Upregulation of Na⁺/Ca²⁺ exchanger and TRPC6 contributes to abnormal Ca²⁺ homeostasis in arterial smooth muscle cells from Milan hypertensive rats

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Zulian A, Baryshnikov SG, Linde CI, Hamlyn JM, Ferrari P, Golovina VA. Upregulation of Na⁺/Ca²⁺ exchanger and TRPC6 contributes to abnormal Ca²⁺ homeostasis in arterial smooth muscle cells from Milan hypertensive rats. Am J Physiol Heart Circ Physiol 299: H624–H633, 2010. First published July 9, 2010; doi:10.1152/ajpheart.00356.2010.—The Milan hypertensive strain (MHS) of rats is a model for hypertension in humans. Inherited defects in renal function have been well studied in MHS rats, but the mechanisms that underlie the elevated vascular resistance are unclear. Altered Ca²⁺ signaling plays a key role in the vascular dysfunction associated with arterial hypertension. Here we compared Ca²⁺ signaling in mesenteric artery smooth muscle cells from MHS rats and its normotensive counterpart (MNS). Systolic blood pressure was higher in MHS than in MNS rats (144 ± 2 vs. 113 ± 1 mmHg, P < 0.05). Resting cytosolic free Ca²⁺ concentration (measured with fura-2) and ATP-induced Ca²⁺ transients were augmented in freshly dissociated arterial myocytes from MHS rats. Ba²⁺ entry activated by the diacylglycerol analog 1-oleoyl-2-acetyl-sn-glycerol (a measure of receptor-operated channel activity) was much greater in MHS than MNS arterial myocytes. This correlated with a threefold upregulation of transient receptor potential canonical 6 (TRPC6) protein. TRPC3, the other component of receptor-operated channels, was marginally, but not significantly, upregulated. The expression of TRPC1/5, components of store-operated channels, was not altered in MHS mesenteric artery smooth muscle. Immunoblot also revealed that the Na⁺/Ca²⁺ exchanger-1 (NCX1) was greatly upregulated in MHS mesenteric artery (by ~13-fold), whereas the expression of plasma membrane Ca²⁺-ATPase was not altered. Ca²⁺ entry via the reverse mode of NCX1 evoked by the removal of extracellular Na⁺ induced a rapid increase in cytosolic free Ca²⁺ concentration that was significantly larger in MHS arterial myocytes. The expression of α1/α2c Na⁺ pumps in MHS mesenteric arteries was not changed. Immunocytochemical observations showed that NCX1 and TRPC6 are clustered in plasma membrane microdomains adjacent to the underlying sarcoplasmic reticulum. In summary, MHS arteries exhibit upregulated TRPC6 and NCX1 and augmented Ca²⁺ signaling. We suggest that the increased Ca²⁺ signaling contributes to the enhanced vasoconstriction and elevated blood pressure in MHS rats.

adducin; hypertension; Milan normotensive rats; C-type transient receptor potential channels; receptor-operated calcium entry

PRIMARY OR ESSENTIAL HYPERTENSION is a multifactorial disorder resulting from the complex interplay between genetic predisposition (genetic heritability, ~30%) and multiple environmental factors (1, 44, 71, 74). The major difficulty in identifying genes contributing to hypertension is the etiological heterogeneity of hypertension (1). Genetic, physiological, and biochemical studies reveal that an alteration in the genes encoding adducin, a ubiquitously expressed membrane-skeletal protein, is associated with a salt-sensitive form of hypertension and renal dysfunction in rats and humans (1, 10, 18).

Adducin is a tetramer comprised of either αβγ- or αγ-heterodimers (50). It is localized at spectrin-actin junctions affecting the assembly of the actin-based cytoskeleton and, thereby, the normal turnover and incorporation of plasma membrane (PM) ion transporters (29, 50) since the latter can be tethered to the membrane cytoskeleton (48, 56). The α-, β-, and γ-subunits of adducin are encoded by genes (ADD1, ADD2, ADD3 or Add1, Add2, and Add3 in humans and rats, respectively) that map to different chromosomes (29, 50). The polymorphism of ADD1 (G460W/S586C) alone (or in combination with those of ADD2) and angiotensin-converting enzyme is associated with either essential hypertension (18, 45, 78) or with the related cardiovascular and renal complications (51, 54, 60, 70, 75).

The Milan hypertensive strain (MHS) of rats is a genetic model of hypertension in which cardiovascular phenotypes seem to be dependent, at least in part, on adducin gene polymorphisms (11, 62). In the MNS × MHS F2 hybrid population, the mutation of the Add1 gene accounts for the 50% of the blood pressure (BP) difference between MHS and its normotensive counterpart (MNS) (9, 11). Adducin polymorphisms are linked with higher Na⁺ pump activity and enhanced constitutive tubular Na⁺ reabsorption in the kidney in both rats and humans (22, 26, 27, 47, 66). Sodium retention should cause a plasma volume expansion and an increased cardiac output (17, 34). In the chronic phase, however, plasma volume and cardiac output are usually normal, and the elevated BP is the result of a blood flow-induced compensatory shift (whole body autoregulation) to increased vasoconstriction and total peripheral vascular resistance (17, 34). A relationship between the alteration in the adducin genes and renal dysfunction has been extensively studied (8, 18, 46, 64, 78), but the mechanisms involved in cardiovascular functional abnormalities in MHS rats are still unclear.

Chronic hypertension is typically associated with increased peripheral vascular resistance (65). This is due, in part, to enhanced arterial smooth muscle contractility, which is regulated by intracellular Ca²⁺ influx through PM store- and receptor-operated Ca²⁺ channels (SOCs and ROCs, respectively), along with voltage-gated Ca²⁺ channels, may play a role in...
regulating myogenic tone and vasoconstriction (30, 53, 67, 73). Transient receptor potential canonical (TRPC)1, TRPC4, and/or TRPC5 proteins, mammalian homologs of the Drosophila transient receptor potential channel, form the endogenous SOCs that are activated by sarcoplasmic reticulum (SR) Ca\(^{2+}\) depletion (6, 57, 76, 77). In contrast, TRPC3 and TRPC6, which are components of ROCs, can be activated by diacylglycerols in a store depletion-independent manner (36). Several reports indicate that Ca\(^{2+}\) homeostasis in arterial smooth muscle cells (ASMCs) is influenced not only by direct Ca\(^{2+}\) entry through TRPC channels but also by Na\(^+\) entry through these nonselective cation channels. The entering Na\(^+\) apparently then also promotes Ca\(^{2+}\) entry through Na\(^+\)/Ca\(^{2+}\) exchanger type 1 (NCX1) (4, 23, 58, 82).

TRPC channels (28, 43, 61) and NCX1 (39, 61, 82) have been implicated in the pathogenesis of various forms of hypertension. For example, TRPC1 and TRPC6 are involved in hypoxic pulmonary hypertension (42, 72). TRPC6 (79) and NCX1 (82) are both implicated in human primary pulmonary hypertension. We demonstrated that upregulated TRPC1, TRPC6, and NCX1 are involved in the augmented Ca\(^{2+}\) signaling in mesenteric artery myocytes from ouabain hypertensive rats (61).

Recent findings indicate that adducin polymorphisms are associated not only with renal dysfunction (18, 64, 78) but also with functional alterations in rat mesenteric small resistance arteries (62). Augmented Ca\(^{2+}\) signaling plays a key role in the vascular dysfunction associated with arterial hypertension. Here we explore the mechanisms responsible for altered Ca\(^{2+}\) signaling in MHS mesenteric artery myocytes. Our data demonstrate that upregulated NCX1 and TRPC6 contribute to the augmented Ca\(^{2+}\) signaling in ASMCs from MHS rats.

**METHODS**

Ethical approval. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

Experimental animals. MHS and MNS rats were obtained from Prassis Sigma Tau Research Institute (Milan, Italy) and bred in the Animal Care Facility of the University of Maryland, School of Medicine. Animals were maintained in a temperature- and humidity-controlled room with a 12-h:12-h light-dark cycle. Rats had free access to tap water and were fed standard rat chow ad libitum. Body weight was measured weekly. Genomic DNA was obtained from tail biopsies for genotyping by PCR. We studied male MHS and MNS rats at 10–12 wk of age. Rats were acclimated to the procedures of BP measurement for a week preceding actual data collection. After acclimation to the conditioning of BP measurements, baseline data were obtained over 1 to 2 wk. Systolic and mean BPs in MHS and age-matched MNS controls were recorded by tail-cuff plethysmography using a commercial photoelectric system (model 29 blood pressure meter/amplifier; IITC, Woodland Hill, CA) and a device providing constant rates of cuff inflation and deflation. The average values for BP in each rat were typically obtained from five sequential cuff inflation/deflation cycles.

Dissection of arteries for immunoblotting. The superior mesenteric artery and aorta from a euthanized rat were rapidly removed and inflation/deflation cycles. The imaging system included a Zeiss Axiovert 100 microscope (Carl Zeiss, Thornwood, NY). The dye-loaded cells were illuminated with a two-photon microscope (Feinberg Microscopy Systems) in the presence of a confocal image converter (Model 4050, Hamamatsu). The ratio of fura-2 fluorescent emission (510 nm) = 200 nm) was calculated from the ratio of fura-2 fluorescent emission (510 nm) excited at 380 and 360 nm as previously described (7). Intracellular
Fura-2 was calibrated in situ in freshly dissociated ASMCs (7). Intracellular 

\[ \text{Ba}^{2+} \] measurements are shown as fura-2 340/380 excitation ratios with fluorescent emission at 510 nm (7).

**Immunoblotting.** Membrane proteins were solubilized in sodium dodecyl sulfate buffer containing 5% 2-mercaptoethanol and were separated by polyacrylamide gel electrophoresis as previously described (7). The following antibodies were used: rabbit polyclonal anti-TRPC1, anti-TRPC3, anti-TRPC5, and anti-TRPC6 (dilution 1:200); Alomone; rabbit polyclonal anti-TRPC4 (dilution 1:300); Alomone; mouse monoclonal anti-NCX1 (dilution 1:500; R3F1; Swant, Bellinzona, Switzerland); rabbit polyclonal anti-

\[ \text{Na}^+ \] pump \( \alpha_1 \)-subunit, isoform (dilution 1:200; gift of Dr. Thomas Pressley); rabbit polyclonal anti-

\[ \text{Na}^+ \] pump \( \alpha_1 \)-subunit isofor (dilution 1:750; Millipore, Billerica, MA); mouse monoclonal anti-PMCA pump 1 (dilution 1:500; Affinity Bioreagents). Gel loading was controlled with anti-rabbit horseradish peroxidase-conjugated IgG for 1 hour at room temperature. The immune complexes on the membranes were detected by enhanced chemiluminescence plus (Amersham Biosciences, Piscataway, NJ) and exposure to X-ray film (Eastman Kodak, Rochester, NY).

**Quantitative analysis of immunoblots** was performed by using a Kodak (dilution 1:1,500; Affinity Bioreagents). Gel loading was controlled with anti-rabbit horseradish peroxidase-conjugated IgG for 1 hour at room temperature. The immune complexes on the membranes were detected by enhanced chemiluminescence plus (Amersham Biosciences, Piscataway, NJ) and exposure to X-ray film (Eastman Kodak, Rochester, NY). Quantitative analysis of immunoblots was performed by using a Kodak DC120 digital camera and 1D Image Analysis Software (Eastman Kodak).

**Immunocytochemistry.** ASMCs were immunolabeled, as previously described (32). Briefly, the cells were fixed in cyclohexylamine-formaldehyde fixative consisting of 0.45% (wt/vol) formaldehyde, 75 mM cyclohexylamine, 75 mM NaCl, 10 mM EGTA, 10 mM MgCl₂, and 10 mM PIPES. After fixation, the cells were permeabilized in fixative containing 0.5% polyoxyethylene 20 cetyl ether (Brij 58) and were then incubated (4–17 h) in antibody buffer containing antibodies against TRPC6 (dilution 1:10; Alomone) and NCX1 (dilution 1:10; clone R₃F₁; Swant). FITC-labeled donkey anti-mouse IgG or Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) were used to visualize the primary antibodies. The fluorescence from the secondary antibody in the absence of primary antibody (positive control) did not exceed 2 to 3% of the fluorescence in the presence of antiserum. To identify SR in arterial myocytes, the cells were treated (5 min) with 1 \( \mu \text{M} \) ER-Tracker (Invitrogen Detection Technologies, Eugene, OR).

**Materials.** Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA). All other tissue culture reagents were obtained from GIBCO-BRL (Grand Island, NY). Fura-2 AM and DAPI were obtained from Molecular Probes (Invitrogen Detection Technologies). 1-Oleoyl-2-acetyl-sn-glycerol (OAG) was purchased from Calbiochem (San Diego, CA). Collagenase type 2 was obtained from Worthington Biochemical (Freehold, NJ). ATP, dimethylsulfoxide, \( \beta \)-actin, smooth muscle \( \alpha \)-actin, collagenase type XL elastase type IV, bovine serum album, nifedipine, ionomycin, penicillin G, and streptomycin were purchased from Sigma-Aldrich. All other reagents were analytic grade or the highest purity available.

**Statistical analysis.** The numerical data presented in RESULTS are means ± SE from \( n \) single cells (1 value per cell). Immunoblots were repeated at least four to six times for each protein. The number of animals is presented where appropriate. Data from 6 to 18 rats were obtained for most protocols. Statistical significance was determined
RESULTS

Abnormal Ca\(^{2+}\) homeostasis in freshly dissociated ASMCs from MHS rats. MNS rats (control group) had a mean systolic BP of 113 ± 1 mmHg (n = 16); the systolic BP of the MHS rats was significantly higher (144 ± 2 mmHg, n = 10; P < 0.05) (Fig. 1A). Freshly dissociated mesenteric artery myocytes from the MHS rats had significantly higher resting [Ca\(^{2+}\)]\(_{cys}\) than did MNS myocytes (97 ± 2 vs. 78 ± 3 nM, P < 0.001; Fig. 1, B–D). Furthermore, activation by 5 μM ATP in physiological media induced augmented Ca\(^{2+}\) signals in the MHS ASMCs. Both the peak initial response, believed to be the result of inositol 1,4,5-trisphosphate-mediated SR Ca\(^{2+}\) release (15), and the later plateau, perhaps mediated by Ca\(^{2+}\) entry through ROCs and/or SOCs, were greater in MHS than in MNS rat ASMCs (Fig. 1, B and C). The plateau was frequently accompanied by low-amplitude [Ca\(^{2+}\)]\(_{cys}\) fluctuations in both MHS and MNS arterial myocytes (Fig. 1B). Superimposed records of the ATP-induced Ca\(^{2+}\) response show that the integral of the rise of [Ca\(^{2+}\)]\(_{cys}\) (area under the [Ca\(^{2+}\)]\(_{cys}\) curve) in MHS arterial myocytes was increased 184 ± 8% of the area in MNS ASMCs (n = 28 MNS cells; 2.4 MHS cells, P < 0.05). To eliminate the contribution of extracellular Ca\(^{2+}\), the experiments were repeated in Ca\(^{2+}\)-free medium. Under these circumstances, the peak amplitudes of ATP-induced Ca\(^{2+}\) transients were also greater in MHS than in MNS rat ASMCs (643 vs. 438 nM, P < 0.05, n = 56 cells). In subsequent experiments, we investigated the role of ROCs and SOCs that can contribute to the altered Ca\(^{2+}\) homeostasis and augmented signaling in ASMCs from MHS rats.

Increased ROC-mediated Ca\(^{2+}\) entry and augmented expression of TRPC6 in MHS rat artery smooth muscle. To determine whether ROC-mediated Ca\(^{2+}\) entry (ROCE) is indeed increased in mesenteric ASMCs from MHS rats, freshly dissociated myocytes were stimulated with OAG, the cell-permeable diacylglycerol analog. OAG opens TRPC3 and TRPC6 channels in a protein kinase C-independent manner (36). SOCs have high-Ca\(^{2+}\) selectivity and, unlike ROCs, are virtually impermeable to other alkaline-earth cations, such as Ba\(^{2+}\) (69). Therefore, to distinguish ROCs from SOCs, we measured Ba\(^{2+}\) entry. Ba\(^{2+}\) is not transported by SERCA or PM Ca\(^{2+}\) pumps (41). In the presence of extracellular Ba\(^{2+}\), 100 μM OAG induced significantly larger elevations of cytosolic Ba\(^{2+}\) (fura-2 340/380nm ratio; see METHODS) in myocytes from MHS rats than in those from normotensive rats (Fig. 2, A and B). To eliminate the contribution of voltage-gated Ca\(^{2+}\) channels to OAG-induced Ba\(^{2+}\) entry, all solutions in the experiments contained 10 μM nifedipine. At this concentration, nifedipine blocks not only L-type but also T-type voltage-gated Ca\(^{2+}\) channels (2). The implication is that ROC activity is augmented in MHS arterial myocytes. Whether this was simply due to an increased entry through an unchanged number of channels or to an increase in the number of channels available was tested by immunoblot analysis. The results reveal that the increased ROCE correlates with the approximately threefold augmentation of TRPC6 expression in denuded mesenteric artery (Fig. 2, C and D) and aorta (not shown). In contrast, the expression of TRPC3, which also belongs to the TRPC3/6/7 subfamily of diacylglycerol-activated ROCs, was not significantly different in the MHS arteries (Fig. 2, E and F); messenger RNA for TRPC7 was not detected in rat mesenteric artery smooth muscle (35) and, therefore, was not studied. Notably, the expression of TRPC1 and TRPC5, which are believed to form subunits of SOCs (6), was not altered in ASMCs from MHS rats (Fig. 3, A–D). TRPC4, also a protein component of SOCs in other cell types, is not expressed in rat mesenteric arteries (7). Augmented ATP-induced Ca\(^{2+}\) response in MHS ASMCs (Fig. 1, B and C) can result not only from increased ROCE (Fig. 2, A and B) but also from increased Ca\(^{2+}\) release from augmented SR Ca\(^{2+}\) stores. Indeed, upregulated SERCA2 expression (Fig. 3, E and F) implies that SR Ca\(^{2+}\) concentration is higher in MHS arterial myocytes than in MNS ASMCs.

Fig. 2. Augmented 1-oleoyl-2-acetyl-sn-glycerol (OAG)-induced [receptor-operated channel (ROC) mediated] Ba\(^{2+}\) entry and transient receptor potential canonical 6 (TRPC6) protein expression in freshly dissociated ASMCs from MHS rats. A: representative records show the time course of the fura-2 fluorescence ratio (F\(_{340}/F_{380}\)) signals induced by 100 μM OAG in freshly dissociated ASMCs from MNS and MHS rats. Extracellular Ca\(^{2+}\) was replaced by 1 mM Ba\(^{2+}\) during the period indicated on the graph. Nifedipine (10 μM) was applied 10 min before the trace shown and was maintained throughout the experiment. B: summarized data show the OAG-induced, ROC-mediated Ba\(^{2+}\) entry in 31 MNS and 42 MHS rat mesenteric ASMCs. Each bar corresponds to data from 8 rats. C–F: Western blot analysis of TRPC6 and TRPC3 expression in ASMCs from MNS and MHS rats. C and E: representative immunoblots (30 μg protein/lane). Summarized data from 4 (D) and 5 (F) immunoblots (total of 18 rats) are normalized to β-actin. *P < 0.05 and ***P < 0.001 vs. MNS ASMCs.
Na⁺ pumps in MHS rat artery smooth muscle. Recently, we demonstrated that the expression of the highly ouabain-sensitive α₂ Na⁺ pump isoform is ~2.5-fold higher in mesenteric arteries of ouabain hypertensive rats versus their normotensive controls, whereas the expression of α₁ Na⁺ pumps, which have very low affinity for ouabain (55), was not altered (61). The Na⁺ pump α₂-subunits control myogenic tone and BP in ouabain-induced hypertension (21, 81). Furthermore, the genetic alteration in the adducin genes in rats and humans is associated with the upregulation of renal Na⁺-K⁺-ATPase; this might be implicated in abnormal Na⁺ reabsorption and high BP (22, 26, 27, 66). The aforementioned findings suggest that the expression of α₂ and possibly α₁ Na⁺ pumps might be altered in deendothelialized mesenteric arteries of MHS rats. Figure 4 shows that neither the α₂ nor α₁ Na pump subunits are upregulated in MHS arterial myocytes.

Upregulated NCX in MHS rat artery smooth muscle. The expression of TRPC6 and NCX1 in rat ASMCs is interrelated (61). If TRPC6 is upregulated in MHS mesenteric arteries, it is logical to ask whether NCX1 is also upregulated. Figure 5, A and B, shows that the removal of extracellular Na⁺ [conditions that favor Na⁺ extrusion and Ca²⁺ entry via NCX1 (12)] induced a rapid increase in [Ca²⁺]_{cyt}. The increase was significantly larger in MHS mesenteric artery myocytes (404 ± 28 nM, n = 26) compared with that in MNS rats (309 ± 25 nM, n = 33). This was associated with a robust upregulation of NCX1 expression in MHS mesenteric artery (Fig. 5, C and D). Notably, the upregulation of NCX1 was dramatically greater in MHS mesenteric artery (~13-fold) than in aorta (~3-fold) (Fig. 5, C–F). The expression of PM Ca²⁺-ATPase (PMCA) was not altered in mesenteric arteries (Fig. 5, G and H) or aortas from MHS rats (not shown). Interestingly, the expression of NCX1 substantially varies in different vascular beds. As shown in Fig. 6, in the MNS control rats, NCX1 expression was ~15-fold higher in the aorta than in the mesenteric artery smooth muscle, whereas there was no difference in PMCA and TRPC6 expression in these arteries (not shown).

Expression of NCX1 and TRPC6 in primary cultured MHS mesenteric artery myocytes. The aforementioned changes in NCX1 and ROCs (TRPC6) in MHS mesenteric artery smooth muscle in vivo might be the result of adducin polymorphisms or, alternatively perhaps, the consequence of the elevated BP or some other in vivo factor(s), such as the level of endogenous ouabain that is elevated in MHS rats (24, 25). To explore this issue, we tested the expression of NCX1 and TRPC6 in primary cultured MHS mesenteric artery myocytes. Figure 7 shows that NCX1 and TRPC6 were marginally, but not significantly, upregulated in cultured MHS arterial myocytes when compared with ASMCs from MNS rats.

NCX1 and TRPC6 are localized in the PM in close proximity. As already noted, NCX1 and TRPC6 in rat ASMCs are functionally associated (61). This raises the possibility of their close proximity in the PM. Immunocytochemistry was used to elucidate the relationship between the specific location of NCX1 and TRPC6 proteins in the PM of cultured ASMCs from MNS and MHS rats. High-power images of a portion of an MNS ASMC show that the NCX1 labeling pattern (Fig. 8A, inset) is remarkably similar to the pattern observed with antibodies directed against the TRPC6 (Fig. 8B, inset). Indeed, when Fig. 8C, a (green) is overlaid on Fig. 8C, b (red), an extensive overlap of the labels is observed (Fig. 8C, c), as indicated by the large amount of yellow in the image. Both NCX1 and TRPC6 labels are distributed in a distinct reticular pattern that parallels the organization of the underlying ER.
Tracker-stained SR (Fig. 8 inset). This reticular pattern indicates that clusters of NCX1 and TRPC6 in the PM are organized around the underlying SR. Notably, reactivity was not detected in the PM in the absence of the primary anti-NCX1 (Fig. 8E) or anti-TRPC6 (not shown) antibodies. A similar distribution of NCX1 and TRPC6 was observed in arterial myocytes from MHS rats (not shown).

**DISCUSSION**

Arterial hypertension in MHS rats has been associated with functional alterations of small resistance arteries including increased contractility (62). As intracellular Ca\(^{2+}\) plays a fundamental role in the genesis and maintenance of arterial tone (39, 65, 81), alterations of vascular Ca\(^{2+}\) signaling might be pivotal events for the development and maintenance of adducin-dependent hypertension. The present report describes the first study of some of the key mechanisms involved in the

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**Fig. 5.** Enhanced Ca\(^{2+}\) entry via the reverse mode of Na\(^+/Ca^{2+}\) exchanger-1 (NCX1) and augmented NCX1 expression in freshly dissociated myocytes from MHS rats. A and B: activation of the reverse mode of NCX1 in ASMCs from MNS (black) and MHS (gray) rats. A: representative time course records showing changes in [Ca\(^{2+}\)]\(_{cyt}\) in single ASMCs; time of treatment with Na\(^+\)-free solution is indicated. Nifedipine (10 \(\mu\)M) was added 10 min before the records shown and was maintained throughout the experiment. B: summarized data show the NCX1-mediated Ca\(^{2+}\) entry in 33 MNS and 23 MHS mesenteric ASMCs. *P < 0.05 vs. MNS arterial myocytes. 

C–H: Western blot analysis of NCX1 (C–F) and plasma membrane Ca\(^{2+}\)-ATPase (PMCA; G and H) protein expression (30 µg/lane) in smooth muscle cell membranes from mesenteric arteries (C, D, G, and H) and aortas (E and F) of MNS and MHS rats. C, E, and G: representative blots. Summary data (D, F, and H) are normalized to the amount of β-actin and are expressed as means ± SE from 9 (D), 18 (F), and 5 (H) immunoblots (total of 22 rats). ***P < 0.001 vs. MNS ASMCs.

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**Fig. 6.** NCX1 expression in deendothelialized mesenteric arteries (MAs) and aortas of control normotensive rats. A: representative immunoblot (35 µg protein/lane). B: summarized data from 5 immunoblots (total of 8 rats) are normalized to β-actin. Aorta = ***P < 0.001 vs. mesenteric artery.

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**Fig. 7.** Expression of NCX1 and TRPC6 in primary cultured mesenteric artery myocytes from MNS and MHS rats. A and C: representative immunoblots (30 µg protein/lane). B and D: summarized data from 5 (B) and 4 (D) immunoblots are normalized to β-actin.
Arterial myocyte Na\(^+\) and Ca\(^{2+}\) metabolism in the MHS rat model of hypertension. The results show there is a pronounced expression of NCX1 and TRPC6 and remodeling of Ca\(^{2+}\) signaling in Milan rats carrying the hypertensive adducin phenotype. The identity of the specific transport proteins involved and their direct role in cellular Ca\(^{2+}\) homeostasis is altered in MHS rat arterial myocytes.

\[\text{Ca}^{2+}\text{ homeostasis is altered in MHS rat arterial myocytes.}\]

Arterial contractility is augmented in many forms of hypertension (13, 21, 81), including adducin-dependent hypertension (62). Some evidence points to dynamic increases in contraction that may be associated directly with Ca\(^{2+}\) dysregulation, such as enhanced responses to vasoconstrictors (13, 62, 80).

Based on the evidence for altered arterial function in MHS rats, we studied aspects of Ca\(^{2+}\) homeostasis in ASMCs from the MHS and MNS rats. Indeed, freshly dissociated myocytes from MHS exhibit Ca\(^{2+}\) dysregulation: elevated resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) and augmented ATP-induced Ca\(^{2+}\) signals (Fig. 1, B–D). The MHS myocytes also exhibit increased ROCE (Fig. 2, A and B). Furthermore, immunoblots indicated that the latter effect is a consequence of upregulated TRPC6 protein expression (Fig. 2, C and D). The expression of other TRPCs such as TRPC3 (Fig. 2, E and F), as well as TRPC1 and TRPC5 (Fig. 3, A–D), was not significantly different in the MHS rats. The upregulation of TRPC6 is thus specific and, when taken with the data showing increased ROCE, is consistent with the augmented norepinephrine-evoked vasoconstriction of intact mesenteric small resistance arteries from MHS rats, especially at high norepinephrine concentrations (62). The increased basal [Ca\(^{2+}\)]\(_{\text{cyt}}\), as well as the increased Ca\(^{2+}\) influx through TRPC6-encoded ROCs (20, 37, 73), is likely to contribute to the augmented myogenic reactivity of MHS rat arteries. Indeed, ouabain-induced hypertension (61), mineralocorticoid-salt hypertension (5), and human idiopathic pulmonary hypertension (79) are all associated with a marked upregulation of TRPC6. Thus the biochemistry and physiology appear to be directly linked, and the upregulation of TRPC6 can provide a molecular explanation for the observed augmentation of vascular contractile responses in hypertension (13, 62, 80). Vascular structural remodeling and artery narrowing (49, 52) and increased arterial stiffness (14) have also been linked with sustained increases in peripheral vascular resistance. Recent findings, however, revealed that the structure of MHS mesenteric small resistance arteries is not altered when compared with arteries from MNS rats (62).

It is noteworthy that adducin-dependent hypertension in rats has been associated with elevated plasma levels of an endogenous ouabain-like factor (24, 25). Indeed, the plasma concentration of endogenous ouabain is twofold greater in MHS rats (0.565 ± 0.06 nM, n = 17) than in MNS rats (0.284 ± 0.05 nM, n = 15, P < 0.01) (24). This begs the question, Is the expression of α3 Na\(^+\) pump and NCX1, both of which are upregulated in ouabain hypertensive rats (61), also augmented in the arteries of MHS rats? Here we demonstrate that the expression of α2 and α1 Na\(^+\) pump subunits is not changed (Fig. 4). The absence of upregulated Na\(^+\) pump abundance implies that either the elevated circulating ouabain-like factors, previously reported in MHS (25), were mitigated or that some other factor is involved. In addition, our results do not exclude the possibility that the activity of the Na\(^+\) pumps in MHS arteries might be increased. NCX1 is greatly upregulated in both deendothelialized mesenteric artery (Fig. 5, C and D) and aorta (Fig. 5, E and F) from MHS rats. Interestingly, the upregulation of NCX1 is more pronounced in MHS mesenteric artery than in aorta. This may be important because resistance arteries develop pressure-induced tone and thus play a major role in the maintenance of arterial BP (19), whereas aorta is primarily considered to be a conduit blood vessel.

The mechanism(s) by which the upregulation of TRPC6 and NCX1 occurs is (are) not yet understood. PM ion transporters and channels can be tethered to the membrane cytoskeleton (29, 50). For example, a direct interaction between human TRPC4 and the spectrin cytoskeleton is involved in the regulation of human TRPC4 surface expression and activation (56). Moreover, NCX activity is modulated by changes in the actin cytoskeleton (16). Similarly, a modulation of actin-spectrin assembly by adducin polymorphisms can affect turnover and the incorporation of PM ion transporters and channels, including NCX1, TRPC6, and Na\(^+\) pumps. In fact, mutant adducin variants lead to a lowering of endocytosis of Na\(^+\) pump and thereby an increase in the number of basolateral Na\(^+\) pumps in kidney epithelial cells (22, 68). It is not clear whether TRPC6 and NCX1 in arteries of MHS rats are simultaneously affected or whether an upregulation of one of them affects the expression of the other protein. As the expression of NCX1 and TRPC6 in rat ASMCs is highly interrelated (61), a mutual interaction between the regulation of Ca\(^{2+}\) homeostasis by NCX1 and the regulation of Ca\(^{2+}\) (and Na\(^{+}\)) entry through ROCs, which are nonselective cation channels, is clearly implied (3, 20, 38). Regardless of the mechanism(s) involved in their upregulation, both transporters may play an important role in the maintenance of arterial BP.

Fig. 8. Immunofluorescent localization of NCX1 and TRPC6 in primary cultured mesenteric artery myocytes from normotensive rat. A–D: images of cell triple labeled with anti-NCX1 antibody (A), anti-TRPC6 antibody (B), and ER-Tracker (D). Insets in A, B, and D (enlargements of boxed areas): NCX1, TRPC6, and SR labels show similar distribution. Ca\(a\) and \(b\): pseudocolor images (green, anti-NCX1; red, anti-TRPC6) of enlarged boxes from A and B, respectively. Ca\(c\): colocalization of NCX1 (\(a\)) and TRPC6 (\(b\)) staining; yellow/orange, and yellow/green areas in overlay indicate regions of overlap between the 2 epitopes. E: fluorescence detected from the secondary antibody (Cy3) in the absence of primary anti-NCX1 antibody (control). Scale bar = 50 μm (A and E). N, nucleus. Similar results were obtained in 12 ASMCs.
role in the development and/or maintenance of hypertension (5, 39, 43, 79). This concept is further underscored in ouabain-induced hypertension (61) and in human primary pulmonary hypertension, where NCX1 (82) and TRPC6 (79) are both upregulated.

NCX1 and TRPC6 in primary cultured MHS mesenteric artery myocytes. Another key observation made in this study is that NCX1 and TRPC6 expression is not significantly augmented in primary cell cultures of MHS arterial myocytes when compared with ASMCs from MNS rats. The results imply that the upregulation of NCX1 and TRPC6 in MHS rats in vivo is not directly triggered by adducin polymorphisms per se, because cultured cells from each of the strains continue to express their respective adducins. Thus we suggest that the differences in the in vivo expression of these transporters may be triggered and maintained by the elevated level of endogenous ouabain in MHS rats or other neurohumoral factors that are absent in the cell culture environment. Consistent with this idea, we have shown that prolonged (72 h) treatment of cultured myocytes from control normotensive rats with nanomolar ouabain significantly upregulates NCX1 and TRPC6 (61). While we cannot entirely exclude the possibility that increases in BP trigger the changes in NCX1 and TRPC6 expression, the aforementioned observations suggest that endogenous ouabain likely accounts for most of the increase in NCX1, TRPC6, and BP in the MHS rat.

The functional interaction of NCX1 and TRPC6 in ASMCs (61) raises the possibility of their clustering in the PM-junctional SR regions. Notably, NCX1 (40) and TRPC channels (32) are confined to the PM microdomains that overlie the closely apposed junctional SR. Moreover, coimmunoprecipitation experiments provide evidence for the association of NCX1 with TRPC3 in protein complexes in HEK293 cells (63). In the present study, immunocytochemistry with anti-TRPC6 and anti-NCX1 antibodies revealed the close proximity of TRPC6 and NCX1 proteins in the PM-junctional SR regions of the mesenteric ASMC (Fig. 8). These findings show that PM microdomains which include TRPC6-containing channels and NCX1 function as integrated units help to regulate Ca\(^{2+}\) signals in vascular smooth muscle cells. The buffering of NCX1-mediated Ca\(^{2+}\) entry in the PM-junctional SR regions by the SR and mitochondria limits its diffusion into the cytosol (59). As a result, the estimated transient increase in the sub-PM Ca\(^{2+}\) concentration upon the substitution of extracellular Na\(^+\) by NMDG in rat ASMCs is 13-fold greater than the observed increase in [Ca\(^{2+}\)]\(_{cyt}\) (59). In this regard also, it is noteworthy that the expression of SERCA2 is ~2.5-fold higher in arterial myocytes from MHS rats than in MNS ASMCs (Fig. 3, E and F). The large effect of multiorganelle buffering therefore can explain the relatively modest difference in changes of bulk [Ca\(^{2+}\)]\(_{cyt}\) in response to Na\(^+\)-free solution in ASMCs from MHS and MNS rats despite a robust upregulation of NCX1 in MHS arteries (Fig. 5, A–D).

In summary, this report provides the first evidence for the upregulation of specific molecular mechanisms and augmented Ca\(^{2+}\) signaling in ASMCs from MHS rats. The alterations in Ca\(^{2+}\) entry via the reverse mode of NCX1 and augmented ROC-mediated Ba\(^{2+}\) entry in MHS arterial myocytes are highly consistent with the upregulation of NCX1 and TRPC6 expression, respectively. Similar alterations in Ca\(^{2+}\) signaling and an upregulation of NCX1 and TRPC6 have been observed in ouabain-induced hypertension (61) and in human primary pulmonary hypertension (79, 82). Thus the aforementioned findings indicate that the upregulation of these two transporters is intrinsic to many forms of hypertension. Therefore, the results of this study are likely to be relevant not only to those patients with essential hypertension harboring specific adducin polymorphisms but also to a broader population of patients with hypertension.

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DISCLOSURES
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