Abstract—In vascular smooth muscle cells (SMCs), several mechanisms act in concert to regulate the intracellular calcium concentration \([\text{Ca}^{2+}]_i\), which may in turn affect vascular tone. One such mechanism is the extrusion of \(\text{Ca}^{2+}\) by the plasma membrane calcium ATPase (PMCA). To address, in particular, the role of the neuronal nitric oxide synthase (nNOS)-associating isoform PMCA4b in regulating vascular tone, a doxycycline-responsive transgene for human PMCA4b was overexpressed in arterial SMCs of mice. Overexpression of hPMCA4b resulted in a 2-fold increase in total aortic PMCA4 protein expression and significant real-time RT-PCR-documented differences in the levels of endogenous mouse PMCA1, PMCA4, SERCA2, and IP3R1 gene expression in arterial SMCs. Whereas no significant difference in basal \([\text{Ca}^{2+}]_i\), or \(\text{Ca}^{2+}\) sensitivity was observed in vascular SMCs or mesenteric arteries, respectively, from hPMCA4b-overexpressing versus control mice, hPMCA4b-overexpressing mice revealed a reduced set-point and increased extent of myogenic response and heightened sensitivity to vasoconstrictors. Treatment of arteries with an nNOS inhibitor resulted in a reduced set-point and increased extent of the myogenic response in control but not hPMCA4b-overexpressing mice. Moreover, aortic SMCs from hPMCA4b-overexpressing mice exhibited reduced levels of cGMP under both basal and phenylephrine-stimulated conditions. These changes were associated with significant doxycycline-reversible elevations in blood pressure. Taken together, these data show that overexpression of hPMCA4b in arterial SMCs increases vascular reactivity and blood pressure, an effect that may be mediated in part by negative regulation of nNOS. (Circ Res. 2003;93:614-621.)

Key Words: transgenic mice • blood pressure • nitric oxide synthase • intracellular calcium • myogenic tone
of SERCA and the inositol 1,4,5-trisphosphate–activated calcium channel (IP3R), suggesting that interdependent Ca\(^{2+}\) signaling systems are adaptable and function to maintain constant [Ca\(^{2+}\)].\(^{10}\) Similarly, transgenic overexpression of PMCA4 in rat myocardium failed to significantly alter cardiac function or resting [Ca\(^{2+}\)], compared with wild-type rats.\(^{11}\)

PMCA localizes in caveolae of SMCs, endothelial cells, and cardiomyocytes\(^ {11,12}\) and can concentrate 18- to 25-fold in caveolar membranes.\(^ {12}\) Caveolae are membrane structures that contain a wide variety of effector and signaling molecules,\(^ {13}\) including neuronal nitric oxide synthase (nNOS).\(^ {14}\) Recently, an interaction between hPMCA4b and nNOS (NOS-I) has been described to occur via the PDZ domain of cNOS,\(^ {13}\) including neuronal nitric oxide synthase (nNOS).\(^ {14}\)

To address the role of hPMCA4b in regulating vascular smooth muscle tone, we generated transgenic mice using an arterial SMC-restricted (SM22\(^ {\alpha}\) promoter-driven) tetracycline-controlled transactivator (tTA)\(^ {16,17}\) to effect conditional expression of a tTA-dependent transgene encoding hPMCA4b. We demonstrate that overexpression of hPMCA4b in arterial SMCs is associated with enhanced vascular reactivity and elevated blood pressure that may be mediated in part by negative regulation of nNOS.

Materials and Methods

Construction of the hPMCA4b Transgene

A BamH1-Xba1 fragment (3964 bp) of a human PMCA4b expression cassette\(^ {18}\) was blunt cloned into pBiGNOT.\(^ {19}\) The resulting pBiGNOT-hPMCA4b was used for pronuclear microinjection (Figure 1A) as described.\(^ {16,17}\)

Generation of Binary Transgenic Mice and Genotyping

The Toronto General Hospital Animal Care Committee approved all experimentation. Potential hPMCA4b founders were bred to SM22\(^ {\alpha}\)-tTA mice. Genotyping was performed by polymerase chain reaction (PCR) of tail biopsies. Human PMCA4-specific primers (forward, 5′-GCC-TCC-CTG-AGT-GTA-CTC-CC-3′; reverse, 5′-CCT-GAT-GAC-GGT-GCT-CAT-TG-3′) in a 30-cycle PCR (94°C, 60°C, and 72°C for 30 seconds each) generated a 559-bp hPMCA4-specific product. Detection of SM22\(^ {\alpha}\)-tTA has been described.\(^ {16,17}\)

RT-PCR

Total RNA was extracted\(^ {20}\) and treated with DNAse I and Super Script II reverse transcriptase (Invitrogen Life Technologies Inc). PCR amplification was performed as per genotyping.

Western Blotting

Aortae were homogenized in an ice-cold buffer of 100 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 50 mmol/L HEPES, pH 7.4, and 1 mmol/L EDTA with protease inhibitors. After electrophoresis and transfer to nitrocellulose, membranes were blocked overnight in 3% nonfat milk, 20 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, and 0.1% Tween 20, incubated with the monoclonal anti–hPMCA4b-specific antibody JA3 (1:500; kindly provided by John Penniston, Mayo Clinic, Rochester, Minn\(^ {21}\)) or the monoclonal anti–pan-PMCA antibody 5F10 (1:1000) and an anti-actin antibody (1:500) washed 3 times with Tris-buffered saline and incubated with a goat anti-mouse IgG secondary (Sigma, Mississauga, Ontario, Canada). Immunoreactivity was detected with enhanced chemiluminescence (Amersham Biosciences) or DAB staining (Vector Labs Canada). The processed blots were scanned by densitometry, and band intensities were quantified.\(^ {7}\)

Isolation of Primary Smooth Muscle Cells

Aortic SMCs were harvested and cultured as previously described.\(^ {7}\) To suppress hPMCA4b expression, cultures were grown in the presence of 1 μg/mL of doxycycline (DOX; Sigma-Aldrich). This was removed from the media 7 days before any assay.

PMCA-Dependent ATPase Activity

Thapsigargin-insensitive Ca\(^{2+}\)-dependent ATPase activity in plasma membrane preparations was measured with a coupled enzyme assay.\(^ {11,22}\) Briefly, mesenteric arteries were minced with scissors, homogenized, and centrifuged (400g for 10 minutes, 10g for 60 minutes) at 4°C. The pellet was resuspended in ice-cold buffer,\(^ {11}\) and
5 μg of protein was added to 0.5 mL of ATPase assay buffer. The disappearance of NADH was measured in a spectrophotometer. SERCA- and Ca\(^{2+}\)-independent ATPase activities were assessed by the addition of thapsigargin (5 μmol/L) and EGTA (2 mmol/L), respectively. Thapsigargin-insensitive Ca\(^{2+}\)-dependent ATPase activity was calculated by subtracting the thapsigargin- and EGTA-sensitive ATPase activities from total membrane ATPase activity.

\[ ^{45}\text{Ca} \text{ Efflux} \]

Ca\(^{2+}\) efflux rates were determined as previously described. Briefly, aortic SMCs were incubated overnight in media containing 5 μCi/mL \(^{45}\text{Ca}\). The amount of released \(^{45}\text{Ca}\) was determined over five 30-second intervals, and the remaining intracellular radioactivity was measured after cell lysis. Efflux rates were calculated as described.

\[ \text{Determination of [Ca}^{2+}\text{]} \]

Free \([\text{Ca}^{2+}]\), in isolated mouse aortic SMCs was measured as described. Briefly, aortic SMCs plated on coverslips were incubated with Fura-2-AM (2 μmol/L; Molecular Probes) for 30 minutes at 21°C in PSS, washed 3 times, and mounted on a modified Leiden chamber. The \([\text{Ca}^{2+}]\), was measured using an Image-Master DeltaRAM digital ratio imaging system (Photon Technology International) using an IC-200 intensified CCD camera mounted on an Olympus DX70 inverted microscope. With alternating 340- and 380-nm excitation, Fura-2 emission (510 nm) ratio images were acquired. \([\text{Ca}^{2+}]\), was obtained from acquired ratios as previously described.

\[ \text{Real-Time RT-PCR} \]

DNase-treated total RNA was used. Conditions were optimized to generate a single PCR product of expected size and melting temperature (SYBR GREEN kit; Applied Biosystems). Relative standard curves were generated for each gene as per protocol 4304965 (http://home.appliedbiosystems.com), and the mRNA level (in nanogram) of each sample was normalized to the GAPDH level (in nanogram) of each sample. Gene-specific real-time PCR primers used were as follows: SERCA2a, 5′-TGAGACCCTAAGTTTGGTGG-3′; SERCA2a, 5′-ATGCA-GAGGCTGTGTAAGTG-3′; SERCA2b, 5′-ACAACCGCCAGGAAATG-3′; IP3RI, 5′-ATGGTTGGCAAGATTCTCTG-3′; GAPDH, 5′-CTTTCCGTGACCGTCTTAC-3′; PMCA1, 5′-TCTGCTGGAACATCTTGACG-3′; PMCA4a, 5′-CTTTTCCTCCAACGACTTCT-3′; mouse PMCA4, 5′-ATGCA-GAGGCTGTGTAAGTG-3′; and GAPDH, 5′-GCATGGCCTTCCGTTGTT-3′, 5′-ATGGTCAT-CATACATGTGCGAGCAGTTT-3′.

\[ \text{Vasomotor Studies} \]

Second-order mesenteric arteries were mounted on a pressure myograph (Living Systems), and passive and active vessel diameter perfusion pressure relationships were obtained. Set-point was defined as the lowest perfusion pressure at which significant myogenic constriction was first observed. Extent was defined as the magnitude of the percent myogenic tone at a given perfusion pressure, and strength was defined as the slope of the active diameter–pressure relationship. Some arteries were incubated in the presence of either the non-specific NOS inhibitor L-nitro-arginine-methyl-ester (L-NAME, 0.25 mmol/L; Sigma-Aldrich) or the specific nNOS inhibitor N\(^{\bullet}\)-propyl-l-arginine (300 mmol/L; Cayman Chemical Company) for 30 minutes. Contractile responses to phenylephrine (1 mmol/L) and prostaglandin-F\(_2\alpha\) (1 mmol/L) or KCl (10 to 120 mmol/L; Sigma-Aldrich) were recorded in vessels pressurized to 60 mm Hg. To examine Ca\(^{2+}\) sensitivity, vessels were washed twice in Ca\(^{2+}\)-free PSS (1 mmol/L EGTA) and placed in Ca\(^{2+}\)-free PSS containing 120 mmol/L KCl. Contractile responses to increasing extracellular CaCl\(_2\) (0.5 to 3.0 mmol/L) were recorded.

\[ \text{cGMP Assays} \]

Primary aortic SMCs from passages 1 through 3 (3×10\(^4\) cells per well on 6-well plates) grown in DMEM containing 50 ng/mL PDGF-BB and 10% FCS were treated with 0.1 mmol/L 3-isobutyl-1-methylxanthine (cGMP phosphodiesterase inhibitor; Sigma) for 15 minutes and then 0.1 mmol/L 3-isobutyl-1-methylxanthine with or without 1 μmol/L phenylephrine for 15 minutes at 37°C. Cells were lysed, and total cellular cGMP levels were measured according to protocol-4 of the BioTrak cGMP enzyme immunoassay kit (Amersham).

\[ \text{Blood Pressure Determination} \]

Blood pressure was assessed as previously described. Briefly, mice were anesthetized with ketamine and xylazine (100 and 10 mg/kg IP) and placed supine on a warming pad. The right carotid artery was cannulated with a 1.4F high-fidelity micromanometer catheter (Model SPR-671, Millar Instruments), and systemic blood pressure was recorded.

\[ \text{Data Analysis} \]

All data are expressed as mean±SEM. Statistical analysis was done using either one- or two-way ANOVA with Bonferroni posttests and Student’s t test where appropriate.

\[ \text{Results} \]

\[ \text{Generation of Binary Transgenic Mice} \]

Mice harboring a SM22α promoter-directed transgene for a DOX-responsive tTA\(^{16}\) were mated with mouse lines carrying a tTA-dependent bidirectional transgene encoding both hPMCA4b and β-galactosidase (Figure 1A). Of the 8 distinct PMCA4b:β-gal lines crossed with SM22α-tTA mice, 6 showed absent, weak, or inconsistent transactivation of PMCA4b:β-gal and were terminated. Two others, 167P4 and 192P4, showed consistent tTA-dependent and DOX-responsive activation of the nuclear-localizing β-gal reporter in arterial SMCs. In crosses with SM22α-tTA mice, binary transgenic (BT) (tTA\(^{+}\)/PMCA4b\(^{-}\)) progeny from both 167P4 and 192P4 lines appeared normal. They exhibited no overt developmental defects and appeared with expected autosomal frequency (25%) in crosses between heterozygotes (tTA\(^{+}\)/PMCA4b\(^{-}\); n=87; tTA\(^+\)/192P4\(^{-}\), 26%, n=74; P=NS for both lines).

\[ \text{Expression and Activity of Human PMCA4b in Arterial SMCs of Binary Transgenic Mice} \]

RT-PCR on RNA from thoracic aorta revealed hPMCA4b expression in BT (tTA\(^{-}\)/PMCA4b\(^{-}\)) but not non-BT (NBT) littermates (tTA\(^{-}\)/PMCA4b\(^{-}\), tTA\(^{-}\)/PMCA4b\(^{-}\), tTA\(^{-}\)/PMCA4b\(^{-}\) or BT animals fed DOX (0.2 mg/mL in drinking water) (Figure 1B). Western blot using the JA3 anti-hPMCA4b antibody demonstrated a band of expected size (∼136 kDa) in aortic lysates from BT but not NBT littermates (Figure 1C). Western blot with the 5F10 anti-PMCA antibody was used to estimate levels of PMCA protein expression (Figure 1C). Although actin-normalized levels of a band believed to represent mPMCA1 (∼144 kDa) did not reveal significant differences between BT and NBT aortae, total PMCA4 protein expression (i.e., endogenous mouse and transgenic PMCA4) was ∼2.0 fold greater in BT versus control mice (Figure 1C). Moreover, tTA-dependent transgene activation resulted in a significant decrease in the ratio of putative mPMCA1:total PMCA4 protein expression (BT ∼1:3 versus NBT ∼2:1).

Thapsigargin-insensitive Ca\(^{2+}\)-dependent ATPase activity was increased by a mean of 33% (range, 27% to 42%) in BT mice compared with controls (Figure 2A). Assessment of
45 Ca efflux in cultures of isolated aortic SMCs revealed a similar range of increased PMCA activity in tTA/H11001/P4/H11001 samples compared with controls (Figure 2B). However, in isolated aortic SMCs, no significant difference in basal \([Ca^{2+}]_{i}\) was observed between tTA/H11001/P4/H11001 and control cells (Figure 2C).

**Myogenic Responses**

Whereas the relationships between passive pressure and diameter were indistinguishable (Figure 3A), the relationship between active pressure and diameter was significantly altered in animals overexpressing hPMCA4b. The set-point of the myogenic response was significantly reduced in BT mice compared with NBT or DOX-treated BT controls (80 versus 100 mm Hg, \(P<0.05\)) (Figure 3B). In addition, the extent of the myogenic response at defined perfusion pressures was significantly increased in BT animals compared with controls (Figure 3B). Analysis of the strength of the myogenic response revealed no significant difference between BT and NBT mice (\(-0.30\pm0.06\%\) versus \(-0.34\pm0.13\%\) per mm Hg, \(P=NS\)). With single-step increases and decreases in perfusion pressure between 60 and 120 mm Hg, the extent of the myogenic response after an increase in perfusion pressure was significantly greater in BT versus NBT mice (28.6\(\pm1.1\%\) versus 18.0\(\pm1.1\%, n=4, P<0.01\)) and similar to that seen with graduated-step increases (Figure 3B). However, after a single-step decrease in perfusion pressure, BT arteries retained a level of myogenic tone not seen before the step increase (3.1\(\pm0.5\%\) versus 3.6\(\pm1.5\%, P=NS, n=4\), which NBT mice did not retain (2.6\(\pm0.5\%\) versus 3.6\(\pm1.5\%, P=NS\)).

**Pharmacomechanical Responses**

Mesenteric arteries from BT mice demonstrated enhanced sensitivity to phenylephrine (Figure 4) and prostaglandin F2\(\alpha\) (data not shown). Phenylephrine induced larger oscillations in the diameter (ie, cycles of contraction and relaxation) of BT arteries than controls (Figure 4A). Near identical oscillations were observed with prostaglandin F2\(\alpha\) (data not shown). Of note, the EC\(_{50}\) for phenylephrine-mediated constriction was almost an order of magnitude lower in BT mice compared with controls (25\(\pm12\) versus 205\(\pm15\) mmol/L, \(P<0.05\), Figure 4B).

An analysis of the maximal change in vessel diameter (ie, difference between maximum constriction and relaxation) confirmed that mesenteric arteries from BT mice had more significant changes in vessel diameter in response to phenylephrine (Figure 4C) and prostaglandin F2\(\alpha\) (data not shown) than control mice.
Expression of Ca\(^{2+}\) Regulatory Genes

RNA isolated from primary aortic SMC cultures and freshly harvested aortae of mice overexpressing hPMCA4b (BT 51.6 versus BT+DOX 75.2). Consistent with this, the modest effect on mouse PMCA1 expression levels observed in cultured aortic SMCs was not evident at the mRNA (data not shown) or protein level (Figure 1C) in freshly isolated aortae. Possible explanations for why GAPDH-normalized values obtained from aortae differed from those of cultured aortic SMCs include the multiple cell types present in aortae (such as endothelial cells, SMCs, and fibroblasts) versus the relatively pure population of SMCs in primary cultures and the systematic effect of tissue culture on gene expression.

Interestingly, BT mice also showed increased levels of expression of SERCA2a and SERCA2b compared with DOX-treated controls (SERCA2a, 10.8 versus 5.9; SERCA2b, 5.1 versus 1.4). Although relative levels of IP3R1 mRNA were considerably lower than those of the other Ca\(^{2+}\) regulatory genes examined, they too seemed to increase in BT mice compared with DOX-treated controls (BT 0.44 versus BT+DOX 0.18). Collectively, these data suggest possible compensatory alterations in other endogenous Ca\(^{2+}\) regulatory gene expression in arterial SMCs in mice that overexpress human PMCA4b.

Contractility and Calcium Sensitivity

In contrast to the oscillations observed in response to G-protein–coupled receptor agonists (phenylephrine and prostaglandin F2α), KCl-mediated contractions were highly stable, with a near-immediate plateau in vessel diameter after each increase in KCl concentration (data not shown). Moreover, no significant difference in the KCl dose response was observed between BT and NBT littermates (Figure 5A).

To determine whether the enhanced vasomotor responses of PMCA4b-overexpressing animals was attributable to heightened Ca\(^{2+}\) sensitivity of the contractile apparatus, we assessed KCl-mediated constriction in the presence of increasing concentrations of extracellular calcium. No significant differences in Ca\(^{2+}\) sensitivity were observed in mesenteric arteries obtained from BT and NBT mice (Figure 5B).

Effect of NOS Inhibitors

Recent studies have demonstrated that hPMCA4b can act as a negative regulator of nNOS activity in cultured cells. To explore whether this was a possible mechanism mediating the phenotype of mice overexpressing hPMCA4b, we examined the effect of NOS inhibition on specific vasomotor responses. Treatment of mesenteric arteries from control mice with L-NAME (a nonspecific inhibitor of NOS) or \(N^\)-propyl-L-arginine (a specific inhibitor of nNOS) resulted in a reduction of the set-point and increase in the extent of the myogenic response similar to that observed in untreated arteries from BT mice (Figures 6A and 6B). In contrast, neither L-NAME nor \(N^\)-propyl-L-arginine altered the set-point or extent of the myogenic response in BT mice (Figures 6A and 6B).

Levels of cGMP

The above data suggested that arterial SMCs from BT mice exhibited depressed nNOS function, which may contribute to their...
vasomotor phenotype. To explore more directly potential differences in NO synthesis, we quantified cGMP levels in isolated SMCs from BT and NBT littermates. Under both basal and phenylephrine-stimulated conditions, aortic SMCs from BT mice exhibit significantly reduced cGMP generation compared with NBT littermates (basal, 27±8 versus 32±6 fmol, n=9, P<0.005; phenylephrine, 5±3 versus 27±9 fmol, n=9, P<0.02).

**Blood Pressure**

Both systolic and diastolic blood pressures were significantly increased in BT mice compared with NBT littermates (Figure 7). DOX had no significant effect on blood pressure in NBT mice but completely normalized systolic and diastolic blood pressures in BT mice (Figure 7). Heart rates did not differ significantly between BT and NBT mice (BT 209±12 versus NBT 199±10 bpm, P=NS), and DOX had no effect on the heart rate of any genotype (BT+DOX 211±12 versus NBT+DOX 223±12 bpm; P=NS).

**Discussion**

Modulation of [Ca\(^{2+}\)] through altered expression or function of the PMCA represents a potential regulatory mechanism in vascular pathophysiology. In this study, we show that DOX-regulated arterial SMC-specific overexpression of the nNOS-associating isoform hPMCA4b increased vasomotor responsiveness and blood pressure in association with altered expression of Ca\(^{2+}\) regulatory genes and depressed activity of nNOS.

More specifically, mice overexpressing hPMCA4b demonstrated a decreased set-point and increased extent of the myogenic response. Given that age- and salt-induced hypertension in mice is associated with an increased set-point and reduced extent of the myogenic response,\(^{27}\) the abnormalities observed in mice overexpressing hPMCA4b may reflect an inability to adapt (ie, blunt) their myogenic tone in response to elevated blood pressure.\(^{27}\) This adaptive failure may contribute to the maintenance of elevated blood pressure in BT mice.

The finding that NO plays a role in modulating myogenic tone is not unique.\(^{28,29}\) Others have shown that L-NAME can reduce the set-point and increase the extent of the myogenic response under no-flow conditions.\(^{28,29}\) However, our finding...
that this effect was confined to arteries from control mice and not observed in arteries overexpressing PMCA4b suggests that NOS activity in BT arteries was already maximally depressed. Indeed, the significantly reduced cGMP levels of BT arteries additionally supported this interpretation.

Incubation of arteries from NBT mice with the nNOS-specific inhibitor Nω-propyl-L-arginine also resulted in a reduction in set-point and increase in extent of myogenic tone development. Again, inhibition of nNOS in BT arteries had no effect on their myogenic response. It is important to note that the concentration of Nω-propyl-L-arginine (300 nmol/L) used was 30-fold lower than the reported K, for endothelial NOS (eNOS).30 Indeed, in preliminary experiments, phenylephrine-preconstricted arteries treated with the same concentration of Nω-propyl-L-arginine were still able to vasodilate in response to acetylcholine (data not shown), suggesting that increased myogenic responses observed with Nω-propyl-L-arginine were not attributable to inhibition of eNOS. Moreover, myogenic tone was decreased in mesenteric arteries from eNOS knockout mice.29

Whereas Boulanger et al31 demonstrated expression of nNOS in rat arterial SMCs and showed that >40% of calcium-dependent NOS activity in rat arteries was endothelium-independent, they did not assess the importance of this finding to vasomotor function. Other studies also support the importance of nNOS in vascular tissues.14,32–34 However, to our knowledge, the direct demonstration that nNOS can modulate myogenic tone is a new finding. Indeed, it is tempting to speculate that the depletion of nNOS from the sarcosome of mice overexpressing an α-syntrophin mutant35 and the increased nNOS activity of mice overexpressing a caveolin-3 mutant36 may be associated with a vasomotor phenotype. Such studies in these models are now indicated.

However, it should be noted that mice with disruption of nNOS exon-2 do not display elevated blood pressure.37,38 These mice lack the PDZ-domain of nNOS, which associates with the PMCA4b isoform, but retain low levels of noncaveolar localized functional nNOS splice variants.39 Whereas the vasomotor properties of these mice are beginning to be examined, it remains possible that developmental or physiological adaptations in the non–tissue-specific and nonconditional nNOS exon-2 knockout may mask a hypertensive phenotype. Although defects in reproductive function have been described in exon-6 nNOS knockout mice, cardiovascular studies have not been reported.40 Nevertheless, the finding that conditional and tissue-specific overexpression of hPMCA4b does manifest elevated blood pressure suggests that other contributing factors present in our model, such as the altered expression of Ca2+ regulatory genes in arterial SMCs, work collectively to generate the phenotype.

Despite overexpression of a functional Ca2+ efflux pump, we did not observe a significant decrease in resting [Ca2+]i in isolated aortic SMCs. This may, in part, reflect compensatory mechanisms active in restoring [Ca2+]i. Indeed, real-time RT-PCR analyses demonstrated decreased expression levels of endogenous PMCA in response to overexpression of hPMCA4b. This may explain the very modest increases in membrane-bound Ca2+ ATPase activity and Ca2+ efflux noted in our model. Furthermore, the direction of altered SERCA2a/2b and IP3R1 expression was consistent with a compensation for enhanced Ca2+ efflux. Whether these adaptations play a more important role than depressed nNOS activity on the phenotype of our model has not been addressed. Determining this may depend on future experiments involving a PMCA that does not associate with nNOS or crossing the current model with mice lacking nNOS.39,40

Importantly, we have not excluded the possibility that significantly reduced [Ca2+]i exists within specific intracellular microdomains. Indeed, PMCA pumps are known to concentrate in the caveolae of vascular SMCs,12 and local changes in Ca2+ efflux activity or free [Ca2+]i may exceed those seen in total cell preparations. Because caveolae are also rich in signaling molecules such as the Ca2+-responsive nNOS,41 it remains possible that overexpression of PMCA4b is sufficient to inhibit the activity of nNOS by reducing microdomain [Ca2+]i without affecting global [Ca2+]i. Such regulation of nNOS by PMCA4b at the microdomain level is supported by previous work using cell culture systems.15 Of note, PMCA4b overexpression did effect changes in the arterial contractile responses to phenylephrine and prostaglandin F2α. Because these agents function through G-protein–coupled receptor activation, generation of IP3, and release of the SERCA-maintained Ca2+ store in vascular SMCs,42 it is tempting to speculate that the enhanced amplitude oscillations observed in vessels were in part mediated by the observed increases in SERCA2a/b and IP3R1 expression.

We conclude that overexpression of hPMCA4b in the vascular smooth muscle of transgenic mice resulted in enhanced vasoconstrictor responses and elevated blood pressure. Our animal model represents the first putative example of an abnormality in the myogenic response contributing to the maintenance of elevated blood pressure and the first to suggest that neuronal NOS can modulate myogenic tone. Our studies also reveal that an intricate transcriptional compensatory process is active in vivo in restoring global [Ca2+]i, and suggest that the genes involved in this homeostasis may participate in the regulation of vasomotor tone.

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