Na\textsuperscript{+}-H\textsuperscript{+} Exchanger Expression in Vascular Smooth Muscle of Spontaneously Hypertensive and Wistar-Kyoto Rats

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Abstract  The Na\textsuperscript{+}-H\textsuperscript{+} exchanger has important modulatory effects on vascular smooth muscle cell proliferation and contractility. Increased Na\textsuperscript{+}-H\textsuperscript{+} exchange activity is a general property of many tissues, including mesenteric artery and cultured vascular smooth muscle cells, in the spontaneously hypertensive rat (SHR). In the present work, we investigated whether alterations in the steady-state levels of specific Na\textsuperscript{+}-H\textsuperscript{+} exchanger mRNA isoforms (NHE-1 through NHE-4) are associated with the observed increases in exchanger activity. Poly(A\textsuperscript{+}) mRNA prepared from 12-week-old hypertensive SHR and normotensive Wistar-Kyoto (WKY) aorta, kidney, and intestine was hybridized to cDNAs specific for each NHE isoform. By Northern blot analysis, NHE-1 was detected in all tissues as well as cultured vascular smooth muscle cells and was not regulated differently in SHR compared with WKY tissues.

There was no expression of NHE-2, NHE-3, or NHE-4 in SHR and WKY aortas or in cultured vascular smooth muscle cells from SHR and WKY aortas. Stimulation of NHE-1 mRNA expression by growth factors was similar in cultured SHR and WKY vascular smooth muscle cells. We conclude that the previously observed increase in exchanger activity in blood vessels and cultured vascular smooth muscle cells of the SHR is not caused by induction of the NHE-2, NHE-3, and NHE-4 isoforms or by alterations in steady-state NHE-1 mRNA expression. These findings suggest that posttranslational regulation of the Na\textsuperscript{+}-H\textsuperscript{+} exchanger is responsible for increased activity in the SHR. (Hypertension. 1994;24:734-738.)

Key Words  • ion exchange • muscle, smooth, vascular • gene expression • hypertension, spontaneous

The cellular and molecular mechanisms that cause hypertension in spontaneously hypertensive rats (SHR) remain unknown. Alterations in the transmembrane movement of Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} have been observed in intact arteries and cultured vascular smooth muscle cells (VSMCs), but these changes have not been directly linked to increased vascular tone or contractile responses.1-3

The Na\textsuperscript{+}-H\textsuperscript{+} exchanger has important effects on vascular smooth muscle growth and contractility.4-6 Recent evidence indicates that Na\textsuperscript{+}-H\textsuperscript{+} exchanger activity is increased in lymphocytes, platelets, and skeletal muscle from SHR compared with normotensive Wistar-Kyoto (WKY) rats.6,8 Our laboratory has demonstrated that cultured VSMCs from SHR exhibit increased growth and Na\textsuperscript{+}-H\textsuperscript{+} exchange.9 Foster et al10 found that mesenteric arteries from SHR have increased Na\textsuperscript{+}-H\textsuperscript{+} exchange activity. These findings have led to the hypothesis that an increase in Na\textsuperscript{+}-H\textsuperscript{+} exchange may play an important role in the development or maintenance of the hypertensive state.

The cellular regulatory mechanisms responsible for this alteration in VSMC Na\textsuperscript{+}-H\textsuperscript{+} exchange are not yet defined. The finding that there is no linkage between the human Na\textsuperscript{+}-H\textsuperscript{+} exchanger gene and hypertension11 suggests that the abnormality in exchanger function is not caused by mutations in the Na\textsuperscript{+}-H\textsuperscript{+} exchanger gene. Thus, the observed increases in Na\textsuperscript{+}-H\textsuperscript{+} exchanger activity in hypertension might be caused by increased gene expression (transcriptional regulation) and/or increased function of the exchanger protein (posttranslational modification). We chose to focus initially on steady-state mRNA expression as an indicator of transcriptional regulation for two reasons. First, we have previously reported that mitogens affect exchanger activity by directly increasing expression of the Na\textsuperscript{+}-H\textsuperscript{+} exchanger gene in cultured VSMCs.12 Second, there is evidence that hypertension stimulates the expression of several genes in vascular tissue.13-15

Four genes encoding highly homologous Na\textsuperscript{+}-H\textsuperscript{+} exchanger isoforms (NHE-1, NHE-2, NHE-3, and NHE-4) have been identified in rat and shown to be expressed in a tissue-specific manner.16,17 These different isoforms exhibit different sensitivity to amiloride, different affinities for Na\textsuperscript{+} and H\textsuperscript{+}, and appear to be differentially regulated by growth factors and hormones.18 Thus, alterations in isofrom expression (either quantity or type) may cause tissue-specific alterations in Na\textsuperscript{+}-H\textsuperscript{+} exchanger activity.

In the present study, we examined the expression of Na\textsuperscript{+}-H\textsuperscript{+} exchanger isoforms in vascular tissue from WKY rats and SHR and determined whether an increase in isofrom expression accounted for the increased exchanger activity in SHR. To do this, we compared the steady-state levels of Na\textsuperscript{+}-H\textsuperscript{+} exchanger isoform mRNA in intact aorta and cultured aortic VSMCs from SHR and WKY rats. The results indicate that NHE-1 is the only exchanger isoform present in vascular tissue and suggest that transcriptional regulation does not account...
for the observed functional differences in Na⁺-H⁺ exchanger activity in the SHR model of hypertension.

Methods

Cell Culture and Cell Growth

Rat VSMCs were isolated from the thoracic aortas of 12-week-old male rats by enzymatic digestion as previously described. SHR and WKY rats were obtained from Harlan Sprague Dawley Inc, Indianapolis, Ind. All experimental procedures conformed to National Institutes of Health regulations and were approved by the Institutional Animal Care and Use Committee. Institutional guidelines for animal care were followed to limit stress to the animals as much as possible. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 10% (vol/vol) heat-inactivated fetal calf serum (WKY rats and SHR). For all experiments, cells at passage levels 2 through 5 were plated onto 100-mm culture dishes at a density of 2×10⁶ cells per dish. Cell growth was arrested at 70% to 80% confluency for 72 hours by replacement of the medium with DMEM containing 0.4% fetal bovine serum.

For cell growth studies, VSMCs were plated in six-well cluster plates (Costar Corp) at a density of 1×10⁶ to 2×10⁶ cells per well and incubated in DMEM with 10% serum. After 24 hours, cell growth was arrested for 48 hours; the medium was then changed to DMEM with 10% serum, and cell counts were performed every 48 hours with a hemocytometer.

RNA Isolation, Gel Electrophoresis, and Northern Blot Analysis

Total cellular RNA was isolated from various tissues (aorta, kidney, stomach, and intestine) by homogenization of each tissue in a denaturing solution containing 4 mol/L guanidine isothiocyanate, 0.1% 2-mercaptoethanol, and 25 mmol/L sodium citrate, pH 7.0. Poly(A⁺) RNA was selected by passing total RNA in high salt containing 20 mmol/L Tris-HCl buffer (pH 7.5) through an oligo(dT)-cellulose column followed by elution with salt-free 20 mmol/L Tris-HCl (pH 7.5). Poly(A⁺) RNA (3 to 5 μg) was size-fractionated by electrophoresis on a 1% agarose/2% formaldehyde gel. After transfer to Nytran membranes, the RNA was cross-linked to the membrane using UV irradiation (Stratalinker, Stratagene). After 4 hours of prehybridization in 50% (vol/vol) formamide, 5× SSC (1× SSC=0.15 mol/L NaCl, 0.015 mol/L sodium citrate), 5× Denhardt’s solution (1×=0.02% wt/vol) each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 50 mmol/L sodium phosphate (pH 6.5), and 250 μg/mL sheared salmon sperm DNA at 42°C, the Nytran membranes were hybridized in the above solution containing 10% (wt/vol) dextran sulfate and 2×10⁴ to 4×10⁵ cpm/mL cDNA probe for 16 hours at 42°C. The cDNA probes for NHE-1 through NHE-4 were radiolabeled with the use of a Gibco/BRL random primer labeling kit following the manufacturer’s protocol with [α-³²P]dCTP (specific activity, 3000 Ci/mmol; Du Pont–New England Nuclear). After hybridization, the Nytran membranes were washed twice with 0.2× SSC(0.1% sodium dodecyl sulfate at 25°C for 20 minutes and once at 60°C for 20 minutes. The membrane was then exposed to Kodak X-Omat AR x-ray film with an intensifying screen at -70°C for 12 to 24 hours. The apparent molecular weights of the mRNA bands were estimated by comparing their relative mobilities to known RNA standards. Densitometric analysis of the autoradiograms exposed in the linear range of film density was made on a Pharmacia Ultrascan XL laser densitometer using National Institutes of Health IMAGE 1.49 software. For comparison of Na⁺-H⁺ exchanger mRNA expression between tissues and between SHR and WKY tissues, the autoradiographic signal for the Na⁺-H⁺ exchanger isoform was normalized to the GAPDH signal from the same sample to correct for mRNA content and transfer efficiency. Results are presented as mean±SEM. Student’s t test for paired observations was performed, and values of P<.05 indicated statistical significance.

Results

Comparison of Na⁺-H⁺ Exchanger mRNA Expression in Normotensive and Hypertensive Tissues

To investigate whether Na⁺-H⁺ exchanger mRNA levels are altered in hypertensive vascular smooth muscle, we compared the expression of Na⁺-H⁺ exchanger isoforms in intact aorta isolated from SHR and WKY rats. Poly(A⁺) RNA was extracted from aorta and analyzed by RNA blot hybridization using Na⁺-H⁺ exchanger isoform-specific cDNA probes. Poly(A⁺) RNA isolated from stomach, intestine, and kidney was used as positive controls. Figs 1 through 3 show representative blots. NHE-1 mRNA was detected in all tissues examined and appeared as a single transcript with a molecular size of approximately 5.0 kilobarn (Fig 1). To compare the steady-state levels of NHE-1 mRNA from SHR and WKY aorta, we normalized the autoradiographic intensity of the 5.0-kb band to the GAPDH signal. The ratio of NHE-1 to GAPDH in SHR aorta was 1.12±0.01, which was not significantly different from the ratio of 1.13±0.01 in WKY aorta (P>.05, n=3). Similar analyses showed no difference in NHE-1 expression between SHR and WKY stomach, intestine, and kidney. Thus, in 12-week-old SHR rats, NHE-1 gene expression is not upregulated compared with WKY rats of the same age.

The distribution of NHE-2 and NHE-3 mRNA showed a more restricted pattern of expression (Fig 2). Although an NHE-2 mRNA transcript of approximately 5.0 kb was detected in intestine and kidney (more apparent at longer exposures), no NHE-2 mRNA was detected in SHR and WKY aorta (Fig 2) or cultured VSMCs (Fig 4). The NHE-3 mRNA transcript of approximately 5.0 kb was present in kidney and intestine (more apparent at longer exposures), but it was not
Blots show expression of steady-state Na\(^+\)-H\(^+\) exchanger mRNA isoforms NHE-2 and NHE-3 in Wistar-Kyoto (WKY) and spontaneously hypertensive rat (SHR) tissues and cells. Poly(A\(^+\)) RNA was extracted from kidney and aorta, and 5 \(\mu\)g was analyzed by RNA blot hybridization as described under "Methods." \(^{32}\)P-labeled NHE-2-specific cDNA (Ava I-Ava I fragment, base pairs 174-2032 of the coding region) and a \(^{32}\)P-labeled NHE-3-specific cDNA (Pst I-Pst I fragment, base pairs 1153-2434 of the coding region) were used as probes. GAPDH mRNA levels were used to correct for differences in sample purity and gel loading. Results are representative of Northern blots from two different RNA preparations.

Detected in SHR and WKY aorta (Fig 2) or cultured VSMCs (Fig 4). These data indicate that the NHE-2 and NHE-3 isoforms are neither present in vascular smooth muscle nor induced by hypertension.

We used a similar analysis to investigate NHE-4 gene expression in hypertensive tissue. This isoform is expressed in visceral smooth muscle, as shown by Orlowski et al.\(^{16}\) The NHE-4 mRNA, with a molecular size of approximately 4.4 kb, was readily detected as a single transcript in stomach from SHR and WKY rats (Fig 3). However, NHE-4 mRNA was not detected in either WKY or SHR aorta, indicating that this isoform is neither present in normotensive vascular smooth muscle nor induced by hypertension.

Comparison of Na\(^+\)-H\(^+\) Exchanger mRNA Expression in Cultured VSMCs from SHR and WKY Aorta

We have previously demonstrated increased Na\(^+\)-H\(^+\) exchanger activity and cell growth in VSMCs cultured from SHR aorta compared with WKY aorta.\(^{9}\) Since Na\(^+\)-H\(^+\) exchanger activity is an important regulator of VSMC proliferation,\(^{21}\) we compared the steady-state mRNA levels of Na\(^+\)-H\(^+\) exchanger isoforms in quiescent and serum-stimulated cells (Figs 4 and 5). In growth-arrested VSMCs (0.1% serum for 24 hours), the steady-state expression of NHE-1 did not differ between SHR and WKY cells (NHE-1/GAPDH = 0.17±0.03 and 0.16±0.04, respectively). In response to growth stimulation (10% calf serum for 24 hours), NHE-1 mRNA increased approximately threefold in both SHR and WKY cells (NHE-1/GAPDH = 0.17±0.03 and 0.16±0.04, respectively). In response to growth stimulation (10% calf serum for 24 hours), NHE-1 mRNA increased approximately threefold in both SHR and WKY cells (NHE-1/GAPDH = 0.17±0.03 and 0.16±0.04, respectively). The relative magnitude of this increase in expression did not differ significantly between VSMCs derived from SHR (3.6-fold) and WKY rats (3.3-fold). In addition, NHE-2 (Fig 4), NHE-3 (Fig 4), and NHE-4 (Fig 5) mRNAs were not detected in cultured VSMCs. Nevertheless, early passage VSMCs...
from SHR exhibited increased cell growth compared with those from WKY rats, as measured by cell number (Fig 6). These results demonstrated that the enhanced Na\(^+\)-H\(^+\) exchange activity and cell growth in SHR VSMCs were not caused by increases in steady-state Na\(^+\)-H\(^+\) exchanger gene expression.

**Discussion**

This is the first article to describe the expression of all four characterized Na\(^+\)-H\(^+\) exchanger isoforms in blood vessels and cultured VSMCs. The major findings are (1) NHE-1 is the only isoform present in aortic smooth muscle; and (2) the previously observed increase in Na\(^+\)-H\(^+\) exchanger activity in arteries\(^{10,22}\) and cultured VSMCs\(^9\) of the SHR is not caused by increased steady-state mRNA levels of NHE-1 or induction of other Na\(^+\)-H\(^+\) exchanger isoforms. This suggests that regulation of Na\(^+\)-H\(^+\) exchanger gene expression is similar in SHR and WKY aortic smooth muscle. This conclusion is based on the observation that there is no upregulation of NHE-1 mRNA in aorta or cultured VSMCs from SHR compared with normotensive WKY controls. In addition, Na\(^+\)-H\(^+\) exchanger isoform–specific regulation does not explain our findings, as induction of the NHE-2, NHE-3, and NHE-4 isoforms does not occur in hypertensive vascular smooth muscle.

The apparent lack of transcriptional regulation of the vascular Na\(^+\)-H\(^+\) exchanger is somewhat surprising because there is evidence that hypertension induces genespecific expression in vascular and cardiac tissues. Herrera et al\(^{13}\) demonstrated isoform-specific modulation of Na\(^+\)-K\(^+\)-ATPase 2-subunit gene expression in hypertensive vessels (uninephrectomized animals treated with deoxycorticosterone acetate–salt) and in rats treated with angiotensin II. Alterations in expression of both renin and atrial natriuretic peptide mRNA have also been observed in SHR.\(^{23,24}\) It should be noted that in the present study all tissues were harvested from 12-week-old rats. It is possible that at earlier ages there may have been alterations in Na\(^+\)-H\(^+\) exchanger gene expression in blood vessels. Finally, there may be alterations in exchanger expression in smaller resistance arteries, which we did not examine in the present study.

We conclude from our data that increased steady-state mRNA expression does not account for enhanced Na\(^+\)-H\(^+\) exchanger function in aortic smooth muscle cells of 12-week-old SHR. Although the exact mechanisms of increased function are unclear, it is likely that posttranslational modification of the Na\(^+\)-H\(^+\) exchanger is important. Sardet et al\(^{25}\) reported that serine phosphorylation of the exchanger, following growth factor stimulation, correlated in time and magnitude with Na\(^+\)-H\(^+\) exchanger activity. These data suggest that activation of the exchanger resulted from phosphorylation-induced conformational changes. It appears that phosphorylation shifts the pH range over which the “intracellular pH sensor” of the exchanger regulates ion transport.\(^{26}\) In vascular smooth muscle, activation of the exchanger appears to involve protein kinase C and a Ca\(^+\)-calmodulin (CaM)–dependent protein kinase.\(^{27}\) We have previously studied the rapid activation of Na\(^+\)-H\(^+\) exchange in VSMCs and have shown that both protein kinase C–dependent and -independent pathways are involved.\(^{28}\) Other studies reported that both CaM antagonists and intracellular Ca\(^+\) chelation significantly inhibited the exchanger as measured by amiloride-sensitive Na\(^+\) flux and cytoplasmic alkalinization.\(^{27}\) Thus, both protein kinase C and CaM-dependent protein kinases play important roles in the regulation of Na\(^+\)-H\(^+\) exchange in vascular smooth muscle.

These findings have led to the speculation that increases in Na\(^+\)-H\(^+\) exchange in hypertension may be caused by alterations in the kinases and phosphatases that regulate its activity. Support for this premise is provided by reports that several kinases are involved in the maintenance of hypertension in SHR.\(^{20,30}\) In addition, the finding that the human Na\(^+\)-H\(^+\) exchanger gene and hypertension are not linked\(^{11}\) suggests that the abnormality in exchanger function is caused...
by the proteins that regulate its activity rather than in the Na\textsuperscript{+}/H\textsuperscript{+} exchanger itself. This may account for the altered kinetics of Na\textsuperscript{+}/H\textsuperscript{+} exchange in SHR mesenteric arteries described by Foster et al\textsuperscript{10} and Ellstrom et al.\textsuperscript{22} Further studies are required to determine whether phosphorylation of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger differs in SHR and WKY vascular smooth muscle.

The results of the present study along with the observations of other researchers\textsuperscript{9,13} indicate that the relation between Na\textsuperscript{+}/H\textsuperscript{+} exchange and cell growth is not simple. Both SHR and WKY VSMCs express increased levels of NHE-1 when stimulated by serum (Fig 5), as we previously observed with Sprague-Dawley VSMCs,\textsuperscript{12} indicating that NHE-1 is a dynamically regulated gene. Cultured SHR VSMCs have increased Na\textsuperscript{+}/H\textsuperscript{+} exchange and cell growth rates compared with WKY VSMCs (Fig 6). However, there is no difference in the steady-state mRNA levels of NHE-1 or induction of other Na\textsuperscript{+}/H\textsuperscript{+} exchanger isoforms in the cultured cells. These findings suggest that translational and posttranslational regulatory mechanisms are important in Na\textsuperscript{+}/H\textsuperscript{+} exchanger function. Possible mechanisms include differences in the kinases and phosphatases that regulate Na\textsuperscript{+}/H\textsuperscript{+} exchanger phosphorylation as well as in the regulatory proteins that modify the affinity of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger for H\textsuperscript{+}. Future studies with isoform-specific Na\textsuperscript{+}/H\textsuperscript{+} exchanger antibodies will be required to resolve this issue.

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