Selective Involvement of Serum Response Factor in Pressure-Induced Myogenic Tone in Resistance Arteries

Kevin Retailleau, Bertrand Toutain, Guillaume Galmiche, Céline Fassot, Reza Sharif-Naeini, Gilles Kauffenstein, Mathias Mericskay, Fabrice Duprat, Linda Grimaud, Jean Merot, Aurelie Lardeaux, Anne Pizard, Véronique Baudrie, Xavier Jeunemaître, Robert Feil, Joachim R. Göthert, Patrick Lacolley, Daniel Henrion, Zhenlin Li, Laurent Loufrani

Objective—In resistance arteries, diameter adjustment in response to pressure changes depends on the vascular cytoskeleton integrity. Serum response factor (SRF) is a dispensable transcription factor for cellular growth, but its role remains unknown in resistance arteries. We hypothesized that SRF is required for appropriate microvascular contraction.

Methods and Results—We used mice in which SRF was specifically deleted in smooth muscle or endothelial cells, and their control. Myogenic tone and pharmacological contraction was determined in resistance arteries. mRNA and protein expression were assessed by quantitative real-time PCR (qRT-PCR) and Western blot. Actin polymerization was determined by confocal microscopy. Stress-activated channel activity was measured by patch clamp. Myogenic tone developing in response to pressure was dramatically decreased by SRF deletion (5.9±2.3%) compared with control (16.3±3.2%). This defect was accompanied by decreases in actin polymerization, filamin A, myosin light chain kinase and myosin light chain expression level, and stress-activated channel activity and sensitivity in response to pressure. Contraction induced by phenylephrine or U46619 were not modified, despite a higher sensitivity to p38 blockade; this highlights a compensatory pathway, allowing normal receptor-dependent contraction.

Conclusion—This study shows for the first time that SRF has a major part to play in the control of local blood flow via its central role in pressure-induced myogenic tone in resistance arteries. (Arterioscler Thromb Vasc Biol. 2013;33:339-346.)

Key Words: myogenic tone ■ resistance arteries ■ serum response factor.

Resistance arteries play a key role in the control of peripheral vascular resistance, tissue perfusion, and hence in cardiovascular homeostasis. They possess a basal (myogenic) tone, which is opposed by flow (shear stress)-mediated dilation. Equilibrium between myogenic tone (MT) and flow-mediated dilation allows efficient control of local blood flow in peripheral tissues and provides a background vascular tone, which increases the efficacy of circulating and locally produced vasoactive agents.

MT is characterized by smooth muscle cell (SMC) contraction in response to increases in pressure or stretch. A rise in MT has been associated with diseases, such as hypertension and diabetes mellitus. Signaling mechanisms contributing to MT require opening of mechanosensitive channels, membrane depolarization, activation of voltage-operated calcium channels, calcium entry, protein kinase C, and phospholipase C activation, as well as calcium-sensitization of the contractile apparatus. The cytoskeleton plays an important role in MT. The decrease, inhibition, or absence of proteins, such as integrins, reduce MT.

Serum response factor (SRF) is a member of the MADS box transcription factor family, which binds directly to the cis-element, CArG-box. It is involved in the control of several extracellular stimuli-regulated genes which are important for cell growth and survival, as well as cytoskeletal genes, including those coding for actins, myosins, calponins, troponin, dystrophin, desmin, and metabolic enzymes, such as muscle creatine kinase. Its role in the resistance arteries is unknown.

Two main families of signal-regulated SRF cofactors have been characterized. First, members of the ternary complex factor family of E-twenty-six domain proteins, switch-activating protein-1 and Net, are activated by mitogen-activated protein kinase phosphorylation, and interact with SRF to...
stimulate the transcription of immediate early genes, such as c-fos or Egr1. The myocardin-related transcription factors, myocardin-related transcription factor-A (also called MAL or MKL1 or BSAC), and myocardin-related transcription factor-B (also called MKL2 or MAL16), form the second family. Activity and nuclear translocation of MAL are controlled by the RhoA-family of GTPases and by actin filament dynamics. On actin polymerization, MAL is released from nuclear G-actin and interacts with SRF to stimulate its transcriptional activity on specific promoters, including actin promoters. In humans, reduced SRF expression has been associated with heart failure. In addition, its increased expression has been associated with a hypercontractile phenotype in the cerebral arteries of Alzheimer patients. However, its role in microvascular mechanotransduction is as yet unknown.

In mouse Cre–loxP models, cardiomyocyte-specific SRF disruption in the adult induces a dilated cardiomyopathy correlated with decreased expression of the proteins involved in force generation and transmission in cardiomyocytes.

The role of SRF in the maintenance of vascular SMC differentiation has been extensively studied, especially in the context of atherosclerosis. However, a direct assessment of the role of SRF through targeted disruption in the adult vasculature has not been carried out so far. We have previously shown that an inducible SMC-specific SRF mutation in adult mice leads to a severe motility disorder resembling chronic intestinal pseudo-obstruction in humans. This demonstrates that SRF plays a crucial role in maintaining visceral SMC contractility. The involvement of SRF in cytoskeletal protein gene regulation led us to hypothesize that SRF might modulate resistance arteries mechanotransduction, especially in response to pressure (which is the strongest mechanical force acting on the vessel wall). We therefore investigated pressure-induced MT in adult smooth muscle-specific SRF knockout mice (SRF<sup>omk</sup>).

**Materials and Methods**

**Animals**

Experiments were carried out in accordance with the guidelines of the Institutional Ethical Committee for Experimental Animals and conform to Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The study was approved by the local University Review Committee for ethical and safety issues. The procedure followed in the care and euthanasia of the study animals was in accordance with the European Community standards on the care and use of laboratory animals (authorization #00577).

We crossed floxed SRF mice with mice expressing tamoxifen-inducible Cre recombinase under the control of a smooth muscle-specific gene. Adult (3–4 months old) male SMC-inducible (SRF<sup>omk</sup>, n=10) and control wild-type mice (WT; n=10) were treated with intraperitoneal injection of tamoxifen (50 mg/kg per day) for 3 days. After 14 days, the mice were anesthetized by isoflurane inhalation (2.5% of isoflurane) and then euthanized with a minimum of 60% of CO<sub>2</sub>. The tail artery was then removed and placed in ice-cold physiologi cal salt solution (PSS) of the following composition (mmol/L): 135.0, NaCl; 15.0, NaHCO<sub>3</sub>; 4.6, KCl; 1.5, CaCl<sub>2</sub>; 1.2, MgSO<sub>4</sub>; 11.0, glucose; and 10.0, N-2-hydroxy-ethylpiperazone-N-2-ethylsulfonic acid. The pH was 7.4.

Several arterial segments from each mouse were used for functional and biochemical studies, as described below.

In a separate series of experiments, mice were also anesthetized (2.5% of isoflurane) and then euthanized with a minimum of 60% of CO<sub>2</sub>. The tail artery was then removed and placed in ice-cold PSS. We used dissociated SMCs isolated from tail arteries from smooth muscle-inducible SRF knockout mice for electrophysiological studies.

**Pressure Myography**

A segment of tail artery was cannulated at both ends and mounted in a video-monitored perfusion system, as previously described. Briefly, cannulated arterial segments were bathed in a 5-mL organ bath containing a PSS maintained at pH 7.4, with P<sub>0</sub> and P<sub>O</sub> of 160 mm Hg and 37 mm Hg, respectively. Arterial diameter was measured and recorded continuously using a video-monitoring system (Living System Instrumentation Inc, Burlington, VT). Pressure and flow rate could be changed independently. To measure MT, stable diameter changes were measured in each segment when the intraluminal pressure was set at 10, 25, 50, 75, 100, and 125 mm Hg. At the end of each experiment, arteries were perfused and superfused with a Ca<sup>2+</sup>-free PSS containing ethylenebis-(oxyethylenenitrilo) tetracacetate (EDTA, 2 mmol/L), sodium nitroprusside (10 µmol/L), and papaverine (10 µmol/L). Pressure steps (10–125 mm Hg) were then repeated to determine passive arterial diameter, that is, in the absence of smooth muscle tone. MT was calculated as percentage of passive diameter. Dilution to acetylcholine (Ach) was tested by adding cumulative concentrations of Ach (10<sup>−6</sup>–10<sup>−4</sup> mol/L). Before each experiment, the contractility of the muscle was tested using phenylephrine (PE, 1 µmol/L), and the integrity of the endothelium was assessed by testing the relaxing effect of Ach (1 µmol/L).

**Wire Myography**

Two-millimeter-long segments of tail arteries were mounted on a wire myograph (DMT, Aarhus, DK), as previously described. Two tungsten wires (40 µm in diameter) were inserted into the artery lumen and fixed to a force transducer and a micrometer, respectively. Arteries were bathed in PSS. Before each experiment, the arteries were tested, as described above. PE (3 µM)- or U46619 (1 µM)-dependent contraction was tested before and after addition of the p38 mitogen-activated protein-kinase inhibitor SB203580 (10<sup>−4</sup> mol/L) to the bath.

In a separate series of experiments, arteries were washed out with Ca<sup>2+</sup>-free PSS containing EDTA, and then bathed in a Ca<sup>2+</sup>-free PSS containing KCl (80 mmol/L, 80K PSS). Contraction to calcium was tested by adding cumulative concentrations of calcium (0.1–1.2 mmol/L). Data were expressed as a percentage of the maximum response obtained with KCl (80 mmol/L).

**Electrophysiological Analysis**

**Dissociation of Vascular SMCs**

Mice were anesthetized using isoflurane. A segment of tail artery (300 µm internal diameter, 3–5 mm length) was dissected and incubated (20 minutes at 37°C) with papain (1 mg/mL) and dithiothreitol (1 mg/mL), followed by a second incubation (3 minutes at 37°C) in collagenase F (0.7 mg/mL) and collagenase H (0.3 mg/mL). Papain, dithiothreitol, and collagenase were dissolved in a SMCs dissociation solution (smooth muscle cells dissociation) of the following composition (in mmol/L): NaCl 140, KCl 5.6, MgCl<sub>2</sub> 2, HEPES 10, glucose 10, CaCl<sub>2</sub> 0.1, and BSA (1 mg/mL). Arteries were washed 3 times in cold smooth muscle cells dissociation solution and triturated. Cell suspensions were placed on 35-mm poly-o-lysine-coated dishes and kept at 4°C for at least 45 minutes. Recordings were only performed on elongated cells.

**Patch Clamp**

The electrophysiological procedure has been previously described. Briefly, single-channel cell-attached or inside-out recordings were...
performed on acutely dissociated vascular SMCs at a holding potential (Vhold) of ~80 mV. Unless stated otherwise, the pipette solution contained the following (in mmol/L): NaCl 140; KCl 5; CaCl2 1; MgCl2 1; TEA-Cl 10; 4-AP 5; and HEPES 10; the pH was 7.35. The bath solution contained (in mmol/L): KCl 140; MgCl2 1; HEPES 10; and glucose 10; the pH was 7.25. The osmolality of all solutions was adjusted to 320 mOsmol/kg. Membrane patches were stimulated with brief negative pressure pulses through the recording electrode using a pressure-clamp device (ALA High Speed Pressure Clamp-1 system, ALA-scientific).

**Histological Analysis**

**Actin Polymerization Ratio on Pressure Myograph**

Segments of tail resistance arteries were fixed in formaldehyde 4% for 30 minutes at 37°C and at 75 mm Hg, 10 mm Hg, or in the presence of PE (10^(-6) M), and were mounted in embedding medium (Tissue-Tek, Miles, Inc); they were then frozen in isopentane precooled in liquid nitrogen, and stored at -80°C. Transverse cross sections (7 μm thick) were incubated with a globular (G) actin marker, DNase I conjugated with Alexa Fluor 488, and a filamentous (F) actin marker, Phalloidin, conjugated with Alexa Fluor 546 dye (Invitrogen). Fluorescence was visualized using confocal microscopy (Nikon, Eclipse TE2000S and Solamere Technology, Salt Lake City, UT). Image analysis was performed, as previously described, using Histolab (Microvision, France).28

**Isolation and Culture of SRF-flex2neo (Sf/Sf) SMCs**

Primary cultures of SMCs were isolated from the aorta of adult Sf/Sf mice. Briefly, under sterile conditions, the media muscle layer of each aorta was scraped from the endothelial intima layer, minced into small pieces, and pooled and digested with bacterial collagenase (1 mg/mL, Roche) and elastase type III (0.5 mg/mL; Sigma). After inactivation of the enzyme with fetal bovine serum, cells were allowed to attach to a plastic tissue culture flask for 6 days. Cells were maintained in Dulbecco modified Eagle’s medium (DMEM; Lonza, Belgium) supplemented with 20% fetal bovine serum (Gibco) and antibiotics 100 U/mL Pen/Strep (Cambrex) in a humidified atmosphere containing 5% CO2 in air at 37°C. Cells between their fifth and sixth population-doubling level were used for all experiments.

**SMC Differentiation and Adenovirus Infection**

To restore the differentiated properties of SMCs, the medium was replaced with a serum-free medium containing a 1:1 (vol/vol) mixture of HAM-F12/DMEM (Lonza, Belgium) complemented with 0.2 mmol/L sodium ascorbate, 1X insulin-transferrin-selenium cocktail (Gibco), and antibiotics for 48 hours. For adenovirus infection, mouse SMCs containing LoxP elements flanking the second exon of SRF were treated with either adenovirus containing Cre-recombinase associated with a green fluorescent protein (Ad-CRE-GFP) to mediate excision of SRF-floxed exon 2, or adenovirus containing green fluorescent protein alone (Ad-GFP) as control. The Ad-CRE-GFP and Ad-GFP virus mediates transgene expression through a human cytomegalovirus promoter. The SMC infection was performed at 37°C with gentle rocking for 5 hours in serum-free medium. Cells were kept in this medium for 7 days awaiting further analyses.

**Immunostaining of SMCs**

The SMCs were fixed in 4% formaldehyde solution for 5 minutes at 37°C. After being washed 3 times with physiological buffer solution, cells were permeabilized in 0.2% Triton X-100 for 5 minutes at room temperature. Non-specific sites were blocked with 2% BSA in physiological buffer solution. SMA and F-actin staining were performed by incubating the cells with polyclonal filamin A antibodies (1:100, Santa Cruz, H300, Ref: SC13029). Secondary antibodies were then added (1 hour 30 minutes, 25°C, DyLight 649 Jackson laboratory 1:300). Slides were mounted in MOWIOL. Immunofluorescence images were collected with an SP5 confocal laser-scanning microscope (DM6000; Leica, Inc). Images were processed using Image J.

**qPCR Analysis**

After dissection, tail arterial segments were kept in 100 μL RNA-later (Sigma) at ~20°C, until RNA extraction using the RNeasy micro kit (Qiagen). Two hundred nanograms of total RNA extracted from each artery were subjected to reverse transcription with the QuantiTect Reverse Transcription kit (Qiagen). Real-time PCR assays were carried out on an ABI 7300 Real-Time PCR System (Applied Biosystems) with Sybr Green Jumpstart Taq Readymix kit (Sigma) and gene-specific primers. All data were normalized to the hypoxanthine guanine phosphoribosyl transferase mRNA. Differences in transcript level were determined using the cycle threshold method, as described by the manufacturer. Messenger RNA levels are expressed as the log (base 2) of the ratio of the SRF<sup>flx2neo</sup> mutant to the control mice.

**Western Blot Analysis of Protein Expression**

Western blot analysis was performed on tail artery segments. The segments were homogenized using a lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L sodium orthovandate, and protease inhibitor cocktail). Extracts were incubated at 4°C for 30 minutes and then centrifuged (22,000 g, 15 minutes, 14°C). Protein concentrations were determined using the Micro BCA Protein Assay Kit (Pierce). After denaturation at 95°C for 5 minutes, equal amounts of proteins (25 μg) were loaded on a 9% polyacrylamide gel and transferred to nitrocellulose membranes for 90 minutes (100 V, 4°C). Membranes were blocked with 5% BSA in tris-buffered saline and tween 20 (20 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, and 0.1% Tween-20) for 60 minutes and were then incubated with primary antibody in 5% BSA in tris-buffered saline.
and tween 20 overnight at 4°C. After extensive washing in tris-buffered saline and tween 20 at room temperature, membranes were incubated with either the antirabbit or the antimouse horseradish peroxidase antibody (dilution 1:10000, Pierce) for 90 minutes at room temperature. After a further 3 washes with tris-buffered saline and tween 20, immunocomplexes were detected by chemiluminescent reaction (SuperSignal West Femto, Pierce) using a computer-based imaging system (Fuji LAS 3000 plus; Fuji Medical System). Quantification was performed by densitometric analysis.29

RhoA-GTP Measurement
RhoA-GTP was measured in tail artery segments using a commercially available kit (G-LISA RhoA Activation Assay Biochem Kit; BK124, cytoskeleton).

Statistical Analysis
Results are expressed as means±SEM. Significance of the differences between groups was determined by analysis of variance (1-factor ANOVA, or ANOVA for consecutive measurements, when appropriate) or unpaired Student's t test. Probability values <0.05 were considered to be significant.

Results
Vascular Reactivity
We first verified, after 14 days of posttamoxifen injection, that SRF was decreased (cf RNA and protein analysis of the resistance artery section). Blood pressure, measured through a catheter (WT=108±6 mmHg; SRFsmko=101±6 mmHg; n=10) or by telemetry (Figure I in the online-only Data Supplement), and body weight, heart rate, heart weight, or weight of mice (Figure I in the online-only Data Supplement) were not affected by the decrease of SRF in SRFsmko mice at 14 days posttamoxifen injection. The absence of SRF did not affect passive arterial diameter (Figure 1A). Increasing pressure by step (10–125 mm Hg) in WT and SRFsmko arteries induced stable contraction in time and amplitude (MT) in resistance arteries. The absence of SRF strongly reduced MT in isolated tail arteries (Figure 1A). However, PE- and U46619-induced contractions, as well as dilation induced by Ach, were unaffected by the absence of SRF (Figure 1B and Figure II in the online-only Data Supplement).

Mechanosensitive Channels
We then examined stretch-activated channel (SAC) activity in vascular SMCs freshly dissociated from tail arteries of WT and SRFsmko mice. In cell-attached patches, brief negative pulses applied through the recording electrode produced reversible opening of SACs (Figure 2A, left panel). The amplitude of averaged SAC currents was significantly lower in SRFsmko myocytes recorded in cell-attached mode (Figures 2A and 2D; P=0.0002 at –60 mm Hg) or in inside-out mode (Figure 2E; P=0.027 at –60 mm Hg). Channel conductance was identical in both genotypes (Figure 2B), and the percentage of patches with at least one active channel was also the same (Figure 2C). We quantified the mRNA expression level of 2 putative mechanosensitive channels, transient receptor potential cathepsin (TRPC) and TRPC6, but found no differences between SRFsmko and WT mice (data not shown). We observed an increase in SAC activity recorded from WT mice, when going from the cell-attached configuration (black squares, Figure 2A, left panel) to the inside-out configuration (red circles, Figure 2A, right panel).

Figure 2. A, Representative single-channel recordings of stress-activated channel (SAC) activity in the cell-attached configuration in vascular smooth muscle cells (SMCs) dissociated from a wild-type (WT) artery (left) and smooth muscle–specific SRF knockout mice (SRFsmko) mice. Downward deflections represent current response because of the opening of single SACs, caused by the application of a brief (0.3 second) negative pressure pulse (indicated on the left of the traces) through the recording electrode. Average pressure (0, –30, and –60 mm Hg)-induced SACs response in vascular SMCs from WT (n=104 cells, middle), and SRFsmko (n=130 cells, right) mice. B, Conductance of active channels at –80 mV. C, Percentage of patches with at least one active channel. Statistical significance was tested using a Student's t test, probability values are as indicated. Mean±SEM is shown (N=10 mice per group). D and E, SAC current as a function of pressure in myocytes recorded in (D) cell-attached configuration (WT, n=120 cells; SRFsmko, n=149 cells) and in (E) inside-out configuration (WT, n=60 cells; SRFsmko, n=53 cells; cells were from 10 mice per group). **P<0.05, SRFsmko SMCs compared with WT SMCs.
Actin Polymerization

As actin dynamics have been implicated in the development of MT and SAC activation,29,30 we examined the F/G actin ratio in vascular SMCs. Increasing pressure from 10 mm Hg to 75 mm Hg induced MT, and an increased F/G actin ratio in arteries isolated from control mice. This change was not observed in SRFsmko mice arteries. This change was not observed in SRFsmko mice arteries. This change was not observed in SRFsmko mice arteries.

Immunofluorescence staining of SRF and filamin A (blue), and F-actin staining (red) by phalloidin on isolated vascular smooth muscle cells from wild-type (WT) and SRFsmko mice. SRF, actin polymerization, and filamin A staining are decreased in SRFsmko smooth muscle cells (SMCs). *P<0.05, WT vs SRFsmko (n=8 mice per group), Bars, 20 µm.

MAPK p38 Activity

The expression level of p38 was similar in SRFsmko and WT mice, whereas the activity reflected by the ratio of phosphop38/p38 was significantly increased in the absence of SRF (Figure 6A). The effect of the p38 inhibitor SB203580 on PE- and U46619-induced contraction was higher in arteries from SRFsmko mice than control mice (Figure 6B).

Calcium and Specific Endothelial SRF Knockout Mice Analysis

The contractile apparatus’s sensitivity to calcium, illustrated by the vasoconstriction induced by exogenous calcium in 80 mmol/L KCl-rich PSS, was similar in SRFsmko and WT mice (Figure III in the online-only Data Supplement). Furthermore, on measuring the baseline calcium concentrations in freshly dissociated SMCs, there was no difference between SRFsmko and WT mice, with calcium concentrations (in nmol/L) of 83.60±7.65 (n=56 cells) and 79.94±11.29 (n=21 cells), respectively (P=0.8, permutation test; see Figure III in the online-only Data Supplement).
In endothelial-specific SRF knockout mice (SRF endo), neither concentration-dependant contraction to PE and U46619 or dilatation by Ach, nor pressure-induced MT were different compared with WT mice (Figure IV and V in the online-only Data Supplement).

**Discussion**

In this study, we have shown that SRF has a selective involvement in pressure-dependent mechanotransduction in small resistance arteries. The absence of SRF in SMCs impaired both pressure-induced (myogenic) tone and mechanosensitive channel activity in the tail artery. The deletion did not, however, alter U46619 and PE-induced contraction, thus demonstrating the selectivity of the effect.

As SACs have an important role in MT, we examined their characteristics in arteries isolated from SRF smko mice. We found that in these mice, both the sensitivity (first opening channel) and the activity (mean ionic current) of SACs were decreased. The conductance was identical in both conditions and similar to previously published values, confirming that we were recorded from the same channels. Interestingly, the percentage of patches in which at least one SAC was observed (termed active patches) was the same in the 2 genotypes, indicating that the distribution of SACs did not change (ie, there was no clustering). The molecular identity of SACs is still unclear, and TRPC channels have been proposed in various studies. We found no differences in the mRNA expression level of TRPC1 and TRPC6 between SRF smko and WT mice. These results suggest that the reduced SAC activity (NPo) in SRFsmko myocytes is a result of either a lower number (N) of SACs in active patches, or to a reduced probability of opening (Po) of SACs in these active patches.

There is, under control conditions, a constant repressive effect of the cytoskeleton on SAC activity, and this effect can be reduced when the patches are excised to the inside-out configuration. We observed this reduction in repressive effect on WT, but not SRFsmko myocytes. This suggests that either the cytoskeleton was already weak in the cell-attached configuration in the absence of SRF, or that a cytoskeleton-independent regulation of SACs by SRF is also present. Those results suggest a relationship between SACs and SRF in MT.

In addition, we found that actin filament polymerization was altered in SRFsmko. This finding is consistent with previous

---

**Figure 5.** Protein expression levels normalized to tubulin in tail resistance arteries from control and smooth muscle–specific SRF knockout mice (SRFsmko) mice (n=10 mice per group). *P<0.05, wild-type (WT) vs SRFsmko. FAK indicates focal adhesion kinase.
The decrease in MT found in SRFsmko mice could therefore be, at least in part, a result of this decrease in actin polymerization with cytochalasin D inhibited the development of MT.30

We thus measured the activity of the 2 mitogen-activated protein-kinases, extracellular signal-regulated kinases (ERK)1/2 and p38, both potentially involved in arterial contractility, but not involved or minimally involved in MT. ERK1/2 activity was not affected by the absence of SRF in tail arteries. This is consistent with our previous study showing that ERK1/2 is not involved in MT.32 However, we found that the ratio of phospho-p38/p38 was increased in SRFsmko arteries. Interestingly, the expression of FAK, an upstream activator of p38, was also significantly increased in SRFsmko arteries, although the ratio of phosphorylated FAK/FAK was only slightly increased in SRFsmko SMCs, because of the high level of FAK. We have previously shown that the angiotensin II increases phosphorylation of FAK and p38 in vascular SMCs,33 and that p38 is strongly involved in U46619-induced contraction,34 but minimally involved in MT.35 Furthermore, p38 inhibition with SB203580 attenuated PE- and U46619-induced contraction without significantly reducing MT in arteries from SRFsmko mice. These findings suggest that increased FAK and p38 activity in SRFsmko mice could reflect a compensatory mechanism allowing agonist-dependent contraction to remain active in arteries from SRFsmko mice. This preservation of agonist-dependent contraction appears to be incompatible with the impressive changes in the expression of genes encoding smooth muscle contractile proteins at mRNA level. In fact, the decrease of some smooth muscle contractile protein level is less important than that at mRNA level at 14 days after induction of SRF deletion by tamoxifen injection, probably because of turnover rate and posttranslational modifications of each protein. For example, the level of protein smooth muscle-myosin heavy chain in SRFsmko mice is similar to the control mice (data not shown) at this stage. We hypothesize that the preservation of agonist-dependent contraction could be partially linked to the slow downregulation of some contractile proteins.

However, SRF activity is implicated in the hypertensive rat (spontaneously hypertensive rat); in fact, spontaneously hypertensive rat contains a 12-base pair insertion adjacent to the SRF-binding site CArG box. This insertion increases SRF binding to the CArG box, and increases the smooth muscle myosin light chain kinase expression. In vivo, inhibiting mitogen-activated protein kinase kinase decreased smooth muscle myosin light chain kinase expression and blood pressure in spontaneously hypertensive rat partly by decreasing SRF binding to the smooth muscle myosin light chain kinase promoter.36 However, in Alzheimer patients, it has been shown that SRF-MYOCDC overexpression in small cerebral arteries results in an arterial hypercontractility, with resulting cerebral blood flow reductions and dysregulation.20

This study demonstrates a novel SRF–SAC pathway involved in the MT of resistance arteries. In previous studies, we have demonstrated a mechanism through which the reinforcement of subcortical F-actin led to the silencing of membrane SACs. This silencing could be abolished by removing the F-actin cytoskeleton, either by treatment with cytochalasin or latrunculin, or by recording channel activity in the inside-out configuration, where the F-actin contribution is removed. Our results in the current study indicate that SRF deletion in SMCs reduces the number of SACs in the plasma membrane, as opposed to inhibiting their activity at the

The mRNA expression level of several genes involved in SMC contraction was altered in SRFsmko mice. In addition, both MLCK and MLC levels were significantly decreased in arteries from these mice. These proteins are involved not only in MT, but also in the contractions induced by the pharmacological agents, PE and U46619. As PE- and U46619-induced contractions were not modified by the SRF deletion in vascular SMCs, we further analyzed the pathways potentially involved in contraction.

Figure 6. A, Protein expression levels of mitogen-activated protein (MAP) kinase p38 and phosphorylated form Pp38 in resistance arteries from control and smooth muscle-specific SRF knockout mice (SRFsmko) mice. The activity is reflected by the ratio of phospho-p38/p38. B, Contraction induced by phenylephrine and U46619 determined after MAP kinase p38 inhibition by SB203580 in resistance arteries isolated from control and SRFsmko mice. Values are mean±SEM (n=6 mice per group). *P<0.05, control vs SRFsmko, drug treatment vs control.

studies on adult cardiac and visceral SMCs lacking SRF.21,23

The decrease in MT found in SRFsmko mice could therefore be, at least in part, a result of this decrease in actin polymerization and filamin content. This is in agreement with a previous study showing that the inhibition of actin polymerization with cytochalasin or latrunculin, or by recording channel activity in the inside-out configuration, where the F-actin contribution is removed. Our results in the current study indicate that SRF deletion in SMCs reduces the number of SACs in the plasma membrane, as opposed to inhibiting their activity at the
membrane without affecting their numbers. This is supported by the observation that removing the F-actin influence by transiting to the inside-out configuration does not affect SAC activity. Our data indicate that SRF is implicated in the tuning of SMC mechanosensitivity. Furthermore, in our mouse model, the role of SRF in agonist-dependent contraction seems to be dispensable, probably because of a compensatory mechanism partially involving the higher p38 MAPK activity.

In conclusion, our present findings indicate a central role for SRF in the tuning of SMC mechanosensitivity in local control of microvascular blood flow through autoregulation.

Acknowledgments

We thank the local Animal Care Unit of the University of Angers and Jérôme Roux, Pierre Legras, Dominique Gilbert for their kind help in treating the rats.

Sources of Funding

This work was supported in part by a grant from the Agence Nationale de la Recherche (ANR), Paris, France, and by the association française contre les myopathies (AFM). Kevin Retailleau was supported by Angers-Loire Metropole. Reza Sharif-Naeini was supported by a long-term fellowship from the Human Frontier Science Program.

Disclosures

None.

References

Selective Involvement of Serum Response Factor in Pressure-Induced Myogenic Tone in Resistance Arteries

Kevin Retailleau, Bertrand Toutain, Guillaume Galmiche, Céline Fassot, Reza Sharif-Naeini, Gilles Kauffenstein, Mathias Mericskay, Fabrice Duprat, Linda Grimaud, Jean Merot, Aurelie Lardeux, Anne Pizard, Véronique Baudrie, Xavier Jeunemaitre, Robert Feil, Joachim R. Göthert, Patrick Lacolley, Daniel Henrion, Zhenlin Li and Laurent Loufrani

Arterioscler Thromb Vasc Biol. 2013;33:339-346; originally published online December 20, 2012;
doi: 10.1161/ATVBAHA.112.300708

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/33/2/339

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIEL

MATERIALS AND METHODS

Blood pressure measurement by telemetry data acquisition (I)
Anaesthesia on mice was induced with 5% isoflurane in an oxygen stream and maintained with 2-3% isoflurane. Mice were kept on a heating pad throughout implantation of the blood pressure (BP) telemeter (model TA11PA-C10; Data Sciences International, St Paul, Minnesota, USA). The catheter was inserted into the aorta via the left common carotid artery with the tip in the aortic arch, and the telemetric transmitter probe was positioned subcutaneously on the right flank[4]. To reduce infection and pain, the mice received one dose (20 mg/kg i.p.) of amoxicillin (Clamoxyl; Smithkline Beecham Laboratories, Nanterre, France) and one dose (5 mg/kg i.p.) of ketoprofen (Profenid; Sanofi Aventis, Paris, France). They were housed in individual cages, positioned on top of the telemetric receivers (model RPC-1; Data Sciences, St. Paul, MN), in a light-dark cycled recording housing (08:00–20:00 h) for a 1-week recovery period before the initiation of experiments. Mice were provided with ad libitum access to water and standard rodent chow (M20, SDS, Argenteuil, France). After recovery, cardiovascular parameters and locomotor activity were recorded daily, continuously over 24 h under basal conditions (1 day before tamoxifen ip injection) and up to 15 days after injection. The telemetry BP signal was digitized at 1,000 Hz. The experimental data were collected continuously in real time, stored on the local hard disk, and analyzed using the Data Sciences acquisition system and the Dataquest ART analysis software.

Calcium analysis (III)
Fura-2AM Ca\(^{2+}\) imaging:
Freshly dissociated smooth muscle cells (SMCs) from tail arteries were incubated for 20 minutes in darkness at room temperature, with 2 \(\mu\)M Fura-2AM (molecular probes) and 0.002% pluronic acid. A modified tyrode solution (containing [in mM]: NaCl 140, KCl 4, CaCl\(_2\) 1, MgCl\(_2\) 1, glucose 5, HEPES 10; and at pH 7.4) was used throughout the experiment. After 10 minutes of washing, ratiometric imaging of the SMC internal calcium level was performed on a microscope (lens x40, Axio Observer, Zeiss) equipped with a CCD camera (CoolSNAP, Roper scientific). An appropriate field of cells was identified and stimulated with wavelengths of 340 and 380 nm. Images were collected and stored every second at each wavelength, with 150 ms exposure. The ratio of fluorescence emitted at 510 nm was analyzed after subtraction of background fluorescence levels during 5 minutes using data acquisition software (Metafluor, Roper Scientific). At the end of the experiment, SMCs were perfused with a tyrode solution containing ionomycin (4 \(\mu\)g/ml) and then with a calcium-free solution containing (in mM): EDTA 100, KCl 4, MgCl\(_2\) 2, HEPES 10; and at pH 7.4. This allowed the ratios to be converted to calcium concentrations using standard calculations[1]. Images were analyzed offline with imaging software (Metafluor, Roper Scientific).
The role of serum response factor (SRF) in the endothelial cell (IV)

It has been shown that transgene (end-SCL-Cre-ERT) mice induce efficient Cre-mediated recombination in most organ endothelial cells after tamoxifen injection. We crossed endothelial-SCL-Cre-ERT transgene (end-SCL-Cre-ERT) mice with floxed SRF (Sf mice). The presence of end-SCL-Cre-ERT transgene was detected by PCR (primers 5’-GATCTCGAGCCATCTGCTG-3’ and 5’-GGTCGGCCGTCAGGGACAA-3’, amplifying a 110-bp fragment of Cre-ERT), as described previously [2]. Endothelial-specific tamoxifen-dependent recombination was triggered by intra-peritoneal injection of tamoxifen (1mg/mouse/day) for five days into the adult male mice (3-4 month old). Deletion of the SRF gene was detected in the tamoxifen-treated double transgenic mice (Sf/Sf: end-SCL-Cre-ERT), but not in the untreated double transgenic mice, nor in the treated or untreated control mice (Sf/Sf). The PCR reaction used primers SF1, SF2 and SF3, as described previously[3]. SF1 (5’-CTGTAAGGGATGGAAGCAGA-3’) and SF2 (5’-TAAGGACAGTGAGGTCCCTA-3’) gave rise to the 492-bp fragment for floxed SRF alleles, while SF1 and SF3 (5’-TTCGGAACTGCGGGGCGACTAAA-3’) allowed amplification of a 310-bp DNA fragment when floxed SRF alleles had been recombined by Cre recombinase.


Legends for Supplementary Figures:

**Figure I**
Effect of intra-peritoneal tamoxifen injection on systolic blood pressure, mean blood pressure, diastolic blood pressure, heart rate, heart weight, and weight of mice in wild type (WT, open square, n=12) and SRF knock-out mice (SRF<sup>smko</sup>, filled square, n=6). Results are expressed as % of baseline (% of value before injection). Arrow: tamoxifen injection.

**Figure II**
Dilation induced by a dose response curve by Acetylcholine after phenylephrine precontraction (50% of max contraction). Arteries were isolated from control and SRF<sup>smko</sup> mice or with L-NAME (10<sup>-4</sup>M, 30 min) : NO inhibition or L-NAME(10<sup>-4</sup>M, 30 min) + INDOMETACINE (10<sup>-5</sup>M, 30min) : NO and prostaglandin inhibition or L-NAME(10<sup>-4</sup>M, 30 min) + INDOMETACINE (10<sup>-5</sup>M, 30min) + KCL (10 mM) : NO and prostaglandin and EDHF inhibition.
Values are mean±s.e.m; *p<0.05, SRF<sup>smko</sup> arteries compared to control arteries (N=9 mice per group)

**Figure III**
Left: Calcium-induced contractility in tail arteries from wild type (WT) and SRF<sup>smko</sup> mice.
Right: Baseline calcium concentrations (in nM) in freshly dissociated smooth muscle cells from WT and SRF<sup>smko</sup> mice.
Values are mean±s.e.m; *p<0.05, SRF<sup>smko</sup> arteries compared to control arteries

**Figure IV**
(A) Contraction induced by phenylephrine and U46619.
(B) Pressure-diameter (myogenic tone and passive arterial diameter) relationship determined in resistance arteries. Arteries were isolated from control and SRF<sup>endo</sup> mice.
Values are mean±s.e.m; *p<0.05, SRF<sup>smko</sup> arteries compared to control arteries (N=10 mice per group)

**Figure V**
Dilation induced by a dose response curve by Acetylcholine after phenylephrine precontraction (50% of max contraction). Arteries were isolated from control and SRF<sup>endo</sup> mice.
Values are mean±s.e.m; *p<0.05, SRF<sup>endo</sup> arteries compared to control arteries (N=10 mice per group)
Supplemental data I: Blood pressure measurement by telemetry data acquisition

Systolic blood pressure

% of baseline vs time (day)

Mean blood pressure

% of baseline vs time (day)

Diastolic blood pressure

% of baseline vs time (day)

Heart rate

% of baseline vs time (day)

weight of mice (g)

heart weight (g)
Supplemental data II: SRF^{smko} mice

Acetylcholine

% Passive Diameter

Log[M]

WT

WT + LN

WT+LN+INDO

WT+LN+INDO+KCL

SRF^{smko}

SRF^{smko}+LN

SRF^{smko}+LN+INDO

SRF^{smko}+LN+INDO+KCL
Supplemental data III : Calcium analysis

![Graph showing calcium analysis for WT and SRFsmko](image)

- WT (n=21)
- SRFsmko (n=56)
Supplemental data IV: endothelium SRF KO mice

A  Phenylephrine  U46619

B  Myogenic Tone  Passive Diameter
Supplemental data V: endothelium SRF KO mice

- Graph showing the effect of Achetylcholine on % Passive Diameter with Log [M] on the x-axis and % Passive Diameter on the y-axis.
- Two curves are plotted: black square for WT and white square for SRFendo.
- The graph indicates a concentration-dependent response to Achetylcholine.