophied myometrium during pregnancy[11,16] and in the megacolon in Hirschsprung's disease,[12] decreased expression of the SM-MHC-B form was observed, while maximal shortening velocity increased. At least in the megacolon, this increased shortening velocity was independent of changes of 17-kDa MLC isoforms.[12]

Another important finding of the present study is that the amount of the relevant cardiac vessels that contained the 5′-inserted MHCs was significantly lower in SHRSP than in normotensive age-matched controls (WKY). This difference is most likely due to a decreased level of SM-MHC-B in SHRSP, which may result from elevated splicing activity in this hypertensive rat strain. On the other hand, it cannot be excluded that in SHRSP a reduction in SM-MHC transcription occurs, resulting in less of each isoform. However, this seems unlikely because, for example, pressure-induced hypertrophied SMCs revealed no reduction in force generation[20] or myosin content.[21] Although the physiological role of 5′-inserted MHC is not completely clear, some speculations may be allowed because of the finding that in the hypertrophied myometrium[10,16] and colon[12] the SM-MHC-B form expression decreased, while maximal shortening velocity increased. Since maximal shortening velocity reflects the detachment rate constant of myosin cross-bridges (gapp[22]), we suggest that the SM-MHC-B isoform remains for a longer time in the force-generating state than the alternatively spliced A form. Any shift of the ratio between the attachment rate (fapp) and the detachment rate (gapp) (fapp/gapp) changes the force-calcium relationship.[23] Our prediction, therefore, is a leftward shift of the force-calcium relationship (increased Ca^{2+} sensitivity) of cardiac vessels expressing the B form compared with those with the A form. Furthermore, since both ATP consumption (which equals fappgapp/[fapp+gapp]) and force (which equals fapp/[fapp+gapp]) (Reference 22) depend on gapp, a more economical force production in the presence of the B form would be allowed. In essential hypertension, this economizing capacity would be reduced as a result of decreased abundance of the SM-MHC-B forms.

In summary, we observed expression of the intestinal SM-MHC isoform in the cardiac arteries located farthest downstream, namely, the precapillary arterioles. The level of the isoform is reduced in the hypertrophied heart of hypertensive animals.

Acknowledgments

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