Increased arterial smooth muscle Ca\(^{2+}\) signaling, vasoconstriction, and myogenic reactivity in Milan hypertensive rats

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Submitted 26 September 2011; accepted in final form 28 November 2011

Linde CI, Karashima E, Raina H, Zulian A, Wier WG, Hamlyn JM, Ferrari P, Blaustein MP, Golovina VA. Increased arterial smooth muscle Ca\(^{2+}\) signaling, vasoconstriction, and myogenic reactivity in Milan hypertensive rats. Am J Physiol Heart Circ Physiol 302: H611–H620, 2012. First published December 2, 2011; doi:10.1152/ajpheart.00950.2011.—The Milan hypertensive strain (MHS) rats are a genetic model of hypertension with adducin gene polymorphisms linked to enhanced renal tubular Na\(^{+}\) reabsorption. Recently we demonstrated that Ca\(^{2+}\) signaling is augmented in freshly isolated mesenteric artery myocytes from MHS rats. This is associated with greatly enhanced expression of Na\(^{+}\)/Ca\(^{2+}\) exchanger-1 (NCX1), C-type transient receptor potential (TRPC6) protein, and sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) compared with arteries from Milan normotensive strain (MNS) rats. Here, we test the hypothesis that the enhanced Ca\(^{2+}\) signaling in MHS arterial smooth muscle is directly reflected in augmented vasoconstriction [myogenic and phenylephrine (PE)-evoked responses] in isolated mesenteric small arteries. Systolic blood pressure was higher in MHS (145 ± 1 mmHg) than in MNS (112 ± 1 mmHg; P < 0.001; n = 16 each) rats. Pressurized mesenteric resistance arteries from MHS rats had significantly augmented myogenic tone and reactivity and enhanced constriction to low-dose (1–100 nM) PE. Isolated MHS arterial myocytes exhibited approximately twofold increased peak Ca\(^{2+}\) signals in response to 5 μM PE or ATP in the absence and presence of extracellular Ca\(^{2+}\). These augmented responses are consistent with increased vasoconstrictor-evoked sarcoplasmic reticulum (SR) Ca\(^{2+}\) release and increased Ca\(^{2+}\) entry, respectively. The increased SR Ca\(^{2+}\) release correlates with a doubling of inositol 1,4,5-trisphosphate receptor type 1 and tripling of SERCA2 expression. Pressurized MHS arteries also exhibited a ~70% increase in 100 nM ouabain-induced vasoconstriction compared with MNS arteries. These functional alterations reveal that, in a genetic model of hypertension linked to renal dysfunction, multiple mechanisms within the arterial myocytes contribute to enhanced Ca\(^{2+}\) signaling and myogenic and vasoconstrictor-induced arterial constriction. MHS rats have elevated plasma levels of endogenous ouabain, which may initiate the protein upregulation and enhanced Ca\(^{2+}\) signaling. These molecular and functional changes provide a mechanism for the increased peripheral vascular resistance (whole body autoregulation) that underlies the sustained hypertension.

adducin; ouabain; myogenic tone; hypertension; Milan normotensive rats

PRIMARY (ESSENTIAL) HYPERTENSION is a multifactorial disorder that leads to severe cardiovascular and renal complications (39). Hypertension is caused by the complex interplay between genetic predisposition (genetic heritability ~30%) and multiple environmental factors, including excess dietary salt (1, 46, 70). One major difficulty in identifying genes contributing to hypertension is the etiological heterogeneity of hypertension (1). Polymorphisms in genes encoding proteins involved in the renin-angiotensin-aldosterone system and volume and/or Na\(^{+}\) homeostasis have been the most extensively studied (1). Accumulating evidence indicates that alterations in the genes encoding adducin, a cytoskeleton protein, are associated with enhanced salt retention by the kidneys and hypertension in rats and humans (8, 17, 49, 68).

The Milan hypertensive strain (MHS) of rats is a genetic model of hypertension in which cardiovascular phenotypes apparently depend, at least in part, on adducin gene polymorphisms (11). In the Milan normotensive strain (MNS) × MHS F2 hybrid population, mutation of the Add1 gene accounts for ~50% of the blood pressure (BP) difference between MHS and its MNS counterpart (10, 11). Adducin polymorphisms have been linked to increased renal Na\(^{+}\),K\(^{+}\)-ATPase activity and enhanced constitutive renal tubular Na\(^{+}\) reabsorption in both rats and humans (23, 27–29, 51, 67). A transient phase of increased salt retention in MHS rats, due to the augmented renal Na\(^{+}\),K\(^{+}\)-ATPase activity, is observed in the prehypertensive phase (9). Moreover, after the development of hypertension MHS rats exhibit upregulation of renal apical Na\(^{+}\)-Cl\(^{-}\) cotransporter and basolateral Cl\(^{-}\) channels (15). Thus the MHS rat may be a good model for a salt-dependent form of hypertension. The relationship between the alteration in the adducin genes and the renal dysfunction has been extensively studied (10, 17, 27, 48, 65, 71). The possible vascular functional abnormalities that can contribute to the elevated BP in MHS rats have, however, been largely ignored.

Ca\(^{2+}\) homeostasis plays a crucial role in the genesis of vascular myogenic tone, and increases in arterial smooth muscle cell (ASMC) Ca\(^{2+}\) signaling appear to underlie at least part of the increased peripheral vascular resistance in hypertension (66, 75). Recently we demonstrated that resting cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)) and Ca\(^{2+}\) entry via receptor-operated channels (ROCs) and Na\(^{+}\)/Ca\(^{2+}\) exchanger-1 (NCX1) are augmented in freshly dissociated mesenteric artery myocytes from MHS rats (79). This is associated with greatly upregulated expression of several cation transport proteins including: C-type transient receptor potential protein (TRPC6; ~3-fold), an essential component of ROCs [nonselective cation channels that admit Na\(^{+}\) and Ca\(^{2+}\) (56)], NCX1 (~13-fold), and SERCA2 (~3-fold) in MHS mesenteric arteries, compared with MNS arteries (79). Ouabain, administered either in vivo or in vitro, also increases expression of these proteins (59). Furthermore, MHS rats have elevated plasma levels of endogenous ouabain (EO) (25, 26), an adrenocortical hormone (34) and a Na\(^{+}\)...
pump inhibitor. The implication is that the augmented expression of these Na⁺ and Ca²⁺ transporters in MHS arteries (79) might be triggered by the high plasma EO. This is important because NCX1 normally mediates Ca²⁺ entry, rather than exit, in ASMs of arteries with tone (36). Upregulation of NCX1 should therefore tend to accelerate Ca²⁺ entry and promote net Ca²⁺ gain. The increased ROC (TRPC6) and NCX1 expression and Ca²⁺ signaling in MHS arterial myocytes should enhance myogenic responses and agonist-induced vasoconstriction. This, however, has not been tested.

Here, using the MHS model, we tested the hypothesis that enhanced Na⁺ reabsorption by the kidneys is linked to 1) altered arterial Ca²⁺ transport and signaling proteins, 2) augmented small artery tone and evoked constriction, and, thus, 3) BP elevation. The results revealed augmented myogenic reactivity and phenylephrine (PE)-induced vasoconstriction in MHS arteries. This altered arterial function likely contributes to the increased total peripheral vascular resistance (TPR) and elevated BP in MHS rats. Our results contradict the view that, in hypertension, “functional changes in the vasculature have been looked for but have in general not been found, with the exception of a possible endothelial dysfunction” (55).

METHODS

Ethical approval. All experiments were carried out according to the guidelines of and 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); they were 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. 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 tail-cuff BP measurements for a week preceding actual data collection. Systolic 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) that provided constant rates of cuff inflation and deflation as described (79).

Measurements of arterial function. The rats were euthanized by CO₂ overdose followed by decapitation. The mesenteric arcade from a euthanized rat was rapidly removed and placed in ice-cold physiological salt solution 1 (PSS1) with the following composition (in mM): 140 NaCl, 5.36 KCl, 0.34 Na₂HPO₄, 0.44 K₂HPO₄, 10 HEPES, 1.2 Mgl₂, 1.8 CaCl₂, and 10 D-glucose (pH 7.2). The mesenteric artery was cleaned of fat and connective tissue and digested in low-Ca²⁺ (0.05 mM) PSS1 containing (in mg/ml) 2 collagenase type XI, 0.16 elastase type IV, and 2 BSA (fat free) for 35 min at 37°C (7). A suspension of single cells was obtained by gently triturating the tissue in low-Ca²⁺ PSS1. Dispersed cells were directly deposited on glass coverslips for fluorescence microscopy. Cells were allowed to settle on the coverslips for 20–30 min before loading with fura-2 (79). Freshly dissociated cells that were markedly contracted under resting conditions (less than 5%) were excluded.

Calcium imaging. [Ca²⁺]cyt was measured with fura-2 by using digital imaging as described previously (7). Freshly dissociated ASMCs were loaded with fura-2 by incubation for 35 min in PSS1 containing 3.3 µM fura-2 AM (20°C-22°C, 5% CO₂-95% air). After dye loading, the coverslips were transferred to a tissue chamber mounted on a microscope stage, where cells were superfused for 15–20 min (35°C to 36°C) with physiological salt solution 2 (PSS2) to wash away extracellular dye. The PSS2 contained (in mM) 140 NaCl, 5.0 KCl, 1.2 Na₂HPO₄, 5 NaHCO₃, 1.4 MgCl₂, 1.8 CaCl₂, 11.5 glucose, and 10 HEPES (pH 7.4). In Ca²⁺-free PSS2, 50 µM EGTA was added to chelate residual Ca²⁺. Cells were studied for 40–50 min during continuous superfusion with PSS2 (35°C).

The imaging system included a Zeiss Axiostar 100 microscope (Carl Zeiss, Thornwood, NY). The dye-loaded cells were illuminated with a diffraction grating-based system (Polychrome V; TILL Photonics, Göttingen). Fluorescent images were recorded with a CoolSnap HQ2 charge-coupled device camera (Photometrics, Tucson, AZ). Image acquisition and analysis were performed with a MetaFluo/ MetaMorph Imaging System (Molecular Devices, Downingtown, PA). [Ca²⁺]cyt was calculated by determining the ratio of fura-2 fluorescent emission (510 nm) excited at 380 and 360 nm as described (7). Intracellular fura-2 was calibrated in situ in freshly dissociated ASMCs (7).

Immunohistochemistry. Membrane proteins were separated by polyacrylamide gel electrophoresis (SDS-PAGE) as described (7) and transferred electrophoretically to a nitrocellulose membrane (Amerham BioSciences). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated overnight at room temperature with rabbit polyclonal anti-inositol 1,4,5-trisphosphate (IP₃) receptor type 1 antibodies (IP₃-R-I; dilution 1:500). Image analysis and acquisition were performed with a MetaFluo/ MetaMorph Imaging System (Molecular Devices, Downingtown, PA). [Ca²⁺]cyt was calculated by determining the ratio of fura-2 fluorescent emission (510 nm) excited at 380 and 360 nm as described (7). Intracellular fura-2 was calibrated in situ in freshly dissociated ASMCs (7).

Materials. Fura-2 AM was obtained from Molecular Probes (Invitrogen Detection Technologies, Eugene, OR). Collagenase (type 2) was obtained from Worthington Biochemical (Freehold, NJ). ATP,
Augmented myogenic responses in MHS arteries. MHS rats have significantly higher mean systolic BP (145 ± 1 mmHg, n = 16) than control MNS rats (112 ± 1 mmHg, n = 16; P < 0.001). To determine the contractile properties of the small resistance arteries from control MNS and MHS rats, the diameter changes in pressurized mesenteric fourth-order arteries (~165 μm internal diameter) were measured. The arteries constrict spontaneously (MT) when intralumenal pressure is increased to 70 mmHg during superfusion with Krebs solution at 37°C (74, 75). The mean MT in arteries from control MNS rats corresponded to a constriction of 14.9 ± 1.7% of PD (n = 15). Mean MT in arteries from MHS rats was 19.5 ± 1.4% of PD (n = 16), which was significantly greater (P < 0.05) than in controls.

The myogenic responses to step increases in intraluminal pressure [myogenic reactivity (MR)] were augmented in MHS arteries relative to myogenic responses in MNS arteries (Fig. 1). Figure 1A shows a set of original data from a MNS rat artery; the intraluminal pressure was lowered from 70 to 10 mmHg and then raised to 130 mmHg in 20-mmHg increments. Normalization of the myogenic reactivity curves from MHS and MNS arteries to PD at 70 mmHg of the respective arteries [i.e., classic MR curves (69)] reveals that the MHS rat arteries exhibited significantly augmented myogenic constriction (Fig. 1B).

PE-induced Ca²⁺ responses and vasoconstriction are increased in MHS arteries. Freshly dissociated mesenteric artery myocytes from the MHS rat had significantly higher resting [(Ca²⁺)cyt] than did MNS myocytes (97 ± 2 nM vs. 78 ± 3 nM; P < 0.001) (79). Activation by 5 μM PE, in physiological media, induced augmented Ca²⁺ signals in the MHS ASMCs. Both the peak initial response, which is likely the result of IP₃-mediated sarcoplasmic reticulum (SR) Ca²⁺ release, and the later plateau, apparently mediated by Ca²⁺ entry through ROCs and store-operated channels (SOCs), were greater in MHS than in MNS rat ASMCs (Fig. 2, A and B). The plateau was frequently accompanied by low amplitude [(Ca²⁺)cyt] oscillations or fluctuations in both MHS and MNS arterial myocytes (Fig. 2A). Superimposed records of the PE-induced Ca²⁺ response show that the integral of the rise of [(Ca²⁺)cyt] under the [Ca²⁺]cyt curve in MHS arterial myocytes was increased to 142 ± 9% of the area in MNS ASMCs (n = 18 MNS cells and 14 MHS cells; P < 0.05). Similar results were previously obtained with the purinergic receptor agonist, ATP (79).

ASMC contraction depends directly upon the [(Ca²⁺)cyt] and on modulation of the sensitivity to Ca²⁺ (e.g., by Rho/Rho kinase pathway) (30). To determine whether augmented PE-evoked Ca²⁺ signals are reflected in alteration of PE-induced vasoconstriction of isolated MHS mesenteric small arteries, we compared PE cumulative dose-response curves in pressurized MNS and MHS rat arteries. Figure 3A shows a representative cumulative PE concentration experiment from a control MNS rat artery. The peak vasoconstrictions to 1–100 nM PE were significantly larger in MHS than in MNS arteries (Fig. 3B), but the maximal constriction to 10 μM PE was comparable in MHS and MNS arteries. Thus the apparent EC₅₀ (PE concentration for a half-maximal effect) was significantly lower in MHS than in MNS arteries: 176 ± 39 nM vs. 371 ± 77 nM (P < 0.05).

Augmented SR Ca²⁺ stores and vasoconstrictor-induced Ca²⁺ entry in MHS rat arterial smooth muscle. If agonist-evoked Ca²⁺ responses and vasoconstriction are increased in MHS rat arterial smooth muscle, it is logical to ask what roles SR Ca²⁺ release and vasoconstrictor-induced Ca²⁺ entry play in this augmentation. To address this issue, Ca²⁺ transients were evoked in freshly dissociated ASMCs by unloading the SR Ca²⁺ stores in Ca²⁺-free medium with the
IP$_3$ receptor type 1 (Fig. 4, C and D) and SERCA2 (79). The secondary ATP-evoked rise in [Ca$^{2+}$]$_{cyt}$, observed when extracellular Ca$^{2+}$ was restored (Fig. 4A), correlates with the increased ROC activity (79), a consequence of the approximately threefold upregulated expression of TRPC6 in MHS artery smooth muscle (79).

Increased vasoconstrictor-induced SR Ca$^{2+}$ release and subsequent Ca$^{2+}$ entry in MHS arterial myocytes are associated with augmented PE-evoked vasoconstrictions of pressurized mesenteric small arteries under the same conditions. Figure 5A shows the protocol for the PE experiments in a MNS artery. The results are summarized in Fig. 5, B and C. They reveal that both PE-evoked vasoconstrictions, the initial response in Ca$^{2+}$-

Fig. 2. Augmented phenylephrine (PE)-induced Ca$^{2+}$ transients in freshly dissociated mesenteric artery myocytes from MHS rats. A: representative records show the time course of cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{cyt}$) changes induced by 5 μM PE in arterial smooth muscle cells (ASMCs) from control MNS (blue line) and MHS rats (red line). B. Summarized data show the peak amplitude of PE-induced Ca$^{2+}$ transients (19 MNS cells and 14 MHS cells) in ASMCs from 4 MNS and 4 MHS rats. The data are shown as quantitative difference of PE-induced transient Ca$^{2+}$ peaks relative to the baseline [Ca$^{2+}$]$_{cyt}$ levels (before the treatment). Data are means ± SE. *P < 0.05 vs. ASMCs from MNS rats.

Fig. 3. PE-evoked vasoconstriction in MNS and MHS rat small mesenteric arteries. A: representative experiment illustrates the changes in arterial diameter in response to cumulative doses of PE (10$^{-7}$–10$^{-3}$ M), indicated by dots over the original record, in a pressurized MNS artery. Horizontal bar at bottom indicates time of exposure to PE. Horizontal dashed line indicates PD. B: PE dose-response curves for MNS and MHS rat mesenteric small arteries with myogenic tone (MT). The vasoconstriction is shown as a percentage of PD (see METHODS). The PE-evoked vasoconstriction dose-response curves for MHS and MNS rat arteries are significantly different (ANOVA; P < 0.05; n = 6 MNS and 7 MHS arteries). The vasoconstriction at 1, 10, and 100 nM PE was significantly greater in MHS than in MNS arteries (Student’s t-test; *P < 0.05). EC$_{50}$ values were obtained from each artery (assuming a maximal contraction at 10 μM PE, the highest dose tested); the mean EC$_{50}$ values were significantly different: MNS = 371 ± 77 nM and MHS = 176 ± 39 nM (Student’s t-test; P < 0.05).
free medium, due to SR Ca\(^{2+}\) release, and the second response, due to Ca\(^{2+}\) entry when extracellular Ca\(^{2+}\) was restored, were significantly greater in MHS than in MNS arteries.

Ouabain-induced vasoconstriction is increased in MHS arteries. The plasma EO level is elevated approximately two-fold in MHS (vs. MNS) rats (25, 26), but the EO is washed out when the arteries are isolated. Prolonged treatment of rodents with ouabain also induces an ouabain hypertension (52, 59) that is mediated by high ouabain affinity Na\(^{+}\) pumps (20) [\(\alpha_1\) is ouabain resistant in rodents (57)] and NCX (19, 20). Moreover, NCX1 is greatly upregulated in de-endothelialized mesenteric arteries of ouabain hypertension rats (59), as well as in arteries of MHS rats (79). To determine whether circulating EO might directly affect vasoconstrictor responses of MHS mesenteric arteries, we tested the effects of acute application of 100 nM ouabain (derived from plants) on pressurized arteries from MNS and MHS rats. This dose of ouabain rapidly and reversibly inhibits Na\(^{+}\) pumps in rodents (57) but has a negligible effect on rodent low ouabain affinity Na\(^{+}\) pumps (75). Figure 6 shows that the isolated, pressurized small MHS mesenteric arteries exhibited significantly greater ouabain-induced constriction than did MNS arteries. The effects were

![Diagram A](image1.png)

![Diagram B](image2.png)

Fig. 4. Elevated ATP-induced sarcoplasmic reticulum (SR) Ca\(^{2+}\) release and extracellular Ca\(^{2+}\) entry in freshly dissociated MHS mesenteric artery myocytes, and augmented expression of inositol 1,4,5-triphosphate receptor type 1 (IP, R-I) in de-endothelialized MHS rat mesenteric arteries. A: representative records showing the time course of [Ca\(^{2+}\)]\(_{cyt}\) changes induced by 5 μM ATP in Ca\(^{2+}\)-free and Ca\(^{2+}\)-containing solutions in ASMCs from control MNS (blue line) and MHS (red line) rats. Nifedipine (10 μM) was added 10 min before the records shown and was maintained throughout the experiments. B: summarized data showing the peak amplitude of ATP-induced Ca\(^{2+}\) release and Ca\(^{2+}\) influx (68 MNS cells and 76 MHS cells) in ASMCs from 10 MNS and 9 MHS rats. The data are shown as quantitative difference of ATP-induced transient Ca\(^{2+}\) peaks relative to the baseline [Ca\(^{2+}\)]\(_{cyt}\) levels (before any treatment). ***P < 0.001 vs. ASMCs from MNS rats. C: representative Western blot showing expression of IP, R-I in smooth muscle cell membranes from de-endothelialized mesenteric arteries of MNS and MHS rats. D: summary data are normalized to the amount of β-actin and are expressed as means ± SE from 4 immunoblots (total of 8 rats). ***P < 0.001 vs. ASMCs from MNS rats.

![Diagram C](image3.png)

Fig. 5. Augmented vasoconstrictions evoked by PE-induced Ca\(^{2+}\) release from SR stores and subsequent extracellular Ca\(^{2+}\) entry in MHS rat small mesenteric arteries. A: representative experiment illustrates PE-evoked vasoconstriction in the absence of extracellular Ca\(^{2+}\) (peak 1) and the secondary vasoconstriction when Ca\(^{2+}\) was added back (peak 2) in a pressurized MNS artery. B and C: summary data. The evoked vasoconstrictions due to SR Ca\(^{2+}\) release (B) and due to extracellular Ca\(^{2+}\) entry (C), expressed as a function of PD, in MHS rat arteries are significantly larger than that in MNS arteries (*P < 0.05; n = 5 for both groups).
sion manifested by augmented proximal tubular sodium reabsorption and salt sensitivity (16).

Enhanced Ca\textsuperscript{2+} signaling augments myogenic responses and PE-evoked vasoconstriction in MHS arteries. Altered Ca\textsuperscript{2+} signaling plays a key role in the vascular dysfunction associated with arterial hypertension (36, 66, 75). Ca\textsuperscript{2+} signaling activates Ca\textsuperscript{2+} calmodulin-dependent myosin light chain kinase and triggers vasoconstriction (78). Our findings demonstrate that resting [Ca\textsuperscript{2+}]\textsubscript{cyt} is elevated (79) and vasoconstrictor (PE, ATP)-induced Ca\textsuperscript{2+} signals are augmented in freshly dissociated mesenteric artery myocytes from the MHS rats (Figs. 2 and 4, A and B). The enhanced signals are due to both vasoconstrictor-evoked release of SR Ca\textsuperscript{2+} and Ca\textsuperscript{2+} entry from the ECF. The augmentation of ATP- and PE-mediated SR Ca\textsuperscript{2+} release in MHS myocytes can be explained by the increased SR Ca\textsuperscript{2+} store content resulting from overexpression of SERCA2 (79) and, perhaps, the higher resting [Ca\textsuperscript{2+}]\textsubscript{cyt} (79) and the upregulation of IP\textsubscript{3} receptor type 1 (Fig. 4, C and D), which should enhance mobilization of the larger Ca\textsuperscript{2+} store.

ROCs and NCX1 mediate the enhanced Ca\textsuperscript{2+} entry in MHS arteries. The increase in Ca\textsuperscript{2+} entry from the ECF in MHS myocytes is associated with ROCs and NCX1. The augmented ATP-induced Ca\textsuperscript{2+} entry (Fig. 4, A and B) correlates with increased activity of ROCs, measured as Ba\textsuperscript{2+} influx from the ECF activated by the cell-permeable diacylglycerol analog, 1-oleoyl-2-acetyl-sn-glycerol (OAG) (79). These effects are a consequence of greatly upregulated expression of TRPC6 (79), an essential component of ROCs in various ASMCs (35) including rat mesenteric artery myocytes (59). The upregulation of TRPC6 is specific, since expression of other TRPC proteins is not altered in MHS arteries (79).

Several reports indicate that Ca\textsuperscript{2+} homeostasis in ASMCs is influenced not only by direct Ca\textsuperscript{2+} entry through TRPC channels but also by Na\textsuperscript{+} entry through these nonselective cation channels. The entering Na\textsuperscript{+} and consequent depolarization should promote Ca\textsuperscript{2+} entry not only via L-type Ca\textsuperscript{2+} channels but also through NCX1 (3, 22, 24, 58). We cannot, however, exclude the possibility that other Na\textsuperscript{+}-selective channels (6, 21) might also contribute to the Na\textsuperscript{+} entry that promotes NCX-mediated Ca\textsuperscript{2+} entry. In contrast with the heart, where NCX1 primarily mediates Ca\textsuperscript{2+} extrusion (18), in ASMCs, which are partially depolarized in arteries with tone, NCX1 primarily mediates Ca\textsuperscript{2+} entry (36, 40, 76). The implication is that the upregulated TRPC6 and NCX1 (79) contribute to the enhanced Ca\textsuperscript{2+} entry and elevated [Ca\textsuperscript{2+}]\textsubscript{cyt} (79). This augments MR (Fig. 1) and arterial tone (36, 40, 75) and, thus, increases peripheral vascular resistance and BP in MHS rats. It is especially noteworthy that ouabain-induced hypertension (59) and human idiopathic pulmonary hypertension also are associated with a marked upregulation of both NCX1 (77) and TRPC6 (73). In mineralocorticoid-salt hypertension, in which endogenous ouabain has been implicated (34), TRPC6 is also upregulated (5) (NCX1 was not studied). This indicates that the upregulation of these Na\textsuperscript{+} and Ca\textsuperscript{2+} transporters is a clue to the origin of the augmented arterial responsiveness in many forms of hypertension (31).

We observed not only augmented myogenic constriction of MHS mesenteric resistance arteries (Fig. 1), but also enhanced contractile responses to 1–100 nM PE (Fig. 3). The augmented PE-evoked vasoconstrictions in the absence and presence of extracellular Ca\textsuperscript{2+} in MHS arteries (Fig. 5, A–C) are consistent
with, respectively, the increased vasoconstrictor-evoked SR Ca\(^{2+}\) release and Ca\(^{2+}\) entry from the ECF (Fig. 4, A and B). Enhanced vascular contractile responses to high dose (>5 \(\mu\)M) norepinephrine were observed previously in mesenteric small resistance arteries from MHS rats (61).

Functional versus structural remodeling of arteries in MHS rats. Some authors have suggested that vascular structural remodeling, including artery narrowing (53, 55), and increased arterial stiffness (14) are responsible for the increased peripheral vascular resistance in hypertension. The structure of MHS mesenteric small arteries is not altered, however, when compared with MNS arteries (61). In our study, both the passive diameter and the maximal response to PE were identical in MHS and MNS arteries. This also implies that the artery structure and contractile apparatus are normal in the MHS arteries. Thus the enhanced responses of MHS small resistance arteries to stretch and to low-dose PE are more likely associated with augmented Ca\(^{2+}\) signaling or functional remodeling than with structural remodeling of the arteries. Functional changes (augmented MR- and PE-evoked vasoconstriction) in the MHS rat arteries are small (Figs. 1 and 3), even though TRPC6 and NCX1 protein expression (79) and Ca\(^{2+}\) signaling are greatly altered (Figs. 2 and 4, A and B). This can be explained by the fact that the relationship between [Ca\(^{2+}\)]\(_{\text{cyt}}\) and vasoconstriction is nonlinear and that in intact arteries, numerous other factors including endothelial dysfunction, with altered release of endothelium-derived relaxing factors, also influence arterial constriction, peripheral resistance, and BP. For example, endothelium-dependent vasodilation in mesenteric small resistance arteries of MHS is significantly impaired compared with age-matched MNS (61). Nevertheless, from Poiseuille’s equation (42) even a modest 5% augmentation of constriction in a 100- \(\mu\)m-diameter artery will increase the resistance to flow by >20%—a large effect when translated into total peripheral resistance and BP.

Vasoconstrictor effects of ouabain. Nanomolar ouabain exerts its acute vasotonic effects by inhibiting the ASM Ca\(^{2+}\) pump-protein kinase cascade (43). The upregulation is ligand specific. For example, digoxin, another cardiac steroid/Na\(^{+}\) pump inhibitor that also augments ASM Ca\(^{2+}\) signaling (4), does not upregulate NCX1 and TRPC6 expression (80) and does not raise BP (50). Furthermore, when arteries are removed from ouabain-induced hypertensive rats, the aforementioned changes in protein expression remain and contribute to the ex vivo enhanced Ca\(^{2+}\) signaling (59) and the vascular contractile responses (74).

As described here and in our previous report (79), the same proteins are upregulated in arteries isolated from MHS rats. Crucially, primary cultured arterial myocytes from these arteries do not, however, exhibit upregulation of NCX1 and TRPC6 (79). This is consistent with the view that an in vivo circulating factor (EO), responsible for the protein upregulation, is absent from the culture environment. The implication is that the elevated circulating EO in MHS rats in vivo plays an essential role in the induction and maintenance of the observed functional alterations of the rat arteries, including the augmented TRPC6- and NCX1-mediated Ca\(^{2+}\) signaling and vasoconstriction. In this way, chronic and acute nanomolar ouabain affects arterial smooth muscle function in MHS rats in a coherent, integrated fashion. The net effect is larger constric tions in response to PE (Fig. 3B) and nanomolar ouabain (Fig. 6) in arteries from MHS than from MNS rats. In addition, impaired endothelial vasodilator mechanisms in MHS mesenteric small arteries (61) should contribute further to the amplification of arterial constriction by sympathetic (60) and other vasoactive agents.

A mechanism for whole body autoregulation. Numerous reports implicate alterations in various Ca\(^{2+}\) homeostasis and signaling proteins in the pathogenesis of hypertension (2, 32, 59, 62, 64, 72). We have identified a critical subset of these proteins that link the enhanced reabsorption of Na\(^{+}\) to the sustained elevation of BP. Our study shows that arterial contractility is enhanced in MHS rats, which carry the hypertensive adducin phenotype and have elevated plasma EO. The augmented myogenic and agonist-evoked vasoconstrictions are consistent with the amplified Ca\(^{2+}\) signaling in ASMCs from MHS rats that results from the upregulation of several Na\(^{+}\) and Ca\(^{2+}\) transporters, including NCX1, TRPC6, SERCA2, and IP\(_3\)R. This appears to be the first report to tie a genetic defect that enhances renal tubular Na\(^{+}\) reabsorption to: 1) altered expression of arterial Ca\(^{2+}\) transporters and signaling proteins, 2) augmented small artery myogenic and agonist-evoked vasoconstrictor responses, and, thus 3) the elevation of BP.

Nearly a half century ago, Borst and Borst de Geus proposed the concept of whole body autoregulation (13). Guyton and colleagues later showed that plasma volume expansion, as may occur with salt retention, initially elevated BP by increasing cardiac output (CO), but the hypertension was then sustained by high TPR with a normal CO (33). The conversion from high CO to high TPR, termed whole body autoregulation, was attributed to the arterial adjustment needed to prevent tissue overperfusion, but no mechanisms for this phenomenon have been identified. We suggest that the upregulated NCX1, TRPC6 channels, and IP\(_3\)R, together with increased sympathetic drive (41, 47), are some of the key mechanisms responsible for the whole body autoregulation and high TPR in hypertension.

The mechanisms that lead to the altered arterial protein expression are still unknown, but the changes in protein expression appear to be initiated by the elevated plasma EO. This implies that Na\(^{+}\) pump-protein kinase cascade signaling (44,
45) is intimately involved in these processes. These results emphasize the importance of vascular molecular and functional abnormalities in the development and maintenance of a genetically induced hypertension that is associated with renal dysfunction in tubular Na\textsuperscript+ reabsorption.

ACKNOWLEDGMENTS
This work was supported by National Institutes of Health/National Heart, Lung, and Blood Institute Grants P01-078870 Project 2 (to V. A. Golovina), Project 1 (to M. P. Blaustein) and Project 3 (to W. G. Wier), R01-045215 (to M. P. Blaustein and J. M. Hamlyn), and R01-HL01969 (to W. G. Wier); by a Japan Heart Foundation/Bayer Yakuhin Research grant (to E. Karashima); and by funds from the University of Maryland School of Medicine. Present address of Eiji Karashima: Dept. of Cardiology, Shimonsoseki City Central Hospital, Shimonsoseki, Japan. Present address of Alessandra Zalian: Dept. of Biological Science, Univ. of Padova, Padova, Italy.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


