Expression of $\text{Ca}^{2+}$ Transport Genes in Platelets and Endothelial Cells in Hypertension

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Abstract—Altered $\text{Ca}^{2+}$ handling is observed in different cells in essential hypertension. We investigated the expression of sarco(endo)plasmic reticulum $\text{Ca}^{2+}$-ATPase (SERCA) and inositol 1,4,5-trisphosphate receptor (IP$_3$R) isoforms in platelets and aortic endothelial cells (EC) isolated from spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats by ratio reverse-transcriptase—polymerase chain reaction (RT-PCR) analysis and Western blotting. SERCA2b and SERCA3 were assessed at mRNA (EC and platelets) and at protein level (platelets). IP$_3$R1, IP$_3$R2, and IP$_3$R3 mRNAs were demonstrated in both cell types, but only IP$_3$R1 and IP$_3$R2 proteins were detected in platelets. Compared with WKY, SHR EC and platelets showed higher SERCA3 and IP$_3$R2 expression and lower IP$_3$R1 expression. We then investigated the effect of lisinopril (20 mg $\cdot$ kg$^{-1} \cdot$ d$^{-1}$; 10-week treatment of 4-week-old rats or 2-week treatment of adult rats) and captopril (100 mg $\cdot$ kg$^{-1} \cdot$ d$^{-1}$; 2-week treatment of adult rats). Consequently, expression patterns of SERCAs and IP$_3$Rs were significantly modified. Except for SERCA mRNA in platelets, all differences between SHR and WKY disappeared. However, SERCA3 remained the predominant isoform. Both EC and platelets demonstrated a high equal expression of IP$_3$R2 mRNA. IP$_3$R1 was the predominant platelet protein isoform, as it was in untreated WKY. mRNA was also isolated from pancreatic islets of WKY and SHR, but no effect of either rat strain or of lisinopril treatment was observed on the expression of the studied genes. We hypothesize that the identical expression pattern of SERCAs and IP$_3$Rs after treatment with ACE inhibitors represents a different nonhypertensive configuration, which, through changes in intracellular $\text{Ca}^{2+}$ handling, improves endothelial and platelet dysfunction in SHR but has no effect in WKY. (Hypertension. 2001;37:135-141.)

Key Words: inositol 1,4,5-trisphosphate receptor $\bullet$ sarco(endo)plasmic reticulum $\text{Ca}^{2+}$-ATPase $\bullet$ receptors, angiotensin $\bullet$ angiotensin-converting enzyme inhibitors $\bullet$ platelets $\bullet$ endothelium $\bullet$ hypertension, experimental

High blood pressure (BP) is a major risk factor for cardiac and vascular diseases, which are among the most frequent causes of morbidity and mortality in Western countries. There is strong evidence that platelets and vascular endothelial cells (EC) play a central role in the pathogenesis and progression of hypertension.1-3 Endothelium is strategically located between the circulating blood and the vascular smooth muscles and is known to synthesize many factors involved in either inhibition or promotion of platelet and smooth muscle activation. Conversely, platelets release substances vital for normal EC function.4

All pathways leading to platelet activation result in an elevation of the cytosolic $\text{Ca}^{2+}$ concentration ([Ca$^{2+}$]). Increased platelet sensitivity to agonists is observed in hypertension, which may be related to the significantly higher resting levels of platelet [Ca$^{2+}$], found in patients with borderline or established hypertension.4,5 In addition, basal and stimulated [Ca$^{2+}$]$_c$ was elevated in platelets isolated from 4-week-old spontaneously hypertensive rats (SHR), indicating that abnormalities in platelet $\text{Ca}^{2+}$ metabolism might precede development of overt hypertension in SHR.6 In disease states, endothelial dysfunction contributes to enhanced vasoconstrictor responses, adhesion of platelets and monocytes, as well as proliferation and migration of vascular smooth muscle cells.7 In aortic EC of SHR, resting [Ca$^{2+}$]$_c$, was found to be significantly lower than that of EC from normotensive Wistar-Kyoto rats (WKY). Moreover, bradykinin and thapsigargin-induced increases in [Ca$^{2+}$]$_c$, in SHR EC were selectively and markedly impaired.8 These data could explain the decreased release of nitric oxide in SHR EC and, consequently, the increased basal vascular tone.9

According to Kuo et al.,10 genes encoding major $\text{Ca}^{2+}$ transport pathways, including Ca$^{2+}$ pumps and inositol 1,4,5-trisphosphate receptor (IP$_3$R) channels are regulatorily linked. This link is provided by the Ca$^{2+}$ load of the
endoplasmic reticulum. Hence, expression of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) might be a more sensitive indicator of the free-exchangeable Ca\(^{2+}\) in cells (in particular in platelets, which lack voltage-dependent Ca\(^{2+}\) channels) than resting [Ca\(^{2+}\)]\(_{i}\). SERCA3 has already been found to be overexpressed in SHR platelets. In addition, SERCA3 knockout mice showed an impairment of both endothelium-dependent relaxation of aortic smooth muscle and endothelial Ca\(^{2+}\) signaling in response to acetylcholine. The mere magnitude of agonist-evoked [Ca\(^{2+}\)]\(_{i}\) responses may poorly reflect the true nature of the cellular Ca\(^{2+}\) signals. These signals spread from discrete domains within cells, and their frequency, duration, and temporal distribution determine different cell functions. Temporal patterns of Ca\(^{2+}\) signals depend critically on the expressed set of IP\(_3\) R subtypes. Angiotensin II stimulation observed in essential hypertension has been related to a defect in the phosphatidylinositol/ Ca\(^{2+}\) signaling pathway.

The aim of the present study was to explore the molecular basis of altered Ca\(^{2+}\) handling observed in essential hypertension. We therefore analyzed the expression patterns of the SERCA and IP\(_3\) R isoforms both in platelets and in EC isolated from SHR and WKY. Effects of angiotensin-converting enzyme (ACE) inhibitors applied at different time points in animals.

**Methods**

Experiments were performed in male, age-matched WKY and SHR (Harlan UK Ltd., Oxon, UK). Because WKY and SHR have numerous genetic characteristics not related to the difference in BP, age-matched male Wistar rats (WR) were used as a control for the WKY strain (Proefdierencentrum, K.U. Leuven, Leuven, Belgium). BP levels and expression patterns of SERCAs and IP\(_3\) Rs were identical in WR and WKY (data not shown). Animals were assigned to one of the following protocols (where \(n\) is number of rats per strain): 14-week-old untreated WKY and SHR (\(n=10\)); 4-week-old WKY and SHR treated for 10 weeks with 20 mg \(\cdot\) kg \(^{-1}\) \(\cdot\) d \(^{-1}\) lisinopril (Sigma-Aldrich; \(n=6\)); 14-week-old WKY and SHR treated for 2 weeks with 20 mg \(\cdot\) kg \(^{-1}\) \(\cdot\) d \(^{-1}\) lisinopril (Sigma-Aldrich; \(n=12\)); 14-week-old WKY and SHR treated for 2 weeks with 100 mg \(\cdot\) kg \(^{-1}\) \(\cdot\) d \(^{-1}\) captopril (Sigma-Aldrich; \(n=6\)). Rats were placed in metabolic cages in a room maintained at 22°C with a 12-hour dark-light cycle. Animals were fed standard rat chow (Muracon-G, Carfil Huybrechts) ad libitum. Water intake was monitored daily, and rats were weighed regularly. Drugs were dissolved daily in drinking water, to which rats had free access. Drug concentration was adjusted according to body weight and water consumption. Systolic BP was measured by a standard tail-cuff method (Harvard Apparatus Ltd). Experimental procedures were approved by the local ethics committee for experiments in animals.

**Cell Isolation**

Rats were anesthetized with 100 mg/kg phenobarbital. Abdominal aortas were cannulated, and blood was withdrawn in heparinized syringes. Platelets were separated by NycoPrep animal 1.077 (14.1% Nycodenz solution, Nycodemed Pharma AS). EC\(^{17}\) and pancreatic islets\(^{18}\) were isolated by collagenase digestion as previously described. Pancreatic islets were chosen because they are a tissue not related to BP regulation. Cells were collected by centrifugation, quickly frozen in liquid N\(_{2}\), and kept at \(-80°C\) until use. Ratio reverse-transcriptase–polymerase chain reaction (RT-PCR) analysis for \(\alpha\)-smooth muscle actin was used to demonstrate the absence of smooth-muscle-cell contamination in isolated EC (data not shown).

**mRNA Isolation and Ratio RT-PCR Analysis**

PolyA RNA was prepared with Micro-FastTrack kit (Invitrogen Co). Oligo(dt)-primed first-strand cDNA was synthesized by Moloney murine leukemia virus reverse-transcriptase (Gibco-BRL). Ratio RT-PCR used in the present study was based on simultaneous amplification of \(\geq 2\) targets that comprised identical primer template sequences. Amplified products were discriminated by restriction enzymes with specific cleavage sites in diverging areas of the targets as previously published. Primer sets and PCR protocols were described earlier. Amplification was performed with AmpliTaq Gold (Perkin Elmer) in 50-\(\mu\)L amplification reaction mixture. After the primary PCR reaction was complete, radioactive labeling was done by 20-fold dilution of the reaction product in fresh amplification mixture containing 15 nCi/\(\mu\)L [\(\alpha\)-\(\beta\)]dATP (Amersham Pharmacia Biotech), and four additional PCR cycles were given. Labeled amplification products and their restriction fragments were separated on a 6% polyacrylamide gel.

**Microsomes, Antibodies, and Western Blotting**

Platelet microsomal membranes were prepared as previously described. Protein concentration was determined by the bicinchoninic acid method (Pierce), with BSA as standard. Microsomal proteins were separated by SDS-PAGE on 3% to 12% linear gels and transferred to Immobilon-P (Millipore Co). The isoform-specific polyclonal antibodies raised in our laboratory against N-terminus of SERCA3 (N89), C-terminus of SERCA2b, IP\(_3\) R1 (Rbt03), or IP\(_3\) R2 (Rbt02), and the commercial monoclonal antibody against IP\(_3\) R3 (Transduction Laboratories) were previously described. For determination of the ratio of SERCA2b/SERCA3, we developed a new antibody (TRY2), with equal affinity for SERCA2a, SERCA2b, or SERCA3. This antibody was raised against a peptide (PDPRAVNQDKKN) corresponding to the common amino acids stretch 195-206 of rat SERCA2a/SERCA2b/SERCA3 coupled to keyhole limpet hemocyanin. This epitope corresponds to a well-exposed tryptic cleavage site, and certainly after SDS-PAGE it is expected to be equally accessible in the different isoforms. Validation of TRY2 is shown in Figure 1, in which its immunoreactivity is compared with that of the isoform-specific antibodies against N-terminus of SERCA3 (N89) and the C-terminus of SERCA2b, respectively. An alkaline phosphatase–coupled anti-rabbit antibody was used as a secondary antibody. Immunoreactivity was visualized by conversion of the Vistra ECF substrate into a fluorescent probe (Amersham Pharmacia Biotech). The 2 SERCA isoforms present in platelets (SERCA2b and SERCA3) differ in electrophoretic mobility, and their ratio was calculated by direct comparison of the signals obtained with the TRY2 antiserum. For the various IP\(_3\) R isoforms, the intensity of the signals recorded with the isoform-specific antibodies was compared with those obtained in RBL-2H3 mast mucosal cells, known to express the 3 IP\(_3\) R isoforms at a protein ratio of 10/70/20.

**Quantification of SERCA and IP\(_3\) R Isoforms**

Phosphorimaging (for radioactively labeled products) and fluorimaging (for fluorescently labeled products) were chosen as very sensitive methods for the quantitative analysis. For accurate volume quantification of the signals, both imaging techniques were performed using the Storm 840 polyvinyldene fluoride membranes equipped with the ImageQuant 4.2 software (Mo-
molecular Dynamics). Volume was defined as the integrated intensity of all the pixels in the spot after subtraction of the background intensity and was proportional to the amount of the mRNA or the protein present in the band. The validation of this method, the linearity of the signal with the quantity of the applied material (over a 10-fold concentration range for fluorescent samples and over a 100-fold range for radioactive samples), was published previously.\(^19,22\) Because \(\alpha-\delta^3\text{P}\)dCTP was used for DNA labeling to calculate isoform ratios, band intensities were corrected for CG content of amplified sequences. To investigate absolute changes in expression in platelets and in EC, mRNA levels of SERCA and IP\(_3\)R were correlated to these of 2 different housekeeping enzymes, GAPDH and cyclophilin. No discrepancy in the results depending on the use of either standard has been found. In the pancreatic islets, only cyclophilin was used as a control housekeeping gene, because it has been shown that the GAPDH level in these cells depends critically on small changes in plasma glucose concentration.\(^17\)

Results are expressed as mean±SEM. Statistical analysis was accomplished by a \(t\) test, and values were considered significantly different at \(P<0.05\).

**Results**

**BP Measurements**

Systolic BP of untreated adult SHR was significantly higher than that of WKY (Figure 2A). At 4 weeks, BP of all rats was normal (Figure 2B). After lisinopril treatment (for 2 or 10 weeks), BP of SHR was, respectively, either normalized (Figure 2C) or its rise was prevented (Figure 2B). A 2-week captopril treatment was as effective as lisinopril application (Figure 2D).

**Expression Pattern of SERCA Isoforms**

In platelets, the SERCA3 mRNA level was considerably higher than that of SERCA2b, but the difference was significantly larger in adult nontreated SHR versus WKY (Figure 3A). Captopril and lisinopril treatment (2 or 10 weeks) did not change the expression pattern of SERCA mRNA in either strain. However, at the protein level, the situation was different (Figure 3B). Although SERCA3 proteins were also more elevated in adult untreated SHR versus WKY, this difference disappeared after application of either lisinopril or captopril.

EC were studied only at the mRNA level (Figure 3C), because the amount of EC that could be isolated from rat aorta was insufficient for microsomal preparation and protein analysis. In addition, we previously demonstrated that the expression pattern of both SERCA and IP\(_3\)R rapidly changed when EC were brought into culture.\(^17\) EC of WKY had approximately equal levels of the 2 SERCA mRNAs, whereas in SHR EC (as in platelets) SERCA3 was significantly higher. Lisinopril or captopril treatments eliminated the difference in expression between the 2 rat strains, which left SERCA3 as the predominant isoform. In pancreatic islets, SERCA2b and SERCA3 mRNA levels were equal in both rat strains, and their expression pattern was not changed on lisinopril application (Figure 3D).

**Expression Pattern of IP\(_3\)R Isoforms**

At mRNA level, a large difference in IP\(_3\)R expression patterns was observed between platelets isolated from WKY and SHR.
Although in WKY IP3 R1 was predominant, IP3 R2 was the main isoform in SHR. After lisinopril or captopril treatment, the relative expression pattern became identical in both strains, with a predominance of IP3 R2 (Figure 3A). At the protein level (Figure 3B) we detected only IP3 R1 and IP3 R2. IP3 R3 remained undetectable, even when a 10-times-higher amount of microsomal proteins was used in the assay. Similar to what was observed at the mRNA level, IP3 R2 protein was higher in adult SHR, although IP3 R1 was the main isoform in WKY. Notably, after lisinopril or captopril treatment, the expression pattern in both strains was identical to that of untreated adult WKY (ie, IP3 R1 was predominantly expressed).

mRNA distribution in EC closely resembled that of platelets: IP3 R1 was predominant in WKY, whereas in SHR it was IP3 R2. Lisinopril or captopril treatment, the relative expression pattern became identical in both strains, with a predominance of IP3 R2 (Figure 4A). At the protein level (Figure 4B) we detected only IP3 R1 and IP3 R2. IP3 R3 remained undetectable, even when a 10-times-higher amount of microsomal proteins was used in the assay. Similar to what was observed at the mRNA level, IP3 R2 protein was higher in adult SHR, although IP3 R1 was the main isoform in WKY. Notably, after lisinopril or captopril treatment, the expression pattern in both strains was identical to that of untreated adult WKY (ie, IP3 R1 was predominantly expressed).

mRNA distribution in pancreatic islets from both strains displayed a similar isoform expression pattern. As was found for the SERCA, a 2-week lisinopril application did not affect the expression in this tissue (Figure 4D). None of the investigated cell types showed significant changes in total mRNA expression levels of SERCAs or IP3 Rs when correlated with mRNA levels of GAPDH or cyclophilin (data not shown).

Discussion

The present study demonstrates different expression patterns of SERCA and IP3 R in platelets and EC isolated from WKY and SHR. Moreover, we show that treatment with 2 ACE inhibitors, lisinopril and captopril, independently of their chemical structure, strongly and identically modified expression of these Ca2+-transporting genes in platelets and EC of both strains. The effect of the ACE inhibitors did not depend on the BP level, given that the same results were obtained when treating prehypertensive, 4-week-old SHR. Observed changes in expression of SERCA and IP3 R isoforms appeared specifically related to cells connected with BP regulation; no modifications were demonstrated in WKY and SHR pancreatic islets.

Untreated Adult Animals

SERCA3 was overexpressed in platelets of SHR compared with WKY, which confirms previous results of Bobe et al.12 IP3 R2 was the main isoform in SHR, whereas IP3 R1 was predominant in WKY. Despite the presence of its mRNA, IP3 R3 protein was undetectable in platelets, as also stated by others.24 The observed differences in the expression patterns of SERCA and IP3 R isoforms might be physiologically significant, because a higher [Ca2+]c, was reported in SHR...
platelets and it is known that SERCA3 is the isoform with the lowest Ca\textsuperscript{2+} affinity, whereas IP\textsubscript{3} R2 is the channel with the highest IP\textsubscript{3} affinity.\textsuperscript{15,21}

Although Wang et al\textsuperscript{8} showed a lower endothelial [Ca\textsuperscript{2+}] in SHR, we observed, for both SERCA and IP\textsubscript{3} R, a similar shift in expression pattern in EC as in platelets. However, note that the study of Wang et al was performed in cultured EC. As already mentioned, a rapid change in SERCA and IP\textsubscript{3} R expression pattern, characterized by a decrease of SERCA3 and an increase of IP\textsubscript{3} R3, was observed once EC were brought into culture.\textsuperscript{17}

On the basis of our results in platelets and EC, we hypothesized that the differences in expression between WKY and SHR could be either a consequence or a prerequisite for BP rise. This assumption was further supported by identical SERCA and IP\textsubscript{3} R expression patterns found in WKY and SHR pancreatic islets, a tissue not related with BP regulation (Figures 3D and 4D).

ACE Inhibitor Treatment
To test the above hypothesis, we first treated WKY and SHR with lisinopril. As an ACE inhibitor, lisinopril has, together with Ca\textsuperscript{2+} entry blockers, the advantage to revert structural changes observed in hypertension. Ca\textsuperscript{2+} entry blockers were not used in the present study because they directly interfere with Ca\textsuperscript{2+} metabolism and thus could be expected to affect the expression of proteins connected with Ca\textsuperscript{2+} homeostasis. Lisinopril either normalized BP in adult SHR or prevented its rise during the 10-week treatment of 4-week-old SHR. Remarkably, the lisinopril application in platelets and EC yielded nearly identical SERCA and IP\textsubscript{3} R expression patterns in both rat strains. In platelets at the protein level, the drug even induced a reversion of the expression pattern to that of untreated, adult WKY. The difference in results obtained at the mRNA and protein levels might be either because the isolated mRNAs do not reflect the actual protein composition (because protein synthesis is impaired in platelets) or in platelets additional posttranslational mechanisms determine final protein expression. Furthermore, the effect of the drug did not correlate with BP level per se, given that it was the same in both groups of SHR with or without developed hypertension.

One plausible explanation of the present data could be that lisinopril has a specific but general action on expression of SERCA and IP\textsubscript{3} R genes. We checked this hypothesis in pancreatic islets, which possess the same 2 SERCA and 3 IP\textsubscript{3} R isoforms and which, as platelets\textsuperscript{27} and EC,\textsuperscript{28} contain angiotensin receptors (AT).\textsuperscript{29} However, as shown in Figures 3D and 4D, the drug treatment did not modify the expression of the studied genes. Therefore, effects of lisinopril seem to be limited to particular cells types involved in BP regulation.
ACE inhibitors differ in chemical structure of their active moieties, which is connected with some of their known side effects.30 Hence, we treated adult WKY and SHR with captopril, which is the prototype of the sulfhydryl-containing ACE inhibitors, in contrast to lisinopril, which contains a carboxyl moiety. However, results were the same as with lisinopril, both concerning the effect on BP and on the modifications in expression patterns of SERCAs and IP,Rs (Figures 3C and 4C). Thus, we assume that ACE inhibitors modified the expression of SERCA and IP,R Ca2+-transporting proteins independently of their chemical structure.

ACE inhibitors, in addition to their effect on BP, reverse endothelial dysfunction observed in hypertension and affect structure of vessel walls.31 In addition to inhibiting the renin-angiotensin system, ACE inhibitors diminish inactivation of bradykinin, thus leading to an augmentation of nitric oxide release. In addition, these compounds stabilize the B2-receptor and reduce oxidative stress and formation of cyclo-oxygenase–dependent endothelium-derived contracting factors.31 Moreover, angiotensin II has growth-modulating actions that depend on the type of angiotensin receptors present on a given cell.26 The growth-promoting effect is mediated through AT1, which through G proteins activate phospholipase C and thus the diacylglycerol and inositol 1,4,5-trisphosphate pathway. This action in turn leads to an elevation of [Ca2+]c in platelets and EC.28 The treatment of the ACE inhibitors leads to an identical expression pattern in WKY and SHR, both at the mRNA and protein levels. An important observation is that at the protein level in platelets, the expression of SERCAs and IP,Rs is far from that found in WKY and is more similar to that of SHR but has no effects in WKY. Further experiments are needed to delineate through which pathway these modifications are related to the level of [Ca2+]c, in platelets and EC.

Acknowledgments

We acknowledge the skillful assistance of Lea Bauwens, Irene Willems, and Yves Parijs. We thank Dr Paul Lijnen (Hypertension and Cardiovascular Rehabilitation Unit, KU Leuven) for stimulating discussions and advice. This project was financially supported by research grants “Levenslijn” 7.0303.98, G.O.A. 99/08, and 3.4552.98 from the “Fonds de la Recherche Scientifique Médicale (Brussels)” and by the Interuniversity Poles of Attraction Program, Belgium State, Prime Minister’s Federal Office for Scientific, Technical, and Cultural Affairs IUAP P4/23. J.B.P. is Research Associate of the FWO, Flanders, and J.-C.J. is Research Associate of the FNRS, Belgium.

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_Hypertension_. 2001;37:135-141
doi: 10.1161/01.HYP.37.1.135

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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