Upregulation of L-Type Ca\(^{2+}\) Channels in Mesenteric and Skeletal Arteries of SHR

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Abstract—An increased Ca\(^{2+}\) influx attributed to dihydropyridine-sensitive L-type Ca\(^{2+}\) channels has been demonstrated in mesenteric vascular smooth muscle cells of spontaneously hypertensive rats (SHR). This study examined whether an upregulation of the pore-forming \(\alpha_{1C}\) subunit of the L-type Ca\(^{2+}\) channel underlies this ionic defect. With the use of mesenteric arcade arteries from 12- to 16-week-old SHR and normotensive Wistar Kyoto (WKY) rats, reverse transcriptase–polymerase chain reaction demonstrated an increased level of amplified cDNA corresponding to the \(\alpha_{1C}\) subunit mRNA in the SHR arteries. Western blots confirmed that the increased mRNA expression was associated with a 3.4-fold increase in the immunoreactive signal of the \(\alpha_{1C}\) subunit protein in SHR compared with WKY mesenteric arteries, and immunocytochemistry confirmed this abnormality at the single-cell level. Finally, isolated mesenteric arteries from SHR were highly reactive to Bay K8644 and developed anomalous Ca\(^{2+}\)-dependent tone, suggesting a functional role for \(\alpha_{1C}\) subunit upregulation in vascular hyperreactivity. To determine if these Ca\(^{2+}\) channel abnormalities extended to the SHR skeletal muscle bed, we repeated a similar series of studies in WKY and SHR hind limb arteries. Skeletal muscle arteries from SHR also expressed higher levels of \(\alpha_{1C}\) subunit mRNA and protein than WKY arteries and developed anomalous Ca\(^{2+}\)-dependent tone attributed to L-type Ca\(^{2+}\) channels. Our data provide the first evidence that the \(\alpha_{1C}\) subunit mRNA and protein are upregulated in SHR arteries and that the increased numbers of L-type Ca\(^{2+}\) channel pores are associated with the generation of abnormal vascular tone. (Hypertension. 2002;40:214-219.)

Key Words: calcium channels ■ muscle, smooth, vascular ■ arteries ■ mesenteric arteries ■ rats, spontaneously hypertensive ■ hypertension, arterial

A growing body of evidence indicates that an elevated Ca\(^{2+}\) entry into vascular smooth muscle cells (VSMCs) through long-lasting (L-type) Ca\(^{2+}\) channels plays a central role in increasing vascular tone in the established stage of hypertension. For example, organic Ca\(^{2+}\) channel blockers profoundly reduce blood pressure in spontaneously hypertensive rats (SHR) but have little hypotensive effect in normotensive Wistar Kyoto (WKY) rats, attesting to the obligatory role that voltage-gated Ca\(^{2+}\) influx plays in elevating peripheral vascular resistance in SHR.1-3 Similarly, the arterioles of the mesenteric and skeletal muscle beds of SHR develop an abnormal Ca\(^{2+}\)-dependent vascular tone and show increased myogenic responsiveness.4-6 Isolated mesenteric and femoral arteries of SHR also develop abnormal Ca\(^{2+}\)-dependent tone, whereas similar arteries from WKY rats are quiescent.7,8 In view of its central role in generating vascular tone, it is surprising that the mechanism of the elevated Ca\(^{2+}\) influx in SHR VSMCs remains unclear. Some evidence suggests that VSMC membranes are depolarized in hypertension, resulting in the activation of L-type Ca\(^{2+}\) channels.9 Alternatively, excitatory stimuli including neurotransmitters and intracellular mediators may preferentially activate vascular L-type Ca\(^{2+}\) channels during hypertension.10,11

Recently, however, studies using mesenteric arteries as a model have shown that VSMCs of SHR, studied in patch-clamp conditions that tightly control cell membrane potential and environment, still exhibit an enhanced voltage-gated Ca\(^{2+}\) influx compared with WKY cells. Ohya et al12 demonstrated a higher membrane density of L-type Ca\(^{2+}\) current in mesenteric VSMC of 5- to 6-week-old SHR compared with age-matched WKY rats, although density was normalized in SHR by 16 to 18 weeks of age. No differences in L-type Ca\(^{2+}\) channel properties were detected between these preparations. In detailed studies, Cox and Lozinskaya13,14 detected a higher density of L-type Ca\(^{2+}\) current in mesenteric VSMC from 6-, 12-, and 20-week-old SHR and observed normal voltage-dependent activation of the SHR Ca\(^{2+}\) channel. Finally, single-channel analysis by Ohya and colleagues15 in cell-
attached patches revealed that single L-type Ca\(^{2+}\) channels in WKY and SHR mesenteric VSMCs have similar unitary conductances and open-time distributions, although SHR membrane patches showed an enhanced number of channel openings. With the notation that a small hyperpolarizing shift in SHR channel activation was reported in 20-week-old SHR that would tend to increase channel availability,\(^1\) all of these findings are consistent with the hypothesis that L-type Ca\(^{2+}\) channels are upregulated in SHR mesenteric VSMC membranes. However, an increased protein expression of the channel pores has not been demonstrated.

The goal of this study was to compare the mRNA and protein expression levels of L-type Ca\(^{2+}\) channel pores between mesenteric VSMCs of WKY rats and SHR and to determine if altered Ca\(^{2+}\) channel expression is associated with abnormalities in the reactivity of isolated arteries. Similar experiments also were conducted in VSMC and segments of rat skeletal muscle arteries to examine the profile of L-type Ca\(^{2+}\) channel alterations in another vascular bed involved in blood pressure regulation.

**Methods**

**Animals**

WKY rats and SHR (4 weeks, or 12 to 16 weeks) were obtained from Taconic Farms (Germantown, NY). Their use was approved by the Animal Care and Use Committee. Rats were anesthetized with ketamine and acepromazine (82 mg/kg and 1.2 mg/kg IM, respectively), and mean arterial blood pressures were measured by cannulation of the carotid artery. Average pressures were 78 ± 3 mm Hg in adult WKY rats (n = 95), 89 ± 3 mm Hg in 4-week-old WKY rats (n = 20), 107 ± 4 mm Hg in 4-week SHR (n = 18), 95 ± 2 mm Hg in adult WKY rats (n = 52), and 163 ± 3 mm Hg in adult SHR (n = 63). After the animals were exsanguinated, the mesenteric arcade arteries and the femoral artery including the saphenous branch were immediately removed, cleaned of adherent tissue, and used in the experiments described below.

**Reverse Transcriptase–Polymerase Chain Reaction**

Total RNA was isolated and pooled from 3 WKY or SHR arteries with the use of an RNaseasy Mini Kit (Qiagen). First-strand cDNA was synthesized from 1 μg of total RNA (First-Strand Beads, Amersham Pharmacia Biotech). Duplex polymerase chain reaction (PCR) was performed according to commercial instructions with 3.5 μL of reverse transcriptase (RT) product and TaKaRa LA Taq polymerase (TaKaRa Biomedicals). Sense and antisense primers (Operon Technologies) were designed from coding regions of the α\(_{1C}\) subunit (nt 5790 to 5809; 6003 to 6022; M59786) and smooth muscle–specific α-actin (nt 151 to 174; 765 to 787; NM007392). Primers for α-actin spanned intronic sequences in the α-actin gene to permit the detection of genomic DNA contamination in PCR reactions. The PCR conditions including annealing temperature and cycle dependency were initially optimized. PCR was begun with a 5-minute denaturation at 95°C followed by 32 to 40 cycles of 95°C (1 minute), 59°C (1 minute), 72°C (2 minutes), and a final extension step at 72°C (5 minutes). Amplified cDNA products were analyzed on a 1% agarose gel with a 100-bp DNA ladder as a marker. The photodensity of amplified cDNA product bands were quantified by densitometry (Alpha Innotech Corp).

**Western Blotting**

Membrane proteins were isolated and pooled from 4 to 8 WKY rats or SHR for use in Western blots.\(^2\) A sequence–specific polyclonal antibody, anti-α\(_{1C}\), raised against residues 818 to 835 of the α\(_{1C}\) subunit, was used. Anti-α\(_{1C}\) was a kind gift from Dr Joerg Striessnig (University of Innsbruck) or was obtained commercially (Alomone Laboratories, Jerusalem). α-Actin (Sigma) was used as an internal standard.\(^3\) The bound antibody was detected by chemiluminescence (ECL, Amersham) and the densities of the doublet bands at 200 and 240 kDa were summed to evaluate the level of α\(_{1C}\) subunit expression.

**Immunocytochemistry**

Single VSMCs were enzymatically isolated\(^4\) and fixed with 95% ethanol on glass coverslips. Immunostaining was performed at 37°C for 45 minutes with antibodies directed against the α\(_{1C}\) subunit or α-actin. Antibodies were diluted 1:50 (α\(_{1C}\)) or 1:100 (α-actin) in phosphate-buffered saline containing 3% normal goat serum and 0.2% Tween 20 (PBS-NGT). After washing, incubations with the secondary Alexa Fluor 594 antibodies (Jackson Immunoresearch) were performed at 37°C for 30 minutes. Finally, nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI; Sigma). Fluorescent technologies (University of Innsbruck) or was obtained commercially (Alomone Laboratories, Jerusalem). α-Actin (Sigma) was used as an internal standard.\(^3\) The bound antibody was detected by chemiluminescence (ECL, Amersham) and the densities of the doublet bands at 200 and 240 kDa were summed to evaluate the level of α\(_{1C}\) subunit expression.

**Figure 1.** A, Relation between cycle number and α\(_{1C}\) product with cDNA from WKY mesenteric arteries. B, Duplex RT-PCR products correlating to α\(_{1C}\) subunit mRNA (233 bp) and α-actin mRNA (837 bp) show higher expression of α\(_{1C}\) in SHR versus WKY at the same cycle number. C, α\(_{1C}\) subunit was recognized in the left lane as a 200- to 240-kDa doublet band in WKY mesenteric arteries (−CP), but was absent in the right lane after competing peptide inhibition (−CP). Similar 45-kDa bands represent α-actin internal standard. D, α\(_{1C}\) subunit was upregulated in adult SHR mesenteric arteries. Inset: Expression of α\(_{1C}\) subunit was similar between mesenteric arteries from 4-week-old WKY rats and SHR.
Vascular Reactivity Assays
Mesenteric and femoral arteries (3-mm length) were mounted in a tension-recording system, and optimal basal tension was established at 1.0 g. After 1 hour, 2 successive contractions to 80 mmol/L KCl were recorded. Subsequently, concentration-dependent responses to the dihydropyridine agonist, Bay K8644 (Sigma), were obtained in one set of arteries. Another set of arteries was maintained in control PSS, and spontaneous changes in vessel reactivity were recorded for 90 minutes or until stable. The level of active basal tone recorded in control PSS, and spontaneous changes in vessel reactivity were obtained in one set of arteries. Another set of arteries was maintained in control PSS, and spontaneous changes in vessel reactivity were recorded for 90 minutes or until stable. The level of active basal tone mediated by L-type Ca$$^{2+}$$ channels was evaluated by application of 1 μmol/L nifedipine (Sigma) at the end of the experiment.

Statistics
Data are expressed as mean±SEM. Significant differences between SHR and WKY preparations were determined by Student’s t test or 1-way ANOVA with repeated measures. A probability value of <0.05 was considered statistically significant.

Results
αIC Gene Is Upregulated in Mesenteric VSMCs of SHR
Initial experiments compared the level of mRNA encoding the αIC subunit between mesenteric VSMCs of WKY rats and SHR. Figure 1A shows the cycle-dependent amplification of 637-bp and 233-bp products corresponding to mRNA for smooth muscle α-actin and the αIC subunit in WKY arteries, respectively. The graph indicates that linear amplification occurred between 28 and 36 cycles. Figure 1B shows that compared with WKY (top panel), the photodensities of the 233-bp bands corresponding to αIC mRNA were substantially higher in SHR arteries (bottom panel). At the same cycle number, α-actin was equally amplified, providing evidence of experimental consistency between reactions. RNA isolations pooled from a total of 6 WKY and 6 SHR arteries demonstrated a 1.53-fold increase in the αIC signal measured in the linear range. Performing amplification without RT reaction or in the absence of specific primers produced no detectable products (data not shown).

αIC Subunit Is Overexpressed in Mesenteric VSMCs of SHR
Western blotting compared the expression of the αIC subunit protein between mesenteric VSMC membranes of WKY rats and SHR. The left lane in Figure 1C reveals the presence of 200- and 240-kDa doublet bands in mesenteric membranes from WKY rats corresponding to the short and long forms of the αIC subunit.16 In the right lane, the doublet bands were abolished by preabsorption of the antibody with 3 μmol/L of its antigenic competing peptide (+CP), confirming antibody specificity for its recognition sequence. The similar immuno-density of the α-actin (45 kDa) internal standard in both lanes verified uniform lane loading of 10 μg protein. Figure 1D displays a subsequent Western blot showing a striking up-regulation of the αIC subunit in SHR compared with WKY mesenteric membranes. The film exposure was minimized to visualize the WKY bands without saturating the SHR signal. In 6 separate comparisons using different protein isolations, the immunoreactive signals associated with the αIC subunit were 3.4-fold higher in SHR compared with WKY, representing a significant elevation of the L-type Ca$$^{2+}$$ channel protein. To determine if the overexpression of αIC subunits is an early feature of SHR arteries, Western analysis was extended to mesenteric arteries pooled from 4-week old WKY rats and SHR. Interestingly, the inset in Figure 1D shows that the αIC subunit was not upregulated in the arteries of the young SHR.

Subsequently, mesenteric VSMCs from adult WKY rats and SHR were freshly isolated for immunocytochemical analysis, and a total of 39 cells were analyzed individually for fluorescent intensities. The left 2 panels in Figure 2A show a Nomarski image of mesenteric VSMCs isolated from WKY rats and SHR arteries, respectively, on the same day. The right 2 panels demonstrate that the fluorescent signals from the SHR cells labeled with the anti-αIC antibody were higher than in the WKY cells when exposed for equivalent times, showing a significantly increased average intensity of 1.6-fold in the SHR cell population. Figure 2B demonstrates that the fluorescent signal for α-actin did not significantly differ between WKY and SHR VSMCs. Notably, cell immunofluorescence was absent in control reactions obtained without primary antibodies. Furthermore, Figure 2C demonstrates that immunofluorescence was absent in WKY and SHR VSMCs exposed to the αIC antibody after it was preadsorbed with its antigenic competing peptide.

SHR Mesenteric Arteries Show More Functional αIC Subunits
Vascular reactivity responses to the L-type Ca$$^{2+}$$ channel agonist Bay K8644 and to the channel antagonist nifedipine
were performed to compare L-type Ca\(^{2+}\) channel-dependent changes in contractility between WKY and SHR mesenteric arteries. The traces in Figure 3A show that cumulative concentrations of Bay K8644 weakly contracted WKY arteries but profoundly contracted SHR arteries. Figure 3B shows that SHR arteries also developed more spontaneous tone than WKY arteries, which was reversed by 1 \(\mu\)mol/L nifedipine at the end of the experiment. The averaged values in Figure 3C show that the maximal responses of WKY and SHR arteries to Bay K8644 were 2±1% and 104±11% of the maximal contraction to 80 mmol/L KCl, respectively (n=5 each). Figure 3D indicates that SHR mesenteric arteries spontaneously developed 1.6-fold more nifedipine-sensitive tone than WKY arteries (n=5, 7).

**Figure 3.** A, Tension recording traces in WKY and SHR mesenteric arteries. Only SHR artery contracted significantly to Bay K8644. B, SHR arteries developed more nifedipine-sensitive spontaneous tone than WKY arteries. C, Concentration-response curves of WKY and SHR arteries elicited by Bay K8644 (n=5). D, Effect of nifedipine on developed tone of WKY and SHR mesenteric arteries (n=5, 7). All values are mean±SEM. Asterisks indicate significant difference (P<0.05) between WKY and SHR arteries.

**Discussion**

Earlier studies have demonstrated that the membrane densities of whole-cell and single-channel L-type Ca\(^{2+}\) currents are elevated in mesenteric VSMCs from SHR compared with WKY rats.\(^{1-15}\) The experiments described here have identified a potential mechanism for this increase and are the first to demonstrate that \(\alpha_{1C}\) mRNA and pore-forming protein are upregulated in SHR mesenteric arteries that show anomalous Ca\(^{2+}\)-dependent tone. Furthermore, our results indicate that \(\alpha_{1C}\) mRNA and protein also are upregulated in SHR VSMCs from skeletal muscle arteries. We interpret these findings to suggest that the upregulation of L-type Ca\(^{2+}\) channels may confer a higher level of Ca\(^{2+}\)-dependent tone to SHR vessels, although other factors including a depolarized membrane potential or abnormal Ca\(^{2+}\) channel properties also may amplify Ca\(^{2+}\) influx in the VSMCs of SHR.

**Potential Mechanisms for \(\alpha_{1C}\) Subunit Upregulation in SHR VSMCs**

The \(\alpha_{1C}\) gene gives rise to both cardiac (\(\alpha_{1C-a}\)) and vascular (\(\alpha_{1C-b}\)) splice variants.\(^{19}\) However, because of the low abun-
durance of the α1c subunit in VSMC membranes, there is little knowledge about the regulation of this subunit by physiological factors. The present study provides initial evidence that the vascular α1c mRNA and protein are upregulated in VSMC of SHR, although further studies will be needed to determine the molecular basis of this abnormality. In this regard, earlier studies in cardiac myocytes have indicated that transcriptional regulation may increase the abundance of α1c subunits in response to glucocorticoids and norepinephrine.\(^{20,21}\) Additionally, posttranslational alterations of the α1c subunit by regulatory β and αβ subunits also may alter L-type Ca\(^{2+}\) channel expression by regulating its formation or its targeting and stability in the plasma membrane.\(^{22,23}\) At the moment, it is unclear to what extent αβ and β subunits are expressed and are functionally important in VSMCs and which factors affect their interaction with the α1c subunit. However, the finding in this study that arterial α1c subunit expression is normal in 4-week SHR but elevated in adult SHR raises the possibility that intraluminal pressure may positively regulate the expression level of arterial L-type Ca\(^{2+}\) channels in vivo. The reports from two other laboratories\(^ {12,14}\) that L-type Ca\(^{2+}\) current is increased in SHR compared with WKY mesenteric VSMCs at 6 weeks of age raise the possibility that there may be a critical period between 4 and 6 weeks, during which blood pressure is sufficiently elevated to upregulate α1c subunits in SHR vessels. Alternatively, the overexpression of L-type Ca\(^{2+}\) channels may be a later event in the pathogenesis of hypertension in the SHR that does not rely on an elevated pressure as a primary mechanism for upregulation.

**Limitations**

Several limitations of the present study should be acknowledged. First, larger arteries rather than arterioles were used to obtain enough vascular tissue to analyze α1c mRNA and protein expression. The possibility exists that α1c mRNA and protein expression may be regulated differently in SHR resistance vessels than in larger arteries, although the abnormally high Ca\(^{2+}\)-dependent tone observed in mesenteric and femoral arterioles of SHR is consistent with upregulation of the α1c transcript.\(^ {4–6}\) Second, the small size of rat arteries made it unfeasible to remove the endothelium from arteries before protein and total RNA were isolated. Thus, some contaminating element of the α1c signal emanating from endothelial cells cannot be ruled out in the Western and RT-PCR studies, although two factors argue against its importance. First, there is little evidence that L-type Ca\(^{2+}\) channels are expressed in endothelial cells, which instead are thought to rely on receptor-operated Ca\(^{2+}\) entry for cell function.\(^ {24}\) Second, we observed an increased fluorescent signal corresponding to the α1c subunit in isolated VSMCs of SHR, although this evidence for α1c overexpression should be viewed as only complementary to the Western blot findings, indicating an upregulation of the L-type Ca\(^{2+}\) channel. Because VSMCs are the major component of larger arteries, most of the α1c signal probably originated from this cell type. Finally, our study did not directly compare L-type Ca\(^{2+}\) current densities between WKY and SHR VSMCs with patch-clamp methods. Instead, because two established laboratories have already provided detailed evidence that whole-cell and single-channel L-type Ca\(^{2+}\) currents are increased in SHR VSMCs,\(^ {12–15}\) we chose to focus on the compelling question of the mechanism of this alteration and relied on vascular reactivity studies to evaluate the level of functional Ca\(^{2+}\) channels. Notably, the 3- to 4-fold increase in α1c subunit expression in SHR compared with WKY arteries detected in the present study would appear to fully account for the <2-fold increase in peak L-type Ca\(^{2+}\) current detected in patch-clamp studies of mesenteric VSMCs from the SHR, as reported by Cox and Lozinskaya.\(^ {13}\)

**Physiological and Therapeutic Implications**

The entry of Ca\(^{2+}\) into VSMCs through L-type Ca\(^{2+}\) channels plays an obligatory role in the development of myogenic tone.
and mediates contractile responses to vasoactive stimuli and drugs. Thus, the upregulation of the \( \alpha_{1C} \) subunit in SHR VSMCs would be expected to enhance myogenic tone in the mesenteric and skeletal muscle circulations that are involved in blood pressure regulation. Additionally, the recent finding that mesenteric arteries from hypertensive patients show an abnormally high component of dihydropyridine-sensitive tone raises the possibility that the \( \alpha_{1C} \) subunit may be upregulated in humans with essential hypertension but has relatively little effect on blood pressure levels in normotensive individuals.27 Thus, the upregulation of the \( \alpha_{1C} \) subunit in some forms of hypertension may predispose an individual to the vasodilator effect of CCBs, and similarly, drug sensitivity differences to CCBs between hypertensive individuals may relate to the further modulation of the \( \alpha_{1C} \) gene by other regulatory factors that alter channel expression.

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