Characteristics of Arterial Myosin in Experimental Renal Hypertension in the Dog

Aravinda Upadhya, Mathew Samuel, Robert H. Cox, Roger J. Bagshaw, and Samuel Chacko

We compared myosin samples isolated from iliac-femoral arteries of control and renal (stenosis) hypertensive dogs to determine the effects of increased blood pressure on the characteristics of the myosin. The ratio of 204-kd (SM-1) to 200-kd (SM-2) myosin heavy chains was approximately 1:0.75 for myosin from the iliac-femoral artery of normotensive dogs. This was not altered significantly in response to hypertension. Both SM-1 and SM-2 myosin heavy chains cross-reacted with antibody against smooth muscle myosin on Western blot analysis. In addition to these heavy chains, purified myosin from both groups showed a very faint protein band slightly below the 200-kd myosin heavy chain on electrophoresis on a highly porous sodium dodecyl sulfate-polyacrylamide gel. This protein band cross-reacted with antibody against nonmuscle myosin but not with smooth muscle myosin antibody. The 20- and 17-kd light chains of myosin isolated from normotensive and hypertensive dogs showed similar results on isoelectric focusing. Peptide maps of tryptic digests of heavy chains revealed both quantitative and qualitative differences. The Ca++-activated myosin ATPase activity measured in high salt (0.5 mol/L KCl) was similar for myosin from both groups, whereas the potassium (ethylenedinitrilo)tetraacetic acid-stimulated ATPase of myosin from hypertensive animals was higher than that from normotensive animals. The actin-activated ATPase activities of the myosin from hypertensive animals was also higher than that of the myosin isolated from normotensive artery (0.11±0.007 and 0.213±0.008 μmol P, per milligram per minute, respectively, for normotensive and hypertensive). These studies indicate that the structural and functional properties of myosin in a muscular artery are altered in hypertensive dogs. (Hypertension 1993;21:624–631)

KEY WORDS • hypertension, renal • myosin • adenosine triphosphatase • muscle, smooth, vascular
pertensive dogs. Data presented in this report indicate qualitative and quantitative differences in the peptide maps of myosin heavy chain from arteries of normotensive and hypertensive animals, although the relative proportion of SM-1 and SM-2 myosin heavy chains is not altered. The difference in the peptide maps of myosin heavy chains from normotensive and hypertensive animals is associated with alterations in the myosin ATPase and actin-activated Mg-ATPase activities.

**Methods**

**Animals**

All procedures followed were in accordance with institutional guidelines for animal experimentation. Fully conditioned and heartworm-free mongrel dogs, all weighing approximately 35 kg, were obtained from a licensed supplier (Quaker Farm, Quakertown, Pa.). The dogs were trained to lie on their right side and to accept a repeatedly inflated blood pressure cuff on the left lower leg.21 Pressure cuffs with a width of approximately 50% of the circumference of the dog's lower leg were used. Systolic and diastolic values were monitored by palpation and auscultation of the dorsalis pedis artery with an ultrasonic stethoscope (model BF5A, Medasonic, Mountain View, Calif.). Initially, systolic pressure was determined by palpation. When these values became constant, systolic and diastolic values were recorded, the systolic by occlusion and diastolic by the occurrence of a second heart sound. Heart rates were determined by dorsalis pedis artery palpation. Blood pressures and heart rates were determined approximately every minute for several minutes. This procedure was carried out twice a day, and the mean of all values was taken as the daily blood pressure and heart rate.

A group of these animals was made hypertensive using a unilateral renal artery stenosis method22 with animals under general anesthesia (sodium pentobarbital, 30 mg/kg i.v.). Each animal's blood pressure was measured on a weekly basis for a period of 3 months. Systolic, diastolic, and mean arterial pressure values were all elevated in the hypertensive group compared with the control animals (Table 1). The iliac and femoral arteries were bilaterally removed for use in these studies. Other arteries were used for experiments unrelated to this study. Arteries from a set of normotensive dogs treated in a similar manner but not subjected to renal stenosis were used for controls.

**Preparation of Contractile Proteins**

The arterial tissues removed from the animals were kept in wash buffer [60 mmol/L KCl, 20 mmol/L imidazole-HCl (pH 6.9), 2 mmol/L (ethylenedinitrilo)tetraacetic acid (EDTA), 2 mmol/L dithiothreitol, 0.4 mmol/L phenylmethanesulfonyl fluoride (PMSF), 1 µmol/L pepstatin A, 5 µmol/L trypsin inhibitor, and 0.01% NaN₃] immersed in ice. The adventitia and intima were removed from the arterial segments with the aid of a dissecting microscope. The tissue was extracted, and ammonium sulfate fractions (0–35% and 35–70%) of the extract were made. Before purification, myosin was phosphorylated using the endogenous light chain kinase present in the 35–70% ammonium sulfate fraction. Myosin from 35–70% ammonium sulfate fractions was purified by gel filtration on Sepharose CL-4B columns as described.23 The profile of the Sepharose CL-4B column is shown in Figure 1. An actin-rich actomyosin was found in peak I; peak II contained pure myosin (see the sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE] on the top of the peak). The third peak contained tropomyosin and other contaminant proteins. The myosin in peak II revealed the heavy chain and two light chains (Figure 1). The myosin fractions purified from arteries of normotensive and hypertensive animals and used for this study were

<table>
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<th>Group</th>
<th>Systolic (mm Hg)</th>
<th>Diastolic (mm Hg)</th>
<th>Mean (mm Hg)</th>
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<tbody>
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<td>80±1</td>
<td>94±1</td>
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<tr>
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<td>167±2*</td>
<td>101±5*</td>
<td>127±2*</td>
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</table>

*p<0.01.

![FIGURE 1. Sepharose CL-4B gel filtration profile. Two milligrams of 35–70% ammonium sulfate fraction of extract of iliac-femoral artery from hypertensive dog was loaded onto a 0.75x60-cm column equilibrated with 0.8 mol/L KCl, 20 mmol/L Tris-HCl (pH 7.5), 5 mmol/L dithiothreitol, phenylmethanesulfonyl fluoride, and antipain. Protein concentrations in the fractions were monitored by a UV absorbance, and 1-mL fractions were collected. Fractions I and II correspond to actin-rich actomyosin and pure myosin, respectively. The third peak (III) contains tropomyosin and other unidentified proteins. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the myosin from peak II is shown above the peak.]
found to be fully phosphorylated on urea gel electrophoresis and isoelectric focusing (data not shown). The myosin peak was pooled and dialyzed against 10 mmol/L imidazole-HCl (pH 7.2), 10 mmol/L MgCl₂, and 2 mmol/L dithiothreitol for 6 hours and was concentrated as described.²⁴ Protease inhibitors (0.4 mmol/L PMSF, 1 µmol/L antipain, 2 µmol/L pepstatin A, 50 µmol/L trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), and 0.02% NaN₃) were present in all buffers. Actin and tropomyosin were prepared from chicken gizzard.²⁵

The yields of myosin from arteries of normotensive and hypertensive animals were similar. Approximately 2 mg 35–70% ammonium sulfate fractions per gram arterial media and 0.2 mg pure myosin per milligram 35–70% ammonium sulfate fraction were obtained from both groups.

ATPase Assay

The myosin ATPase activities were determined in 0.5 mol/L KCl, 20 mmol/L imidazole-HCl (pH 7.5), 2 mmol/L adenosine 5'-triphosphate (ATP), and either 2 mmol/L EDTA or 10 mmol/L CaCl₂, with a myosin concentration of 6–10 µg/mL, as described by Chacko et al.²³ For actin-activated ATPase assay, myosin was reconstituted with tropomyosin-actin (molar ratio of tropomyosin to actin, 1:6) to obtain a 1:100 molar ratio of myosin to actin in 10 mmol/L imidazole-HCl (pH 7.2), 20 mmol/L KCl, 1 mmol/L dithiothreitol, 0.1 mmol/L CaCl₂, and 5 mmol/L MgCl₂. All samples were incubated at 37°C, and assays were initiated by adding [γ-³²P]ATP (10–30 µCi/mol) mixed with cold ATP (final concentration, 2 mmol/L) so as to give 20,000–40,000 cpm in 5 µmol/L of the assay mixture. Aliquots were removed at zero time and two additional times to ascertain linearity of phosphate release. Inorganic phosphate released from ATP was measured as described.²⁶

Gel Electrophoresis and Quantitation of Proteins

The myosin heavy chains were separated by electrophoresis on slow-running, highly porous SDS-polyacrylamide slab gels. These gels were made 3.5–7% (gradient) acrylamide with 0.065% bis-acrylamide using the buffer system of Laemmli.²⁷ These gels were 1 mm thick, 160 mm wide, and 130 mm long, including the 30 mm of stacking gel (3.5% acrylamide). To maximize the resolution of the two myosin heavy chains, we kept the amount of myosin loaded into each well between 0.5 and 1 µg. Electrophoresis was carried out for 12 hours at 8 mA per gel slab. Gels were stained for 30 minutes at 70°C in 0.5% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and were destained with a solution containing 30% methanol and 10% acetic acid. Stained proteins were quantified by densitometric scanning on an UltraScan XL (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.). The relative proportion of each band was calculated from the areas under the peaks in the scans. The relative ratio of SM-1 to SM-2 was obtained by dividing the area under the peak of SM-1 by that of SM-2. The statistical significance was determined by Student's t test. Protein purity was ascertained by electrophoresis on a 5–12% gradient SDS-polyacrylamide gel.²⁷ The urea gel electrophoresis, to determine the extent of phosphorylation of the light chain, was performed as described by Ferrie et al.²⁸

Protein concentration was determined by the procedure of Lowry et al.²⁹

Immunoblotting

Proteins in the 3.5–7% SDS-polyacrylamide gels were blotted onto a nitrocellulose membrane (Millipore Corp., Bedford, Mass.) and assayed immunologically for myosin by the method of Towbin et al³⁰ with slight modifications. The gels were transblotted onto membranes using a mini transblot electrophoretic transfer cell (Bio-Rad Laboratories, Richmond, Calif.). The transfer was done at a constant current of 200 mA for 3 hours. The membranes containing protein blots were treated with 3% gelatin solution containing 20 mmol/L Tris-HCl (pH 7.5) and 0.5 mol/L NaCl for 1 hour to block the nonspecific binding of the antibodies to the membranes. The membranes were then incubated overnight with antibodies diluted in TTBS buffer (20 mmol/L Tris-HCl, 0.5 mol/L NaCl, 0.05% Tween-20, pH 7.5) containing 1% gelatin. After incubation, membranes were washed twice in TTBS and treated with protein A gold solution (Bio-Rad), which gave antigen-antibody complexes a rose-red color in 30 minutes to 1 hour.

Polyclonal antibodies raised in rabbit against myosin from bovine aorta smooth muscle and from human platelets were gifts from Dr. Robert S. Adelstein of the National Heart, Lung, and Blood Institute.

Two-dimensional Peptide Mapping

Radioiodination of the myosin heavy chain, obtained by SDS-PAGE (7.5%) of purified myosin from iliac-femoral arteries from normotensive and hypertensive dogs, was carried out by the method of Elder et al.³¹ In brief, the heavy chains, which migrate as one band in 7.5% SDS-polyacrylamide gel, were stained with Coomassie blue, then sliced, washed, and dried. The gel slices were each incubated separately with [¹²⁵I] (0.2 mCi/30 µL) (Amersham Corp., Arlington Heights, Ill.) in the presence of chloramine T. After the unreacted [¹²⁵I] was removed by extensive washing in 10% methanol with several changes, the gel slices were dried. The dried gel slices were incubated for 20–40 hours at 37°C in 50 µg/mL 1:1-trypsin (200 units/mg) (Cooper Biomedical Inc.) dissolved in 50 mmol/L NH₄HCO₃. The resulting tryptic digests were lyophilized and dissolved in electrophoresis buffer (acetic acid/formic acid/water, 15:5:18). The peptide solution was applied to a Silica Gel 60 thin-layer plate (EM Science, FRG) and was air-dried. The plate was autoradiographed and examined under a UV lamp. The bands were cut out and eluted with 1 mol/L HCl. The eluates were dried and dissolved in 20 µL of water. The radioactivity of radioiodinated myosin and peptide bands was determined by liquid scintillation counting.

Isoelectric Focusing

Mini isoelectric focusing cylindrical gels (2×65 mm) were cast according to O'Farrell.³² Pharmacia ampholine with a pH range of 4–6.5 was used. The gels were prefocused for 1 hour at 200 V and focused at 500 V for
Results

Myosin purified by gel filtration on a Sepharose CL-4B column shows a myosin heavy chain (200 kd) and two light chains with molecular weights of approximately 20 and 17 kd, respectively, on SDS-PAGE 7.5% gel (Figure 1). The two heavy chains of the myosin molecules were not separated by electrophoresis under this condition (Figure 1). In view of the finding that developing chicken gizzard and bovine aorta contain isoforms of 20- and 17-kd light chains, purified myosin was subjected to isoelectric focusing to see if isoforms of the light chains emerged in response to hypertension in the iliac-femoral artery. The 20-kd light chain gave only one band when fully phosphorylated myosin was electrophoresed on isoelectric focusing gel; partially phosphorylated myosin gave two bands (see Figure 2, gel 1). The 17-kd light chain also showed only one band, and the electrophoretic mobilities of this light chain were not altered in response to hypertension (Figure 2).

The difference in the molecular weights of the two heavy chains is demonstrated by electrophoresis of the myosin on highly porous SDS-polyacrylamide gels (3.5–7%). As shown in Figure 3, the mobilities of the two myosin heavy chains are different. There was no remarkable difference between the myosin isolated from the iliac-femoral arteries of normotensive and hypertensive dogs. The ratio of the 204-kd (SM-1) to the 200-kd (SM-2) heavy chains for myosin isolated from normotensive and hypertensive iliac-femoral arteries was not significantly different (data from six normotensive and six hypertensive: normotensive, 0.757±0.038; hypertensive, 0.818±0.036; p=0.05). Both SM-1 and SM-2 heavy chains from control and hypertensive animals reacted with the polyclonal antibody prepared against smooth muscle myosin but not with the antibody prepared against nonmuscle (human platelet) myosin (Figures 3 and 4). A very faint band that migrated below the 200-kd heavy chain was observed in heavily loaded gels. Western blot analysis showed that this band reacted with antibody against platelet myosin (Figure 4B), and it was present in equal quantity in myosin isolated from both normotensive and hypertensive animals.

To determine if the myosin from the arteries of hypertensive dogs was different in chemical structure, we subjected purified myosin to peptide mapping. Two-dimensional electrophoreograms of the tryptic peptides made from iodinated (125I) myosin heavy chains of myosin obtained from normotensive and hypertensive animals are shown in Figure 5. The peptide maps showed that a large number of radioactive spots are common for myosin heavy chains from normotensive and hypertensive animals. However, the peptide maps also revealed that there were well-defined and reproducible (four of four experiments) differences in the peptide maps as noted (arrows in Figure 5B). The smaller peptides (two spots on the right of the peptide map on Figure 5B) were observed only in two peptide maps of four, presumably because of a difference in the degree of proteolysis.

The high salt ATPase activity of myosin was measured to determine if the changes in the peptide maps of the myosin from hypertensive arteries were associated with functional differences. As shown in Table 2, the Ca²⁺-activated ATPase activity of the myosin from hypertensive and normotensive dogs, respectively; gel 3 is myosin from chicken gizzard. The pH range of the IEF gel is from 4 to 8. Positions of the unphosphorylated (u) and phosphorylated (p) 20-kd light chain on the IEF gels are marked. The myosin sample used in gel 3 is unphosphorylated, and the sample in gel 1 contains a small amount of unphosphorylated light chain. The 17-kd light chain (indicated by a line) from all samples migrates at the same pH range.
FIGURE 3. Immunoblotting of SM-1 and SM-2 of femoral-iliac arteries from renal hypertensive (lanes 1 and 2) and normotensive (lanes 3 and 4) dogs. The 3.5–7% sodium dodecyl sulfate–polyacrylamide gradient gel was blotted onto nitrocellulose membrane and immunostained with antibody against aortic myosin. Notice that the relative proportions of SM-1 (upper band) and SM-2 (lower band) heavy chains are equal in both cases.

mol/L KCl), the Mg$^{2+}$-ATPase activity of myosin was very low (0.01–0.015 μmol P$_i$ per milligram per minute). Myosin from normotensive and hypertensive animals was reconstituted with chicken gizzard actin complexed with tropomyosin (molar ratio of tropomyosin to actin, 1:6). The ATPase activity of actomyosin complex was measured to determine whether the differences in the myosin ATPase activities and peptide maps of normotensive and hypertensive animals are reflected in the physiologically relevant, actin-activated ATPase (Table 3). The Mg$^{2+}$-ATPase of myosin from both groups was activated 10- to 20-fold by actin. The actin-activated ATPase activities of myosin from arteries of a total of five normotensive and four hypertensive animals were 0.11±0.007 and 0.213±0.008 μmol P$_i$ per milligram per minute ($p<0.0001$), respectively.

Discussion

Differences in the contractile characteristics of hypertrophied cardiac muscle are correlated with alterations in the distribution of myosin isoform and myofibrillar ATPase.10–14 A correlation between myosin ATPase and the maximum velocity of shortening at zero load has been known to exist for more than two decades.35 Smooth muscle cells exhibit greater functional diversity20; however, it is not known if the functional diversity is due to differences in functional specialization or due to differences in the isoforms of contractile proteins.

The investigation of contractile proteins in arterial smooth muscle is hampered by the small amounts of tissue available and the diversity of the smooth muscle cells along the arterial system. The present study was carried out using the iliac-femoral artery, a highly muscular segment of the arterial system. Furthermore, the basic biochemical methods were modified to enable analysis of small amounts of proteins.

Embryonic gizzard muscle has been shown to contain a smooth muscle myosin with 23- and 20-kd light chains.33 Isoelectric variants of 17 kd have also been reported for mammalian smooth muscle.34 Purified myosin from arteries of both control and hypertensive animals revealed light chains with apparent molecular weights of 20 and 17 kd. A similar observation was made for myosin purified from chicken gizzard,25–36 bovine stomach,37 guinea pig vas deferens,23 and swine pulmonary artery.24 The electrophoretic mobility of these light chains is not altered in response to hypertension either on SDS-PAGE (data not shown) or on isoelectric focusing (Figure 2).

Myosin heavy chains that differ by less than 2% in molecular weight have been observed in intact smooth muscle16–17–38 and in aortic cell cultures.39 The relative proportions of the SM-1 and SM-2 myosin heavy chain isoforms in dog iliac-femoral artery myosin are slightly different (1:0.75 versus 1:1) from those observed for bovine aortic myosin.17,18 As shown in this study, the
Electrophoresis

proportion of SM-1 and SM-2 is not altered significantly by hypertension. Both heavy chains cross-reacted with antibody prepared against bovine aortic myosin, indicating tissue specificity for myosin heavy chains (Figures 3 and 4A). These heavy chains are antigenically different from platelet myosin (Figure 4B). The heavy chain that migrated below 200 kd cross-reacted with antibody against nonmuscle myosin (Figure 4B). This heavy chain may be derived from a small population of nonmuscle cells inadvertently included when the muscular media was dissected out for myosin preparation. It is also possible that the muscular media may have contained a small population of cells that had not differentiated phenotypically into smooth muscle cells. A third possibility is that a small amount of nonmuscle myosin may be present in differentiated smooth muscle cells, as previously reported.40 Further cytological studies aimed at localizing various myosin types are important for a better understanding of the level of differentiation of the muscle cells in the arterial media and the relevance of their response in pathological processes.

It has been shown recently that the hypertrophy of urinary bladder smooth muscle is associated with an alteration of the ratio of SM-1 to SM-2 in favor of SM-1.41 Interestingly, the change in the ratio of SM-1 to SM-2 in the urinary bladder myosin is not associated with a change in the myosin ATPase. A lack of difference in the ratio of SM-1 to SM-2 in response to hypertension indicates that there is no change in the regulation of the synthesis of these isoforms that differ in their C-terminal region.19 However, the difference in the peptide maps of the tryptic digests of the myosin heavy chain from normotensive and hypertensive dogs indicates a difference in the amino acid composition of the myosin heavy chains. Further experiments, using techniques in molecular biology, are essential to determine if the difference in the peptide maps is caused by a posttranslational modification or is due to the expression of a myosin gene that is not expressed in normotensive animals. Published reports on smooth muscle myosin show that the SM-1 and SM-2 myosin heavy chain isoforms are formed by the alternate splicing of the

TABLE 2. Specific Activity of Myosin ATPase

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<th>Preparation</th>
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<th>Hypertension</th>
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Specific activity is shown as micromoles of P₃ liberated per milligram of myosin per minute. Conditions: 0.5 M KCl, 20 mmol/L imidazole-HCl (pH 7.5), 2 mmol/L ATP, and either 2 mmol/L EDTA or 10 mmol/L CaCl₂, with a myosin concentration of 6–10 μg/mL. Each preparation represents iliac-femoral arteries from separate dogs. A total of six normotensive and six hypertensive dogs were used for these experiments.

* p<0.01.
TABLE 3. Actin-Activated ATPase Activities of Purified Myosin

<table>
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<tr>
<td>Mean</td>
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</tr>
<tr>
<td>SD</td>
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ATPase activity is shown as micromoles of Pi liberated per milligram of myosin per minute. Conditions: Assays were carried out in (mmol/L) imidazole-HCl 10 (pH 7.0), KCl 20, dithiothreitol 1, Mg-ATP 2, free Mg²⁺ 2, and CaCl₂ 0.1. Concentrations of myosin, 0.05 mg/mL; myosin-to-actin molar ratio, 1:50; tropomyosin-to-actin molar ratio, 1:6. Preparations refer to individual myosin preparations made from the iliac-femoral arteries from both sides of the dog. Five normotensive and four hypertensive dogs were used for these experiments. p = 0.0001.

The myosin ATPase activity measured in high salt buffer (0.5 mol/L KCl), although not physiological, gives an indication of any differences in the active sites of the enzyme. The Ca²⁺-activated ATPase activity of smooth muscle myosin isolated from a variety of smooth muscles is lower than K⁺-EDTA-stimulated activity. 23

Modification of the sulphydryl group of the myosin is associated with changes in high salt ATPase activities. 43 In the present study, oxidation of the sulphydryl groups was prevented by using dithiothreitol. Furthermore, the finding that the Ca²⁺-activated ATPase activities of the two myosins were similar and lower than K⁺-EDTA-stimulated activity (Table 2) indicates that the sulphydryl groups were not altered.

A difference in the level of phosphorylation of myosin obtained from different preparations may cause a difference in the actin-activated ATPase activity, but the K⁺-EDTA-stimulated ATPase activity is not altered by phosphorylation. 23 The myosin samples for this study, from both normotensive and hypertensive animals, were fully phosphorylated, as evident from urea gel electrophoresis or isoelectric focusing (data not shown). The difference in the K⁺-EDTA-stimulated myosin ATPase was also associated with a twofold difference in the physiologically relevant actin-activated ATPase activity (Table 3). The differences in the peptide maps and the K⁺-EDTA-stimulated and actin-activated ATPase activities suggest a change in the N-terminal, globular region of the myosin molecule, which contains the active sites of the enzyme, the actin binding site, or both.

Findings in cardiac muscle 12-15 show that structural changes in myosin are associated with increased myofibrillar ATPase activity and increased economy of force generation and maintenance. 12-15 The difference in the peptide maps and the ATPase activity of the myosin isolated from hypertensive arteries from those of the myosin from normotensive arteries may be reflected in either the contractile property or motility-associated functions of the smooth muscle cells in the hypertensive arteries. Increased myofibrillar ATPase 44 and force-velocity relations 3 have been reported to be associated with spontaneous hypertension in rats. Additional immunocytochemical studies on the localization of the myosin in the smooth muscle cells of the vascular media will be helpful in understanding the pathogenesis of other lesions, such as atherosclerosis, associated with hypertension.

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16. Rovner A, Thompson M, Murphy R: Two different heavy chains and lower than K⁺-EDTA-stimulated activity (Table 2) indicates that the sulphydryl groups were not altered.

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