Vascular-Specific Increase in Exon 1b-Encoded CaV1.2 Channels in Spontaneously Hypertensive Rats

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Background: The vasculature of the adult spontaneously hypertensive rat (SHR) is known to express more functional L-type Ca channels than the vasculature of normotensive Wistar Kyoto (WKY) rats, but it is not known which CaV1.2 channel isoform is upregulated.

Methods: Western blots and real-time reverse transcriptase–polymerase chain reaction (RT-PCR) were used to compare the expression levels of CaV1.2 channel protein and message in selected tissues of adult SHR and WKY rats.

Results: The results indicate overexpression in SHR vasculature specifically of the short exon 1b-encoded amino terminus CaV1.2 isoform. Brain and visceral smooth muscle expressing the same isoform were not similarly affected. Differences in message levels are insufficient to account for the differences in isoform-specific protein levels.

Conclusions: We conclude that SHR vasculature must regulate the channel posttranscriptionally. Further experiments will be required to determine whether this involves translation of protein from exon 1b-specific transcripts more efficiently, posttranslational chaperoning to the surface membrane more efficiently, or selective degradation of the short amino terminus form of the protein more slowly than in WKY vasculature.

Key Words: Calcium channels, SHR rat, hypertension.

One of the most extensively investigated animal models of hypertension is the spontaneously hypertensive rat (SHR), which progressively develops elevated blood pressure (BP) from birth and shows pronounced hypertension at adulthood. Notably, abnormalities of vascular Ca²⁺ channels are postulated to contribute to the increased vascular tone. By 12 weeks of age, arteries of SHR exhibit greater Ca²⁺ influx and Ca²⁺-dependent contractions than similar vessels from WKY rats, and this abnormality is more evident in arteries of male than female animals. In addition, intracellular free [Ca²⁺] is elevated in SHR rat aorta, and L-type Ca²⁺ channel current is elevated in smooth muscle cells isolated from the SHR vasculature. Finally, BP in the SHR is normalized by Ca²⁺ channel blockers. A similar anti-hypertensive effect of Ca²⁺ channel blockers has been noted in patients with essential hypertension, leading to the suggestion that abnormalities of vascular Ca²⁺ channels also may contribute to the human form of the disease.

Despite these findings, the processes that result in vascular Ca²⁺ channel abnormalities in hypertension have not been identified. Although two earlier reports suggested unaltered or decreased dihydropyridine-binding sites in SHR vasculature, in pulmonary vessels Ricci et al reported a higher density, implying an increased number of channel proteins. Western blots performed by Pratt et al showed a greater abundance of the pore-forming α₁C protein of the L-type Ca²⁺ channel (ie, CaV1.2) in small mesenteric and skeletal arteries of SHR compared to WKY rats. In the same arteries, the expression of mRNA encoding the α₁C subunit was increased. Although these latter findings suggest that an overabundance of CaV1.2 channels may represent a shared abnormality of SHR arteries, it has been difficult to define the molecular basis of this finding due to the diverse processes that regulate CaV1.2 expression. For example, several transcripts for the channel exist, and these may be variably expressed in different tissues. Furthermore, in addition to transcriptional regulation, the final number of functional CaV1.2 channels...
channels relies on the efficiency of post-transcriptional events including translation, trafficking, membrane stability, and the effects of β and α2δ channel subunits on these processes.

In this regard, our laboratory and others have established that smooth muscle cells express a form of the CaV1.2 channel with a different amino terminus than that found in the heart. This finding raises the intriguing possibility that the expression level of the CaV1.2 channel in the vasculature may be altered in a tissue-specific manner during hypertension and other cardiovascular pathologies. Thus, the aim of the present study was: 1) to determine whether the mRNA and protein corresponding to the smooth muscle isoform of the CaV1.2 channel are upregulated in the vasculature of the SHR, and if confirmed, 2) to define whether this abnormality is specific for vascular smooth muscle cells or extends to other nonvascular smooth muscle expressing the same channel isoform.

Methods

Animals

Male SHR and WKY rats were obtained from Taconic Farms (Germantown, NY colony), fed standard rat chow and sacrificed at 16 to 20 weeks of age. Mean arterial BP was measured by the tail-cuff method in a subset of four SHR (183 ± 6 mm Hg) and five WKY rats (109 ± 8 mm Hg).

Western Blotting

Microsomal membranes were prepared from differential centrifugation (pellet from a 1-h at 100,000 g spin of the supernatant from a 5-min at 13,000 g spin) of tissue homogenized in 50 mmol/L MOPS, 250 mmol/L sucrose, 2 mmol/L EDTA, 2 mmol/L EGTA, pH 7.4 with Complete Mini protease inhibitor cocktail pellets (Roche, Indianapolis, IN). Homogenates were prepared from pulverized frozen aorta, mesenteric arteries (Dounce homogenization), left plus right ventricle, brain, and colon (Polytron homogenization) of SHR and WKY rats. In addition, samples were prepared from freshly isolated vascular smooth muscle cells from aorta dissociated in 0.1% collagenase, and sacrificed at 16 to 20 weeks of age. Mean arterial BP was measured by the tail-cuff method in a subset of four SHR (183 ± 6 mm Hg) and five WKY rats (109 ± 8 mm Hg).

Western blotting. The first antibody (ACC-003) directed against residues 848 to 865 of the cytoplasmic II–III linker of the rat brain CaV1.2 channel recognized all three CaV1.2 isoforms (including exons 1a, 1b, or neither) under consideration here. A second antibody (ACC-022) directed against residues 2 to 15 coded for by exon 1b of the human CaV1.2 channel recognized only the short amino terminus encoded for by a short alternative first exon (exon 1b) expressed at very low levels in the heart. The regions of the channel representing the antibody epitopes are boxed in Fig. 1. Also seen in Fig. 1, the exon 1b-specific antibody recognizes channels in aorta well, but not channels in ventricle, whereas the II–III linker antibody recognizes both. Antigenic peptide in excess competes out labeling of aortic samples. In contradistinction to Saada et al, we report that the lower band stained with exon 1b-specific antibody corresponds to the upper band seen with the II–III linker antibody. Thus, three size forms of the channel (see Fig. 1C) are distinguished: full length ~250 kDa, amino- or carboxy-terminally truncated ~220 forms, and an amino- and carboxy-terminally truncated ~190 kDa form. Neither amino-terminally truncated form is detected by the exon 1b-specific antibody. In addition, bands were stripped and reprobed with anti-α-actin (Sigma A-2547) or anti-β-actin (Sigma A-5441). Films were scanned with an Alpha Innotech (San Leandro, CA) 8900 imager using AlphaEase FC software, version 3.2.3. The density values in the upper and lower bands were measured, and background was subtracted from the same size area close to the upper and lower band, respectively, avoiding trailing edge effects. The final values are the total values of the upper and lower bands of each lane.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

RNA was extracted from tissues using the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA) following the manufacturer’s instructions, and quantitated using RiboGreen RNA (Molecular Probes, Eugene, OR). In most cases, DNA standards were generated by polymerase chain reaction (PCR) amplification with the same real-time PCR primers as used for reactions. Those PCR products were quantitated, serially diluted as standard template, and cloned into TOPO cloning vectors. RNA standards were generated by in vitro transcription of linearized plasmid DNA using a MEGAscript T7 Kit (Ambion, Austin, TX). RNA standards were quantitated assuming that a 500-nucleotide standard (30 ng/μL) would yield 1.1 × 10^11 copies. RNA standards were used to control for tube-to-tube differences in reverse transcription. Reverse transcription of 50 ng of RNA samples was carried out using gene-specific primers at 50°C for 30 min, followed immediately by 40 cycles of amplification of the cDNA on a Mx3000P Real-Time QPCR System (Stratagene, La Jolla, CA). In these reactions PCR products were detected quan-
titivatively with the double-stranded DNA-specific dye Sybr Green (Molecular Probes). In certain other cases, RNA was reverse transcribed using random primers, and the resulting cDNA was amplified by real-time PCR. Primers for exon 1b-specific amplification (178-bp product) were forward 796: ccaccaaggttccaactatg, reverse 973: gatggctgcattgccagcac (GenBank, M67516). The same reverse primer (497 in GenBank NM 012517) was used for exon 1a-specific amplification (147-bp product) with forward primer 351: ccagctcatgccaacatgaa (GenBank NM 012517). The Pan CaV1.2 primer pair used was forward 1834: ccgcagtaagtccaacgtc and reverse 2227: gagcgcagtgagtctagcaa (GenBank M59786), generating 394-bp product, amplifying message for IIS1–IIS4. All fluorescence data were analyzed with software provided by Stratagene, a melting curve performed to verify amplification of a single product, and the correct length of PCR product verified on an agarose gel.

Statistical Methods

All data are presented as mean ± SD. Statistical significance was assessed using unpaired Student t test at P < .05.

Results

Expression of the α1C subunit of the channel was compared between aortas of SHR and WKY rats in Western blots, as shown in Fig. 2A. The α1C isoform emanating from exon 1b (α1C/1b) is shown above and compared to general expression of the α1C subunit using a pan-α1C antibody targeting the II–III cytoplasmic linker present in all forms of the subunit (Fig. 2A, second line). Each lane represents an aorta from a different animal. Expression of CaV1.2 channels was apparent in SHR aorta probed with either antibody. However, there was a very marked (94%) difference when the antibody recognizing the channel encoded by exon 1b was used to detect only the exon 1b-encoded isoform (Fig. 2A,C). To verify that increases in the α1C subunit were localized to vascular smooth muscle cells, smooth muscle cells were freshly isolated from these aorta, 93% of which stained positive for α-actin. Again, the SHR sample showed increased staining relative to the WKY sample using the exon 1b-specific antibody (Fig. 2B). Cells demonstrated less post-transla-
FIG. 2. Expression of Cav1.2 protein in SHR and WKY aorta. (A) Samples (15 μg protein, each from a separate rat) from aortic tissue of SHR and WKY rats, together with a control from Sprague Dawley heart (†). (B) Samples from freshly isolated vascular smooth muscle cells from SHR and WKY aorta. (C) Densitometric quantitation of results in (A) and two additional determinations. *P < .05. In the case of the exon 1b antibody data, P = .000009; in the case of II–III linker antibody data, P = .0092. (D) Samples of SHR aorta containing 2.5, 5, 10, and 20 μg protein were compared with samples of WKY aorta containing 10 and 20 μg protein by probing with the exon 1b antibody. (E) Densitometric quantitation of results from (D).
tional processing or proteolysis, as evidenced by a higher ratio of upper to lower bands on Western blots, perhaps due to a different sample preparation that avoids freezing and thawing.

Densitometric scans of the results in Fig. 2A are shown in Fig. 2C, with background near each band subtracted. The ratio of SHR/WKY expression in aorta from Fig. 2C was 4.54 with the exon 1b-specific antibody compared to 2.56 with the II–III linker antibody ($P = .026$). We routinely observed greater actin staining in our SHR aorta blots. Although we suspect this may be due to differential retention of actin in our microsomal aorta preparations, normalization of values by actin yields an SHR/WKY ratio of 2.56 ($P = .0011$) in the case of exon 1b antibody data, $P = .017$. Expression of Ca$_v$1.2 channel protein in SHR and WKY ventricle (20 µg protein samples, C), brain (20 µg protein samples, D), and colon (40 µg protein samples, E). Exon 1b-specific antibody not used for ventricle due to low expression levels of this isoform (see Figs. 1 and 2). The $\beta$-actin control for brain is not shown; it showed lesser loading only for the leftmost lane.

These quantitations assumed linearity, which was explored in a separate experiment in which different amounts of one SHR aorta sample were titrated on a Western blot using the exon 1b-specific antibody against two concentrations of the same WKY sample, as seen in Fig. 2D. The densitometric results are shown in Fig. 2E. Although the SHR results are not linear, 2.5 µg of SHR protein yielded a greater signal than 10 µg of WKY protein. Similarly 5 µg of SHR protein yielded a greater signal than 20 µg of WKY protein. Therefore, a quadrupling of exon 1b-encoded $\alpha_{1C}$ expression appears consistent without actin normalization.

To lend additional support to the finding that the exon 1b-encoded Ca$^{2+}$ channel was more abundant in SHR than in WKY vascular smooth muscle, samples were prepared from mesenteric resistance artery and shown in Fig. 3. As with aorta, the difference in expression was clearly greater...
using the exon 1b-specific antibody than the II–III linker antibody. Densitometric scans of this and a third sample from each rat strain also support the visual impression, although in this case, the enhancement of exon 1b-encoded α1C protein appears to be threefold rather than fourfold. The ratio of SHR/WKY expression in mesenteric arteries was 3.30 with the exon 1b-specific antibody compared to 1.73 with the II–III linker antibody (P = .039).

Subsequently, similar Western blots were used to determine whether the α1C subunit also was upregulated in the cardiac ventricle of SHR. Although Ca2+ channels are plentiful in the heart, the ventricles only sparsely express the exon 1b-encoded α1C isoform (ie, Figs. 1 and 2A). The WKY and SHR ventricles showed similar expression of the Ca2+ channel using the pan-α1C antibody (Fig. 3 C). Thus, the α1C subunit was not upregulated in the SHR heart, which lacks the exon 1b-encoded channel isoform.

Interestingly, the upregulation of the α1C subunit also did not extend to rat brain or rat colon, tissues that express significant levels of the exon 1b-encoded Ca2+ channel. The WKY and SHR brain exhibited no significant difference in α1C (P = .60) or α1C/1b (P = .76) expression (Fig. 3D). Similarly, despite greater variability, no difference in the expression of α1C (P = .37) and α1C/1b (P = .51) was detected between WKY and SHR colon (Fig. 3E). Thus, the Ca2+ channel α1C subunit and α1C/1b isoform show a tissue-specific overexpression in SHR that may be limited to vascular smooth muscle cells.

Because tissue-specific promoters have been reported for the Cav1.2 α1C subunit, real-time RT-PCR was used to compare mRNA encoding the α1C/1b isoform between WKY and SHR aorta. Mesenteric artery from SHR demonstrated 47% higher (using RNA standards) to 64% higher (using DNA standards) exon 1b-specific transcript levels compared to WKY rats (Fig. 4, Table 1). Equivalent determinations from aorta also revealed a significant but small increase (23%) of α1C/1b transcript in SHR relative to WKY (Table 1). However, these increases did not approach the three to fourfold increases in α1C and α1C/1b evident at the protein level (Figs. 2 and 3). No significant difference was noted with a Pan CaV1.2 primer pair or exon 1a-specific transcripts tested (Table 1).

**Discussion**

These results indicate overexpression in SHR vasculature, specifically of the short amino terminus Cav1.2 isoform. Brain and visceral smooth muscle expressing the same isoform are not similarly affected. The modest increases in message levels could be due to increases in transcription or message stability but are insufficient to account for the differences in isoform-specific protein levels. These results

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**FIG. 4.** Relative quantitation of Cav1.2 transcripts in SHR and WKY tissue. (A) Real-time reverse transcriptase–polymerase chain reaction curves of Sybr Green fluorescence from individual mesenteric artery samples of three SHR and three WKY rats. (B) Melting curves of PCR products obtained in (A). Leftmost peak represents negative control (absence of template); all other samples displayed the same higher melting temperature. (C) Blow-up of the boxed region in (A), demonstrating only small differences in cycle threshold between SHR and WKY rat samples.
Using RNA standards 1b-specific form of the channel’s message as before21 and degradation of channel protein. Although Ca channel trafficking to the surface membrane, or a reduced rate of efficient translation of message into protein, more efficient expression and increased L-type Ca current, including more events could in principle explain greater Ca channel ex-
tional, and primarily due to overexpression of the exon cDNA ends) and sequenced the 5′ affect translation, we used RACE (rapid amplification of protein implicated in the pathogenesis of hypertension. for the CaV1.2 Ca channel (process would represent a novel form of regulation, either or selectively degrade the short amino terminus form of the channel. 18 –21 However, if the 
ble increased amino-terminal isoform. tis suggest that some other isoform(s) of the channel must not be upregulated, although relative functional activity among such isoforms is unknown. The results reported here with CaV1.2 would not explain any increases in Ca current reported in cardiac myocytes in some 26 but not all studies,27 because those cells express the exon 1a-encoded form of the channel.18–21 However, if β2 expression were increased in heart of SHR rats, that could result in an increase in functional Ca channels at the surface membrane, and protein kinase C (PKC) may preferentially increase activity in the cardiac form of the channel.18 Pesic et al28 reported that hypertension due to aortic banding causes a vascular smooth muscle cell depolarization, which in turn causes the increased CaV1.2 expression. Although this finding implies that hypertension causes the increase in Ca channel expression, that increased Ca channel expression most likely also contributes to the hypertension, as Ca channel blockers can normalize BP in both SHR rats8 and hypertensive humans.10 Differential CaV1.2 alternative splicing between SHR and WKY has not been assessed and could also contribute to both hypertension and its normalization by blockers.

Table 1. Quantitation of CaV1.2 mRNA levels in SHR and WKY vasculature

<table>
<thead>
<tr>
<th>Exon 1b-encoded</th>
<th>Pan CaV1.2</th>
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<tbody>
<tr>
<td><strong>Using DNA standards</strong></td>
<td></td>
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<tr>
<td>Aorta</td>
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</tr>
<tr>
<td>SHR</td>
<td>1.23 ± 0.06 (n = 4)</td>
</tr>
<tr>
<td>WKY</td>
<td>1.00 ± 0.04 (n = 4)</td>
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<tr>
<td>Mesenteric artery</td>
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<tr>
<td>SHR</td>
<td>1.64 ± 0.25 (n = 3)</td>
</tr>
<tr>
<td>WKY</td>
<td>1.00 ± 0.12 (n = 3)</td>
</tr>
<tr>
<td><strong>Using RNA standards</strong></td>
<td></td>
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<tr>
<td>Mesenteric artery</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>1.47 ± 0.02 (n = 3)</td>
</tr>
<tr>
<td>WKY</td>
<td>1.00 ± 0.12 (n = 3)</td>
</tr>
<tr>
<td><strong>Exon 1a-encoded</strong></td>
<td></td>
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<tr>
<td>Aorta</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>0.89 ± 0.13 (n = 4)</td>
</tr>
<tr>
<td>WKY</td>
<td>1.00 ± 0.10 (n = 4)</td>
</tr>
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n = independent samples, ie, animals.
Values given are mean ± SD.
* P < .05.
† Reactions run with randomly primed cDNA.

are consistent with the report of a 1.53-fold increase in CaV1.2 message accompanied by a 3.4-fold increase in CaV1.2 protein in SHR mesenteric arteries.14 Our results also suggest that the upregulation in SHR vasculature of CaV1.2 protein is primarily translational or post-transla-
tional, and primarily due to overexpression of the exon 1b-encoded amino-terminal isoform.

Modification of any of several post-transcriptional events could in principle explain greater Ca channel expression and increased L-type Ca current, including more efficient translation of message into protein, more efficient trafficking to the surface membrane, or a reduced rate of degradation of channel protein. Although Ca channel β subunits are known to act as chaperones to direct CaV1.2 to the surface membrane,23–25 the crude membrane fraction used in our study for Western blots is unlikely to be enriched in surface membranes as opposed to endoplasmic reticulum, Golgi, or endosomes, and should therefore be incapable of reflecting more efficient trafficking. Consequently, the other two explanations of our results are considered more likely.

We conclude that SHR vasculature must either translate protein from exon 1b-specific transcripts more efficiently or selectively degrade the short amino terminus form of the protein more slowly than the WKY vasculature. Either process would represent a novel form of regulation, either for the CaV1.2 Ca channel (α1C) or for any transport protein implicated in the pathogenesis of hypertension.

Because untranslated regions (UTRs) of mRNA can affect translation, we used RACE (rapid amplification of cDNA ends) and sequenced the 5′ UTR of the exon 1b-specific form of the channel’s message as before21 and found no differences between aortas of SHR and WKY rats. Similarly, sequencing of the 3′ UTR of the channel revealed no sequence differences between SHR and WKY. Thus, it is very unlikely that any genetic modification in SHR involves regulatory elements in the CaV1.2 gene. The relative lengths of poly(A) tails, which can affect message stability, have not been tested.

Our Western blot results do not automatically indicate increased L-type Ca current in SHR vasculature because many of the additional channels could be intracellular or exhibit low channel activity. Quantitation of Western blots indicate greater increases of exon 1b-encoded channel protein than of the reported 1.35 to 1.62 in current. Differences between results with the two antibodies suggest that some other isoform(s) of the channel must not be upregulated, although relative functional activity among such isoforms is unknown. The results reported here with CaV1.2 would not explain any increases in Ca current reported in cardiac myocytes in some26 but not all studies,27 because those cells express the exon 1a-encoded form of the channel.18–21 However, if β2 expression were increased in heart of SHR rats, that could result in an increase in functional Ca channels at the surface membrane, and protein kinase C (PKC) may preferentially increase activity in the cardiac form of the channel.18 Pesic et al28 reported that hypertension due to aortic banding causes a vascular smooth muscle cell depolarization, which in turn causes the increased CaV1.2 expression. Although this finding implies that hypertension causes the increase in Ca channel expression, that increased Ca channel expression most likely also contributes to the hypertension, as Ca channel blockers can normalize BP in both SHR rats8 and hypertensive humans.10 Differential CaV1.2 alternative splicing between SHR and WKY has not been assessed and could also contribute to both hypertension and its normalization by blockers.
The present findings related to SHR may not apply to other animal models of hypertension or to hypertensive humans. Pesic et al. demonstrated that the depolarization associated with high BP in aortic-banded hypertensive rats was also associated with an increased Ca\textsubscript{v}1.2 protein in the affected renal artery vascular smooth muscle cells. However, because channel message levels were not determined, the cause of the increased Ca channel expression could be primarily transcriptional rather than post-transcriptional. Similarly, hypertension induced by the Goldblatt model of renal artery clipping increased Ca current in the basilar artery, but there have been no biochemical studies of vascular Ca channel expression using this model. Pulmonary hypertension in neonatal piglets has been associated with increases in both \alpha\textsubscript{1C} and \beta\textsubscript{2} subunits. Enhanced sensitivity to Bay K 8644 has been reported for vasculature from deoxycorticosterone acetate (DOCA)- and salt-treated Sprague-Dawley rats, to reported for vasculature from deoxycorticosterone acetate and other classes of antihypertensive drugs in spontaneously hypertensive rats. Hypertens Res 1996;19:247–254.

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