Hypertension is a common condition in Western countries, affecting ≈27% of the population worldwide. It is a major risk factor for the development of life-threatening conditions such as coronary heart disease and stroke. However, the cause of increased blood pressure is unknown in most patients.\(^1\) Smooth muscle contractility is one of the primary determinants of vascular resistance, thereby contributing significantly to the maintenance of a physiological blood pressure. Accordingly, molecular defects in the regulation or mechanics of arterial smooth muscle contraction generally cause profound cardiovascular phenotypes. For example, α-smooth muscle actin (α-SMA) knockout mice display impaired vascular contractility and reduced blood flow.\(^2\) Likewise, smooth muscle myosin heavy chain (SM-MHC)-B knockouts show a significant decrease in maximal shortening velocity of vascular smooth muscle,\(^3\) and SM-caldesmon-deficient mice have impaired mean arterial pressure (MAP) regulation.\(^4\)

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Despite the importance of smooth muscle cell (SMC) contraction for the cardiovascular system, the contractile process itself is still incompletely understood. In particular, the functions of regulatory proteins that are connected to the actin-myosin filaments in vascular SMCs remain poorly defined.\(^5\) Candidate thin filament regulatory proteins that have not been studied in this respect are the smoothelins, which are α-SMA–binding proteins that are abundantly expressed in healthy visceral (smoothelin-A) and vascular (smoothelin-B) smooth muscle. Their expression is strongly associated with the contractile phenotype of smooth muscle cells. Analysis of mice lacking both smoothelins (Smtn-A/B\(^−/−\)) mice previously revealed a critical role for smoothelin-A in intestinal smooth muscle contraction. Here, we report on the generation and cardiovascular phenotype of mice lacking only smoothelin-B (Smtn-B\(^−/−\)).

**Methods and Results**—Myograph studies revealed that the contractile capacity of the saphenous and femoral arteries was strongly reduced in Smtn-B\(^−/−\)/B\(^−/−\) compound mutant mice, regardless of the contractile agonist used to trigger contraction. Arteries from Smtn-A/B\(^−/−\) compound mutant mice exhibited a similar contractile deficit. Smtn-B\(^−/−\) arteries had a normal architecture and expressed normal levels of other smooth muscle cell–specific genes, including smooth muscle myosin heavy chain, α-smooth muscle actin, and smooth muscle-calponin. Decreased contractility of Smtn-B\(^−/−\) arteries was paradoxically accompanied by increased mean arterial pressure (20 mm Hg) and concomitant cardiac hypertrophy despite normal parasympathetic and sympathetic tone in Smtn-B\(^−/−\) mice. Magnetic resonance imaging experiments revealed that cardiac function was not changed, whereas distension of the proximal aorta during the cardiac cycle was increased in Smtn-B\(^−/−\) mice. However, isobaric pulse wave velocity and pulse pressure measurements indicated normal aortic distensibility.

**Conclusions**—Collectively, our results identify smoothelins as key determinants of arterial smooth muscle contractility and cardiovascular performance. Studies on mutations in the Smtn gene or alterations in smoothelin levels in connection to hypertension in humans are warranted. (Circulation. 2008;118:828-836.)

**Key Words:** hypertension • hypertrophy • muscle contraction • muscle, smooth • vascular resistance
proteins specifically and abundantly expressed in contractile SMCs. They are encoded by a single-copy gene that generates 2 major isoforms, both containing a troponin T–like domain. The smaller smoothelin-A isoform is expressed most prominently in vascular SMCs. It is encoded by a single-copy gene that generates 2 major isoforms, both containing a troponin T–like domain.10

In recent years, smoothelin-B has been increasingly recognized as an excellent marker of the so-called contractile SMC phenotype of vascular SMCs. Indeed, loss of smoothelin-B expression reliably indicates the disappearance of the contractile SMC phenotype in various vascular disorders ranging from aortic aneurysms to atherosclerosis and restenosis.9,12–16

Functional studies on smoothelins have been hampered by the rapid downregulation of their expression in vitro and their relative insolubility at physiological ionic concentrations. Therefore, despite its relevance for the characterization of the contractile SMC phenotype, the function of smoothelin-B in vascular SMCs has remained elusive. Recently, however, we showed that smoothelin-A plays a crucial role in intestinal SMC contraction in mice.17 Smtn-A/- mice, which lack both smoothelin isoforms, develop fatal intestinal problems as a result of drastically decreased intestinal SMC contractility. The severe impact of smoothelin-A deficiency on visceral smooth muscle contraction suggests that smoothelin-B might play an equally important role in vascular smooth muscle. To test this hypothesis, we generated mice lacking only smoothelin-B (Smtn-B/-) and investigated their cardiovascular physiology. We report that smoothelin-B–deficient mice show reduced arterial contractility, which is paradoxically accompanied by elevated MAP because of increased peripheral vascular resistance.

Methods

Generation of Smtn-B/- Mice
To generate Smtn-B/- mice, we targeted exons 3 through 7 with a neomycin gene under the control of the thymidine kinase promoter in reverse orientation (Figure 1A). Targeting of embryonic stem cells and germline transmission of the targeted alleles were detected by Southern blotting and polymerase chain reaction (PCR) analysis (Figure 1B; see the online-only Data Supplement for a list of primers). Mice were further backcrossed at least 5 times on C57Bl/6 background before initiation of experiments. Because the mice had a mixed background, we used littermates for experimentation. All animal experiments were approved by the Maastricht University animal ethics committee.

Quantitative Reverse-Transcriptase PCR
Total RNA was extracted from jejunum, hearts, or pooled aortas and femoral arteries from Smtn-A/- and Smtn-B/- mice with Tri-reagent (Sigma-Aldrich, Zwijndrecht, the Netherlands). Reverse
transcription was performed with the iScript cDNA synthesis kit (Biorad, Veenendaal, the Netherlands) and 0.5 or 1 μg RNA. Expression of several transcripts was investigated by quantitative PCR (Q-PCR) with the ABI Prism 7700 System (Perkin Elmer, Norwalk, Conn). Applied primers and probes are listed in the supplemental table. The cyclophilin A transcript was used to normalize the amount and quality of the extracted RNA. Smtn-B\textsuperscript{−/−} expression levels were set at 1.

**Histology and Immunohistochemistry**

Organs from mice 2 months and 1 year of age were fixed in 3.7% formaldehyde in PBS, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Samples of aorta and femoral artery were snap-frozen and embedded in optical coherence tomography (Tissue Tek compound (Sakura, Chicago, Ill). Cryostat sections were blocked with 5% normal donkey serum (Jackson ImmunoResearch, Soham, UK) and stained with a polyclonal antibody raised against 2 smoothelin-specific peptides (described below) and a secondary antibody donkey anti-rabbit conjugated with FITC (Sigma-Aldrich, St. Louis, Mo). Polyclonal antibody generation was performed by Eurogentec (Seraing, Belgium). Two rabbits were immunized with the smoothelin-B-specific peptide KRFRAEQDNKENWNL (smoothelin-B residues 52 to 66) and the peptide RQRKRDQKDERERR, which is present in both smoothelin-A and smoothelin-B (smoothelin-A residues 160 to 174, smoothelin-B residues 614 to 628). Synthesized peptides (5 mg) were conjugated to keyhole limpet hemocyanin. The rabbits received 3 booster injections with a 14-day interval. Sera were tested on different tissues for cross-reactivity and smooth muscle specificity.

To determine the staining pattern of the extracellular matrix proteins collagen and elastin, paraffin-embedded cross sections of arteries were stained with Sirius Red and Lawson’s solution. To evaluate α-SMA and SM-MHC expression, sections were incubated with anti–α-SMA (DAKO, Glostrup, Denmark) or anti–SM-MHC antibody (Biomedical Technologies, Stoughton, Mass) diluted 1:200 or 1:40, respectively.

**Vascular Contractility**

Contractility of the thoracic aorta (n=6) and the femoral artery (n=6) was compared between 10-week-old Smtn-B\textsuperscript{−/−} and Smtn-B\textsuperscript{−/−} littermates. For the contractility analyses of Smtn-A/B\textsuperscript{−/−} and Smtn-A/BB\textsuperscript{−/−} littermates, mice ≈8 weeks of age were used (n=7 for thoracic aorta and femoral artery). Isolated arteries were mounted in myograph organ baths as previously described.\(^{19}\)

We examined reactivity in response to K\textsuperscript{+} (40 mmol/L), the thromboxane A	extsubscript{2} mimetic U-46619 (0.1 to 100 nmol/L), and the \(\alpha\)-adrenergic agonist phenylephrine (to 30 μmol/L), all obtained from Sigma-Aldrich. Contractile forces were corrected for vessel segment length and medial thickness and normalized to wild-type values.

**Hemodynamics**

Five-month-old male Smtn-B\textsuperscript{−/−} (n=7) and Smtn-B\textsuperscript{−/−} (n=10) mice were instrumented with catheters as described before,\(^{19}\) and conscious MAP and heart rate (HR) were recorded for 30 to 60 minutes on days 3 and 5 after surgery. In addition, the contribution of several endogenous neurohumoral mechanisms to blood pressure was assessed by the following pharmacological protocol. On day 3, MAP and HR responses were recorded during intravenous injection of phenylephrine (dose-response curve, 0.1 to 10 μg/kg in the presence of atropine [1.2 mg/kg] to block baroreflex-mediated bradycardia) and after administration of the \(\beta\)-blocker metoprolol (2.5 mg/kg). On day 5, MAP and HR responses to the \(\alpha\) blocker prazosin (0.1 mg/kg) and the \(\alpha\) blocker yohimbine (1 mg/kg) were recorded.

Pulse-wave velocity and pulse pressure were measured under isoflurane anesthesia by a high-fidelity catheter-tip micromanometer (Mikro-tip 1.4 F SPR-671, Millar Instruments, Houston, Tex) that was inserted via the left carotid artery into the aorta of Smtn-B\textsuperscript{−/−} (n=7) and Smtn-B\textsuperscript{−/−} (n=5) mice.

Because of their physical condition and short lifespan, hemodynamic parameters of Smtn-A/B\textsuperscript{−/−} and Smtn-A/BB\textsuperscript{−/−} mice were measured in 6-week-old females under 1% to 2% isoflurane anesthesia via a catheter introduced into the right carotid artery (n=5 for each genotype).

**Magnetic Resonance Imaging**

Magnetic resonance imaging measurements were performed in eleven 8-month-old male mice of each genotype with a 6.3-T horizontal-bore animal scanner (Bruker BioSpin, Ettlingen, Germany) and a 3-cm-diameter birdcage radiofrequency coil (Rapid Biomedical, Rimpar, Germany). End-diastolic volume, end-systolic volume, stroke volume, ejection fraction, and left ventricular mass (1.05 g/cm\textsuperscript{3})\(^{19}\) were calculated from a semiautomatic segmentation of the images with the FARM MRV CAAS software provided by Pie Medical Imaging (Maastricht, the Netherlands).\(^{21}\)

To determine the distension of the descending thoracic aorta during the cardiac cycle, a modified fast low-angle shot sequence was used with an in-plane navigator echo.\(^{21}\) The distension of the thoracic aorta was measured manually at a fixed position for 7 Smtn-B\textsuperscript{−/−} and 5 Smtn-B\textsuperscript{−/−} mice. From these data, the relative distension as function of time and the maximum distension were derived.

**Statistical Analysis**

Statistical significance was calculated by 2-tailed Student t tests or repeated-measures 2-way ANOVA as indicated with Graphpad Prism software (version 4.0) and SPSS software (version 15.0; SPSS Inc, Chicago, Ill). Results were considered significantly different at values of \(P<0.05\). Values are expressed as mean±SEM. The online-only Data Supplement provides a more detailed Methods section.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

*Msmtn-B\textsuperscript{−/−} Mice Develop Normally, Expressing Smoothelin-A but No Intact Smoothelin-B*

To generate *Smttn-B\textsuperscript{−/−} mice, we replaced *Smttn* exons 3 through 7 with a neomycin-resistance cassette (Figure 1A and 1B). This deletion disrupts the smoothelin-B gene while leaving the smoothelin-A promoter intact.\(^{22}\) Intercrosses of *Smttn-B\textsuperscript{−/−} mice produced *Smttn-B\textsuperscript{−/−}:offspring at mendelian frequency (*Smttn-B\textsuperscript{−/−}: 28%; *Smttn-B\textsuperscript{−/−}: 50%; *Smttn-B\textsuperscript{−/−}: 22%; n=506), indicating that loss of smoothelin-B does not interfere with embryonic survival. In contrast to *Smttn-A/B\textsuperscript{−/−} mice, which die rapidly after birth,\(^{17}\) *Smttn-B\textsuperscript{−/−} mice appeared normal, had no gross histological abnormalities in any organ as evaluated by an experienced animal pathologist, and had unaltered intestinal function.\(^{17}\)

Q-PCR analyses showed an absence of intact smoothelin-B transcripts in the aorta, femoral artery, and jejunum of *Smttn-B\textsuperscript{−/−} mice (Figure 1C). On the other hand, upregulation of either smoothelin-A or an interrupted smoothelin-B transcript still containing its 3’ smoothelin-A sequence was found in *Smttn-B\textsuperscript{−/−}: blood vessels (Figure 1C). However, smoothelins were not detectable in the aorta of *Smttn-B\textsuperscript{−/−} mice by immunohistological stainings with polyclonal antibodies recognizing both smoothelin-A and smoothelin-B (Figure 1D). Thus, *Smttn-B\textsuperscript{−/−} mice display a null mutation in vascular tissue and can be used to delineate the role of smoothelin-B in vascular smooth muscle function. As predicted, *Smttn-B\textsuperscript{−/−} mice displayed normal smoothelin expression in visceral tissues (Figure 1C).

**Normal Contractile Gene Expression and Normal Arterial Structure in *Smttn-B\textsuperscript{−/−} Mice***

To study whether levels of important contractile smooth muscle–specific genes were altered by the loss of smoothelin-B, we examined the expression of α-SMA, SM-MHC, and SM-calponin in arteries of 6-month-old mice by...
Q-PCR (n=6 for each genotype). The expression of these components of the SMC contractile machinery was not significantly changed at the mRNA level (Figure 2A). In addition, both α-SMA (Figure 2B) and SM-MHC proteins (S.S.R., unpublished data, 2007) were readily detectable in medial SMCs of Smtn-B−/− mice. Moreover, the femoral artery and aorta of Smtn-B−/− mice appeared histologically normal and had a similar medial cross-sectional area and vessel radius compared with Smtn-B+/+ mice (cross-sectional area of aorta, 0.086±0.004 versus 0.075±0.004 mm²; cross-sectional area of femoral artery, 0.0107±0.0005 versus 0.0101±0.0005 mm²; radius of aorta, 367±4 versus 349±10 μm; radius of femoral artery, 145±5 versus 143±5 μm for Smtn-B+/+ versus Smtn-B−/− mice, respectively). The staining patterns of the extracellular matrix proteins collagen and elastin also were normal in Smtn-B−/− mice, showing regular arrangement of elastic fibers and laminae without fragmentation (Figure 2C and 2D). Hence, loss of smoothelin-B does not affect blood vessel architecture or the expression of other major smooth muscle contractile proteins.

Figure 2. Normal contractile gene expression and arterial structure in Smtn-B−/− mice. A, Expression of α-SMA, SM-MHC, and SM-calponin (SM-Calp) was measured by Q-PCR (n=6 for each genotype). Levels were normalized to cyclophilin A expression, and Smtn-B+/+ levels were set at 1. No significant change in expression levels was found between Smtn-B+/+ and Smtn-B−/− mice. B, Representative immunohistochemical staining of α-SMA in the aorta (top; ×100 magnification) and femoral artery (bottom; ×200 magnification) of Smtn-B+/+ and Smtn-B−/− mice showing no differences between the genotypes. C, Representative images of Sirius Red staining of the aorta (top; ×400 magnification) and femoral artery (bottom; ×200 magnification) of Smtn-B+/+ and Smtn-B−/− mice showing no major differences in collagen content or distribution. D, Representative images of Lawson’s elastin staining of the aorta (top; ×400 magnification) and femoral artery (bottom; ×200 magnification) of Smtn-B+/+ and Smtn-B−/− mice showing regular elastic fibers in both genotypes.

Severely Compromised Arterial Contractile Capacity in Smtn-B−/− Mice

To examine the effect of smoothelin-B deficiency on vascular smooth muscle function, we measured contractility of the femoral artery and the saphenous artery, which contain high amounts of smoothelin-B.7 Several contractile agonists were applied to isolated vessel segments in a myograph to assess the integrity of different signal transduction pathways that activate SMC contraction. Because sensitivities to the contractile stimuli did not differ significantly between genotypes, only differences between maximal responses are discussed. Maximal contractile responses generated by aortas of Smtn-B−/− mice were attenuated during stimulation with K⁺, the thromboxane A₂ mimetic U46619, or the α₁-adrenergic agonist phenylephrine, although the difference with Smtn-B+/+ aortas was not significant (Figure 3A). In contrast, maximal contractions produced by both femoral and saphenous arteries of Smtn-B−/− mice were strongly and significantly decreased compared with

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control mice (Figure 3B and 3C). Contractility of the femoral artery in response to phenylephrine was reduced by 50%. Maximal contractile responses of the saphenous artery to all 3 stimuli were reduced by 60% to 70%.

To investigate whether lack of smoothelin-A on top of smoothelin-B deficiency has an additional effect on vascular smooth muscle performance, we tested the same vasoactive compounds on arterial vessels isolated from Smtn-A/B−/− mice. Of note, Smtn-A/B−/− mice had a decreased medial cross-sectional area of the thoracic aorta and femoral artery that was proportional to their smaller body size.17 The reduced smooth muscle volume was corrected for in the contractility analyses. Thoracic aortas of Smtn-A/B−/− mice displayed a more pronounced reduction of maximal responses to phenylephrine (Figure 3D). Femoral arteries of Smtn-A/B−/− mice showed greatly reduced contractility regardless of the type of agonist used to trigger contraction (Figure 3E). In general, the extent of the reductions were comparable to those observed in Smtn-B−/− vessels. Taken together, these data show that smoothelin-B is an important determinant of muscular artery contractility.

Increased MAP and Cardiac Hypertrophy in Smtn-B−/− Mice

The physiological consequences of the reduced arterial contractility in Smtn-B−/− mice were analyzed by measuring several hemodynamic parameters in conscious mice. Surprisingly, basal MAP was significantly higher in Smtn-B−/− mice than in control mice (≈20 mm Hg; P<0.01), whereas HR was not different (Figure 4A).

In line with the elevated MAP, Smtn-B−/− mice developed a significantly higher ratio of heart weight to body weight at 8
months of age (Figure 4B). Morphometric analysis of the hearts of these mice showed that both the left and right ventricular walls were enlarged (left ventricle cross-sectional area, 13.4±0.1 versus 12.9±0.1 mm²; right ventricle cross-sectional area, 5.4±0.6 versus 4.8±0.4 mm² for Smtn-B⁻/⁻ versus Smtn-B⁺/⁺mice). The number of cardiomyocyte nuclei per 1 mm² was similar (79.8±3.0 versus 79.4±4.1 for Smtn-B⁻/⁻ versus Smtn-B⁺/⁺ littermates), consistent with a hypertrophic response. To establish whether the increased ratio of heart weight to body weight indeed reflected cardiac hypertrophy, we measured the expression of the cardiac hypertrophy markers atrial natriuretic factor and brain natriuretic peptide in the hearts of 8-month-old mice (n=11 for each genotype). mRNA levels of both natriuretic peptides were elevated in Smtn-B⁻/⁻ mice compared with Smtn-B⁺/⁺ littermates (Figure 4C).

Increased MAP and higher ratios of heart weight to body weight also were detected in Smtn-A/B⁻/⁻ mice at the young age of only 6 weeks (MAP: 95±5 versus 87±2 mm Hg, P<0.01; ratio of heart weight to body weight: 6.8±0.9 versus 5.8±0.4 mg/g, P<0.01 for Smtn-A/B⁻/⁻ versus Smtn-A/B⁺/⁺ littermates). Collectively, these results show that smoothelin deficiency results in elevated blood pressure, leading to cardiac hypertrophy.

**Normal Blood Pressure Control in Smtn-B⁻/⁻ Mice**
To determine whether the increased MAP in Smtn-B⁻/⁻ mice was due to alterations in endogenous blood pressure control mechanisms, we determined MAP and HR after injection of several autonomic nervous system blockers in conscious mice. Smtn-B⁻/⁻ and Smtn-B⁺/⁺ mice displayed similar changes in MAP and HR after administration of atropine or atropine plus metoprolol (Figure 5A and 5B), indicating that cardiac parasympathetic tone and sympathetic tone were comparable. In addition, the MAP response to the α₁-adrenergic blockers prazosin and yohimbine did not differ between Smtn-B⁻/⁻ and Smtn-B⁺/⁺ mice (Figure 5C). Thus, no difference exists in autonomic control that may explain the different MAP between the genotypes. In line with the myograph data, MAP responses to intravenous bolus injections of the α₁-adrenergic agonist phenylephrine were lower in Smtn-B⁻/⁻ mice than in Smtn-B⁺/⁺ mice, although the differences were not significantly different at any dose (Figure 5D and 5E).

**Normal Cardiac Function but Increased Peripheral Vascular Resistance in Smtn-B⁻/⁻ Mice**
The distensibility of the large arteries affects central arterial pressure. Therefore, we first investigated whether decreased distensibility in Smtn-B⁻/⁻ animals contributes to the elevated
MAP. We measured pulse-wave velocity and pulse pressure in the thoracic aorta. At comparable HRs (573±36 versus 562±38 bpm for Smtn-B−/− and Smtn-B+/+ animals; P=0.80) and comparable MAPs (89±9 versus 86±1 mm Hg for Smtn-B−/− and Smtn-B+/+ animals; P=0.45), no significant difference was found in either pulse-wave velocity or pulse pressure (Figure 6A), demonstrating unchanged aortic distensibility in Smtn-B−/− mice.

MAP is by definition the product of cardiac output and total peripheral vascular resistance. Consequently, the elevated MAP in Smtn-B−/− mice might arise from increases in either or both of these factors. We first analyzed cardiac output by magnetic resonance imaging. Stroke volume, HR, and cardiac output did not differ between Smtn-B−/− and Smtn-B+/+ littermates (Table). Because cardiac output was not increased, the elevated MAP in Smtn-B−/− mice had to be due to increased peripheral vascular resistance. Analysis of the distension of the aorta during the cardiac cycle by magnetic resonance imaging (Figure 6B) revealed increased distension in Smtn-B−/− mice throughout the cardiac cycle (Figure 6C). The maximal distension was almost 2-fold greater for Smtn-B−/− mice (Smtn-B−/−, n=5; Smtn-B+/+, n=7). *Statistically significant differences.

Table. Characteristics of Cardiac Function of Smtn-B−/− (n=11) and Smtn-B+/+ (n=11) Mice as Measured by Magnetic Resonance Imaging

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Smtn-B−/−</th>
<th>Smtn-B+/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>End-diastolic volume, µL</td>
<td>44±2.6</td>
<td>47±2.1</td>
</tr>
<tr>
<td>End-systolic volume, µL</td>
<td>10±0.8</td>
<td>13±1.2</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>76±1.1</td>
<td>73±1.2*</td>
</tr>
<tr>
<td>Left ventricular mass, mg</td>
<td>114±2.7</td>
<td>101±4.5*</td>
</tr>
<tr>
<td>Stroke volume, µL</td>
<td>34±2.0</td>
<td>34±1.1</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>521±11</td>
<td>527±6</td>
</tr>
<tr>
<td>Cardiac output, mL/min</td>
<td>17.7±1.0</td>
<td>18.1±0.7</td>
</tr>
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</table>

*P=0.02.

Discussion

The present study was instigated by our recent demonstration that mice lacking both smoothelin isoforms display markedly reduced intestinal smooth muscle contraction.23 In line with this, we show here that loss of the vascular-specific smoothelin-B protein leads to greatly diminished vascular contractile capacity. Paradoxically, this is accompanied by elevated MAP and cardiac hypertrophy. Because cardiac output, autonomic nervous system activity, and large-artery properties are not altered in smoothelin-B−/− deficient mice, the increased pressure must have its origin in altered microvascular properties. The data obtained in this study provide the first evidence that smoothelin-B is essential for vascular smooth muscle performance.

Reduced contractility of Smtn-B−/− muscular arteries was observed regardless of the signal transduction pathways activated. Together with the binding of smoothelin-B to the contractile filaments,23 this nonselective reduction of contractility suggests that smoothelin-B plays a role at the core of the
vascular SMC contractile machinery. The mechanism by which smoothelin-B affects smooth muscle contraction needs more study. The cardiovascular phenotype of other knockout mouse models of contractile SMC proteins has been shown to be due to downregulation of other proteins involved in SMC contraction, upregulation of related proteins, or expression of different alternatively spliced contractile proteins.\(^2,3,24,25\) We have excluded the possibility that downregulation of the most important SMC contractile genes is responsible for the phenotype of Smtn-B\(^{-/-}\) mice. In addition, smoothelin homologs are not detected by database searches, making it unlikely that upregulation of such proteins can compensate for the loss of smoothelin function. It cannot be completely ruled out, however, that changes in alternative splicing or organization of other contractile proteins occur in Smtn-B\(^{-/-}\) animals.

An important determinant of the amount of contractile force in muscle contraction is the degree of cooperativity between multiple actomyosin cross-bridges. Cooperativity in skeletal muscle is coordinated by tropomyosin and the troponin proteins.\(^26\) However, troponins are not expressed in SMCs. Instead, smooth muscle tropomyosin interacts with h-caldesmon and SM-calponin, which partly take over the role of troponins.\(^5\) Importantly, smoothelins contain a 37–amino acid sequence that is similar to the tail domain of troponin T.\(^10\) In skeletal muscle, this domain not only is required for troponin T interaction with tropomyosin but also is involved in the activation of actomyosin ATPase.\(^27\) Thus, smoothelins might be part of a smooth muscle tropomyosin–troponin–like system. The diminished contractile potential of vascular smooth muscle of Smtn-B\(^{-/-}\) mice may be due to a lack of cooperativity of contraction, which then would depend on a functional smoothelin–tropomyosin system.

Surprisingly, the reduced contractile capacity of smoothelin-B–deficient muscular arteries ex vivo was accompanied by elevated blood pressure in Smtn-B\(^{-/-}\) mice. However, reduced maximal vascular contractility does not necessarily manifest itself in the MAP. Elevation of mean blood pressure may occur as a result of increased cardiac output, increased total peripheral resistance, or their combination. The magnetic resonance imaging measurements in this study show that cardiac output in the Smtn-B\(^{-/-}\) mice is not changed. Therefore, the peripheral resistance of the vasculature must be affected by the mutation, which is conceivable considering the significant expression of smoothelin-B in the smaller vessels. The increased aortic distension despite similar cardiac output and similar aortic structure, diameter, and distensibility in Smtn-B\(^{-/-}\) mice supports that increased resistance to blood flow is brought about by the smaller downstream parts of the vascular tree.

It is unlikely that the increased MAP is caused by overactivity of the autonomic nervous system because neither blockade of muscarinic receptors nor blockade of \(\beta_1\)- or \(\alpha_1\)-adrenergic receptors revealed differences in blood pressure response and because HR did not differ between the genotypes either. However, changes in other neurohumoral vasoressor systems that control blood pressure, changes in arterial relaxation properties, or differences in the total number of vessels might contribute to the altered MAP in Smtn-B\(^{-/-}\) mice. In addition, we cannot rule out that smoothelin deficiency might have a stimulatory effect on arteriole contractility.

Overall, the cardiovascular phenotype of Smtn-B\(^{-/-}\) mice is similar to that of patients with established hypertension. They, too, have a normal cardiac output with a hypertrophic heart, accompanied by increased peripheral resistance.\(^28\) Two other observations in this study deserve further comment. First, we found no indications of SMC phenotype changes such as decreased contractile gene expression or altered cell morphology in Smtn-B\(^{-/-}\) mice. Therefore, it is unlikely that smoothelin-B plays a role in the regulation of SMC differentiation, as was previously suggested on the basis of its strict contractile phenotype–specific expression.\(^23\) Second, arterial contractility was similarly reduced in mice lacking both smoothelins and mice lacking only smoothelin-B, indicating that smoothelin-B is the functional smoothelin isoform in vascular smooth muscle.

**Conclusions**

The data in this study show that smoothelin-B deficiency causes a major decline in the contractile performance of vascular smooth muscle. Instead of merely reflecting the SMC contractile phenotype, smoothelins appear to actively participate in the contractile process itself. Mutations in the Smtn gene or alterations in smoothelin levels may therefore contribute to the development of hypertension and concomitant cardiac hypertrophy in humans.

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**Disclosures**

None.

**References**


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**CLINICAL PERSPECTIVE**

The causes of essential hypertension remain largely unknown, although it is commonly accepted that vascular smooth muscle dysfunction is a potential culprit. Improved insight into the mechanics and regulation of smooth muscle contraction may provide additional therapeutic targets to treat such hypertension. However, our current understanding of these 2 aspects of smooth muscle function is limited. Here, we introduce smoothelin, a protein specifically expressed in fully differentiated, contractile smooth muscle cells, as a crucial component of the vascular smooth muscle cell contractile apparatus. We demonstrate for the first time that smoothelin is necessary for physiological vascular smooth muscle contraction. Smoothelin deficiency in mice resulted in severely reduced contractile potential, particularly in smaller arteries. Paradoxically, this was accompanied by hypertrophy and concomitant cardiac hypertrophy. Analyses of differently sized blood vessels indicated that the cause of the hypertension is likely to be downstream of vessels like the saphenous artery and/or mediated by overcompensation of blood pressure regulatory systems like the renin-angiotensin system. Recently, inimab, a drug used in clinical practice, was shown to specifically promote smoothelin expression in vascular smooth muscle cells. Considering the currently reported data, such an increase in smoothelin concentration not only may indicate a more contractile phenotype of the vascular smooth muscle cell but also may improve vascular smooth muscle contractile potential. The combination of an increased knowledge of smoothelin function and the availability of pharmacological tools that affect smoothelin expression provides interesting opportunities to treat pathologies originating from vascular smooth muscle cell dysfunction.
Smoothelin-B Deficiency Results in Reduced Arterial Contractility, Hypertension, and Cardiac Hypertrophy in Mice


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Supplemental material

Materials and methods

Generation of Smtn-B<sup>−/−</sup> mice
The targeting vector contained a thymidine kinase gene for negative selection. After electroporation of the PvuII-linearized constructs into mouse L129/Sv embryonic stem (ES) cells, we selected neomycin-resistant clones with G418 (Invitrogen, Carlsbad, California) and 1-[2-Deoxy-2-fluoro-β-D-arabinofuranosyl (Invitrogen). DNA from resistant clones was screened by Southern blotting after Sacl restriction digestion using a 5' probe as indicated in Fig. 1a. ES cells from targeted clones were injected into C57Bl/6 blastocysts and implanted into pseudopregnant C57Bl/6 females. Mating of the resulting chimeric males to C57Bl/6 females led to germline transmission. Mice were genotyped by multiplex PCR analysis using the primers indicated in the supplemental table.

Pulse wave velocity and pulse pressure measurements
Under mild isoflurane anesthesia (1.5-2 vol%), the left carotid artery was isolated and a high fidelity micro-manometer catheter (Mikro-tip 1.4 F SPR-671, Millar Instruments, Houston, TX) was inserted into the thoracic aorta. Recordings were made at 2 locations. The first recording was made with the catheter tip placed just above the diaphragm. The second recording was made when the tip was retracted rostrally over a distance of 8-10 mm. The ECG signal was simultaneously digitized with the pressure signal at 5 kHz. The second derivative of the pressure signal was used to identify the exact location of the “foot” of the pressure wave, i.e the location of the initial rise of pressure wave. Using the R-peak of the ECG signal as a reference point, the time difference between the occurrence of the “foot” of the pressure wave at the two locations was used to calculate the pulse wave velocity (supplemental figure). PP was determined by subtracting the systolic pressure from the end diastolic pressure.
MRI experiments

The mice were positioned supine in the MRI scanner with their front paws fixed on copper electrodes used for ECG triggering. During the experiments the mice were sedated using 1.5 vol% isoflurane. Prospective CINE MRI scans were collected using a FLASH sequence with the following parameters: repetition time 7 ms; echo time 2.3 ms; field of view 3×3 cm²; matrix 192×192; slice thickness 1 mm; total acquisition time per slice 5 min. The mice were scanned by acquiring short axis CINE images, covering the heart from apex to base.

To determine the lumen motion of the descending thoracic aorta during the cardiac cycle, a modified FLASH sequence was used with an in-plane navigator echo with the following parameters: flip angle 30°; repetition time 5.4 ms; echo time 2.98 ms; field of view 2.5×3 cm²; matrix 256×192; slice thickness 2 mm; number of repetitions 700; total acquisition time approximately 12 min. The whole thoracic aorta was placed within the slice, with the descending aorta perpendicular to the MRI readout direction. Cardiac triggering and respiratory gating were performed by analyzing the navigator echo data retrospectively and dividing the cardiac cycle into twenty time frames. Before analyzing the motion, all images were zero-filled to a matrix of 512×512 for enhancing the aorta lumen.

Morphometric analyses

To determine the radius and medial cross sectional area (CSA) of arteries, cross sections were stained with Lawson’s solution. The CSA was defined as the area between the internal elastic lamina and the external elastic lamina. To assess the volume of different parts of the heart, surface areas of two different cross-sections of the heart were measured. Total CSA, left and right ventricular wall CSA, and left and right lumen area were measured. To determine the number of cardiomyocytes, three pictures were taken of each heart at different locations, and cell nuclei were counted manually. Images were captured using a Zeiss Axioscope (Zeiss, Göttingen, Germany) and a standard CCD camera (Stemmer Imaging, Puchheim, Germany), and analyzed with Leica QWin image analysis software (Leica Microsystems, Cambridge, UK).
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<tr>
<td>Smoothelin (genotyping)</td>
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<td>CTCGTGCTTTACGGTATCGC (B⁻⁻⁻⁻)</td>
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<td>BNP</td>
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Supplemental figure

- ECG
- 2nd der. pressure (a.u.)
- Pressure (mmHg)
- Time (s)
- Signal
- R-peak
- Foot
- Reflection point
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