Altered Na, K-ATPase mRNA Expression in Spontaneously Hypertensive Rats

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We investigated the expression of Na, K-ATPase α and β isoform mRNAs in spontaneously hypertensive rat (SHR) hearts, aortae and kidneys, compared with findings in normotensive Wistar-Kyoto rat (WKY), to obtain insight into the role of these isoforms in the development of hypertension in SHR. We used 4-, 8-, and 16-week-old SHR and WKY. Systolic blood pressure was recorded by tail sphygmomanometry. The expression of Na, K-ATPase α and β isoform mRNAs was analyzed by Northern blot hybridization, using isoform specific cDNA probes. In the ventricles, pre-hypertensive 4-week-old SHR showed a significant 3 to 4-fold increase in the $\alpha 1$ and $\alpha 2$ isoform mRNA accumulation compared with age-matched WKY. The $\alpha 3$ isoform mRNA expression was detected only in 4-week-old SHR hearts. The pattern of $\beta 1$ mRNA expression resembled that of $\alpha 1$ or $\alpha 2$ mRNAs, and a significant 7-fold increase was observed in 4-week-old SHR compared with age-matched WKY. In the aortae, the $\alpha 1$ and $\alpha 2$ isoform mRNAs were expressed, and 4-week-old SHR exhibited a significant 5-fold increase in the $\alpha 1$ and $\alpha 2$ isoform mRNA levels compared with age-matched WKY. In the kidneys, only the $\alpha 1$ isoform mRNA was expressed, and that of SHR showed a significant 2-fold increase compared with age-matched WKY. We conclude that the expression of Na, K-ATPase α and β isoform mRNAs is significantly increased in the hearts, aortae and kidneys of SHR before the onset of hypertension. These findings suggest that, in this model, the altered expression of Na, K-ATPase gene is an early, if not a primary, event in the development of hypertension. (*Hypertension Res* 1993; 16: 269-274)

Key Words: Na, K-ATPase, Na, K-pump, mRNA, gene expression, spontaneously hypertensive rat

Na, K-ATPase (Na, K-pump), a ubiquitous transmembrane protein, plays an important role in regulating intracellular Na^+ and K^+ levels. This functional enzyme is composed of two subunits, a catalytic α subunit ($M_r = 110,000$), and a glycosylated β subunit ($M_r = 35,000$). It is generally agreed that non-vectorial functions of Na, K-ATPase, such as ATPase activity and cardiac glycoside binding, are localized to the α subunit. At least three α subunit isoforms, $\alpha 1$, $\alpha 2$ and $\alpha 3$, have been characterized in rats (1) and humans (2) by cDNA cloning and sequencing. Although the functional role of the β subunit is unknown, two types of β subunit isoforms, $\beta 1$ and $\beta 2$, have also been identified (3-5). The functional significance of the multiple α isoforms is not clear, although specific differences in sensitivity to cardiac glycosides inhibition and affinity to intracellular Na^+ concentration have been reported (6,7). The mRNA level of the α isoform varies among different tissues and stages of development (8). For example, in the rat heart, the $\alpha 1$ isoform mRNA is the major isoform expressed throughout all developmental stages, while the $\alpha 2$

isoform mRNA is present primarily in juvenile and adult hearts, and the $\alpha 3$ isoform mRNA is found mainly in fetal and neonatal hearts.

The spontaneously hypertensive rat (SHR) is a genetic model of hypertension whose intracellular Na^+ levels might be increased (9-11) which is assumed to contribute to the pathogenesis of hypertension. Previous studies on Na, K-ATPase in SHR tissues have been on measurements of Na, K-ATPase activity or of ouabain binding sites, and various conflicting results have been obtained (12-19). In this study, we investigated the expression of Na, K-ATPase α and β isoform mRNAs in SHR hearts, aortae and kidneys, compared with findings in normotensive Wistar-Kyoto rat (WKY), to obtain insight into the role of these isoforms in the development of hypertension in SHR.

Methods

Materials

SHR and WKY were purchased from Charles River Japan and fed normal rat chow throughout the

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study. Systolic blood pressure was recorded by tail sphygmomanometry.

Northern Blot Analysis

Total RNA was isolated from SHR and WKY left ventricles, aortae, and kidneys by the guanidine isothiocyanate-cesium chloride (GITC-CsCl) method (20,21). Equal amounts of total RNA (5–15 μ g) were size-fractionated by electrophoresis on denaturing 1.0% agarose/formaldehyde gels and transferred to nylon membranes (Hybond N⁺, Amersham, UK). Hybridization was performed for 24 h at 65°C with Na, K-ATPase α 1, α 2, α 3 and β 1 isoform specific cDNA probes (specific activity $> 1 \times 10^8$ cpm/ μ g DNA) labeled with ³²P-dCTP (Amersham, UK), using a multiprime DNA labeling kit (Amersham, UK). The α 1 cDNA probe consisted of a 2.2 kb Nco restriction fragment (1). The α 2 cDNA probe consisted of a 2.9 kb fragment restricted by Sac I (1). The α 3 cDNA probe consisted of a 1.6 kb Sac I-Eco RI restriction fragment (1). The β 1 cDNA probe consisted of a 0.9 kb Pst I restriction fragment (3). Following hybridization, filters were washed once in 2xSSPE (1xSSPE contains 150 mmol/l NaCl, 10 mmol/l NaH₂PO₄ and 1 mmol/l EDTA) with 0.1% SDS for 15 min at room temperature and then twice in 1xSSPE with 0.1% SDS for 15 min at 65°C. Filters were exposed to Kodak XAR-5 film for 1–2 days at –80°C with intensifying screens. Radioactivity was quantitated by densitometric scanning (Immunomedica Image Analyzer TIF-64, Japan) and normalized on the basis of 18 S or 28 S rRNA to correct for RNA loading.

Statistical Analysis

Data are reported as mean $\pm$ SE. Statistical analysis was performed using the unpaired *t*-test, two-tail method, with *p* values less than 0.05 considered statistically significant.

Results

Systolic blood pressure (SBP) of the 4-week-old SHR was 119 ± 2.3 mmHg, not significantly different from that of the age-matched WKY (117 ± 3.5 mmHg) (Fig. 1). However, at 8 weeks of age, the SBP of SHR significantly exceeded that of WKY (177 ± 1.0 vs. 141 ± 1.3 mmHg; $n=12$, $p<0.01$). From this analysis, we observed that SHR exhibited three stages of spontaneous hypertension: pre-hypertensive (4 weeks), early hypertensive (8 weeks) and established hypertensive (16 weeks). We investigated the expression of Na, K-ATPase α and β isoform mRNAs in the left ventricles, aortae and kidneys at each of the three stages of hypertension in SHR and in age-matched WKY.

Na, K-ATPase α 1, α 2, α 3 and β 1 mRNAs were detected in hearts by Northern blot analysis (Fig. 2). The α 1 mRNA signal was detected as a 3.7 kb band, the α 2 mRNA signal was detected as two bands of 5.3 kb and 3.4 kb, the α 3 mRNA was detected as a 3.7 kb band, and the β 1 mRNA was detected as a broad band with an approximate size of 2.0 kb. The levels of both α 1 and α 2 mRNAs re-

Fig. 1. Systolic blood pressure (SBP) of spontaneously hypertensive rat (SHR) and normotensive Wistar-Kyoto rat (WKY) during 4 to 16 weeks of age. SHR exhibited three stages of spontaneous hypertension; pre-hypertensive (4 weeks), early hypertensive (8 weeks) and established hypertensive (16 weeks). Data are the mean $\pm$ SE of 12 samples. * $P<0.01$ compared with age-matched WKY.

mained essentially equivalent in SHR throughout the early (8 weeks) and the established periods of hypertension (16 weeks). In contrast, the levels of α 1 and α 2 mRNAs in WKY increased steadily during the same time period. The neonatal α 3 isoform mRNA was detected only in the pre-hypertensive (4 weeks) SHR ventricles. In the quantitative analysis of Northern blot autoradiography, at the age of 4 weeks, there was a significant 4-fold increase in the amount of α 1 and α 2 mRNAs in SHR compared with WKY (3.30 ± 0.27 and 4.04 ± 0.43 , respectively; $n=6$, $p<0.05$) (Fig. 3). The pattern of β 1 mRNA expression resembled that of α 1 or α 2 mRNAs, and an approximate 7-fold increase was observed in 4-week-old SHR compared with age-matched WKY (6.71 ± 1.54 ; $p<0.01$).

The α 1 and α 2 mRNAs were detected in the aortae (Fig. 4), and their levels in SHR remained essentially equivalent throughout the period of early and established hypertension. In contrast, in WKY, the α 1 and α 2 mRNAs increased steadily during the same period of time. The α 3 mRNA was not detected in the aortae at any stages. As shown in Fig. 5, a significant 5-fold increase in the amount of α 1 and α 2 mRNAs was observed in pre-hypertensive SHR compared with age-matched WKY (4.88 ± 0.60 and 5.46 ± 1.20 , respectively; $n=5$, $p<0.05$).

Only the α 1 mRNA was detected in the kidneys (Fig. 6). Similar to findings in the hearts and aortae, the level of α 1 mRNA in SHR kidneys remained essentially equivalent throughout the 16 weeks, while the level of α 1 mRNA in WKY increased steadily. At 4 weeks of age, the expression of α 1 mRNA in SHR showed a significant 2-fold increase compared with that of WKY (2.39 ± 0.35 ; $n=6$, $p<0.05$) (Fig. 7).

Fig. 2. Expression of Na, K-ATPase $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ isoform mRNAs in SHR and WKY ventricles at the age of 4, 8 and 16 weeks. Total RNA was isolated from rat ventricles by GITC-CsCl method. Equal amounts of total RNA (15 μ g) were size-fractionated by electrophoresis on denaturing 1.0% agarose/formaldehyde gels and transferred to nylon membranes. Hybridizations were performed with an excess of 32 P-dCTP-labeled $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ isoform specific cDNA probes and 18 S rRNA probe. Filters were washed to a final stringency of 1xSSPE with 0.1% SDS at 65°C and exposed to Kodak XAR-5 film for 1-2 days at -80°C with an intensifying screen.

Fig. 3. Relative accumulation of α and β isoform mRNAs in ventricles of SHR at three stages of hypertension to that of age-matched WKY. Levels of α mRNAs were determined by Northern blot analysis and quantified by densitometric scanning. Data are the mean $\pm$ SE of 6 samples. * $p < 0.05$, ** $p < 0.01$.

Fig. 4. Expression of Na, K-ATPase $\alpha 1$ and $\alpha 2$ isoform mRNAs in SHR and WKY aortae at the age of 4, 8 and 16 weeks. Equal amounts of total RNA (15 μ g) were size-fractionated on denaturing 1.0% agarose/formaldehyde gels and transferred to nylon membranes. Filters were hybridized with 32 P-dCTP labeled $\alpha 1$ and $\alpha 2$ specific cDNA probes and 18 S rRNA probe.

Fig. 5. Relative accumulation of α isoform mRNAs in aortae of SHR at three stages of hypertension to that of age-matched WKY. Levels of α mRNAs were determined by Northern blot analysis and quantified by densitometric scanning. Data are the mean $\pm$ SE of 5 samples. * $p < 0.05$, ** $p < 0.01$.

Discussion

Our objective was to determine whether the expression of Na, K-ATPase gene was differentially regulated, and its pattern was altered, during the development of spontaneous hypertension. By Northern blot analysis, we observed the $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoform mRNA expression in the ventricles, the $\alpha 1$ and $\alpha 2$ isoform mRNA expression in the aortae, and only the $\alpha 1$ isoform mRNA expression in the kidneys. We also revealed an increase in the α isoform mRNA expression in the pre-hypertensive 4-week-old SHR tissues, compared with the age-matched WKY controls. In the ventricle, the $\beta 1$ mRNA expression was also increased in SHR compared with WKY. Our results further showed that during the normal development of WKY, there was

Fig. 6. Expression of Na, K-ATPase $\alpha 1$ isoform mRNA in SHR and WKY kidneys at the age of 4, 8 and 16 weeks of age. Total RNA (5 μ g) was isolated from rat kidneys and size-fractionated by electrophoresis on 1.0% agarose/formaldehyde gels and then transferred to nylon membranes. Filters were visualized by UV transillumination (28 S) and hybridized with an excess of 32 P-dCTP labeled $\alpha 1$ cDNA probe.

Fig. 7. Relative accumulation of $\alpha 1$ mRNA in kidneys of SHR at three stages of hypertension to that of age-matched WKY. Data are the mean $\pm$ SE of 6 samples. * $p < 0.05$.

during the normal development of WKY, there was an increased expression of the α and β isoform mRNAs in the hearts, aortae, and kidneys relative to the total cardiac RNA. In SHR, a genetic model of spontaneous hypertension, this developmental pattern of Na, K-ATPase mRNA expression was significantly altered even before the onset of hypertension. These findings suggest that an abnormality in Na, K-ATPase gene expression constitutes an early, if not a primary, event in spontaneous hypertension.

Previous studies on the role of Na, K-ATPase in the pathogenesis of hypertension were based on measurements of Na, K-ATPase activity or of ouabain binding, and conflicting data were obtained

(12-19). Concerning Na, K-ATPase activity in the heart, Godfrained *et al.* (12) reported that such activity was higher in 7-week-old SHR than in age-matched WKY. David-Duflho *et al.* (13) also showed that 3-week-old SHR hearts had a greater number of Na, K-ATPase sites than age-matched WKY hearts. In contrast, Chen *et al.* (14) showed that cardiac Na, K-ATPase activity of SHR was decreased at 6 and 14 weeks of age compared with age-matched WKY. Concerning the aorta, Webb *et al.* (15) showed that Na, K-ATPase activity of SHR was significantly increased compared with WKY at 14 to 18 weeks of age. Bruner *et al.* (16) reported that Na, K-ATPase activity in the tail arteries of SHR was significantly increased compared with age-matched WKY controls. However, Aalkjar *et al.* (17) reported that the superior mesenteric arteries of SHR showed no significant difference in the concentration of [3 H]ouabain binding sites compared with the age-matched WKY controls. There are also conflicting data on Na, K-ATPase activity in SHR and WKY kidneys. Cangino *et al.* (18) reported that Na, K-ATPase activity of 5-week-old SHR kidneys was significantly increased compared with that of age-matched WKY kidneys, while Sowers *et al.* (19) found that Na, K-ATPase activity was significantly reduced in the renal cortex of 9- and 12-week-old SHR compared with that of age-matched WKY. These discrepancies in protein levels may be due to differences in membrane isolation techniques or in enzymatic and ouabain binding assay conditions. Additional factors complicating functional or binding studies are the heterogeneity of the α isoforms expressed and the very low affinity of the rat $\alpha 1$ isoform to ouabain inhibition and binding (6). The heterogeneity of SHR and WKY populations studied may be an additional variable.

It is important to evaluate the difference in the expression of Na, K-ATPase gene between the genetic model of spontaneous hypertension and that of normal rats with experimentally induced hypertension. Herrera *et al.* (22) showed that both the $\alpha 1$ and $\beta 1$ mRNAs in the hearts were unchanged, while the $\alpha 2$ and $\alpha 3$ mRNAs were de-induced in two models of hypertension produced by angiotensin II infusion or by uninephrectomy and treatment with DOC-salt. These investigators speculated that an elevated intraventricular pressure mediated a down-regulation of $\alpha 2$ mRNA expression via an unknown signal transduction mechanism. In contrast, our results showed that the $\alpha 1$ and $\alpha 2$ mRNA levels were elevated in pre-hypertensive SHR ventricles compared with WKY controls, and remained unchanged in response to elevated blood pressure. These two models would differ with respect to the basal, developmental and pressure-mediated regulation of Na, K-ATPase gene expression. Experimentally induced hypertension in normal rats presents a model of the compensatory regulation of Na, K-ATPase gene expression in response to an increase in blood pressure. Alternatively, the differing expression of $\alpha 2$ mRNA in SHR may reflect an abnormality in pressure-mediated signal transduction.

We observed the expression of $\alpha 3$ isoform mRNA only in the ventricles of 4-week-old SHR. The absence of detectable $\alpha 3$ mRNA in 4-week-old WKY ventricles is consistent with the developmental switch from the $\alpha 3$ to the $\alpha 2$ isoform mRNA that occurs between 10–14 days in the normal post-natal rat heart (8). The mechanism involved is not known, but most likely involves the developmentally regulated transcriptional regulation of the $\alpha 2$ and the $\alpha 3$ isoform genes. We previously reported that thyroid hormone may contribute to the developmental switching of these α isoforms (20). The phenomenon of the expression of “fetal” protein in pressure-loaded ventricles has been observed with creatine kinase, myosin heavy and light chains, α -actin and β -tropomyosin (23–27). Charlemagne *et al.* (28) reported that aortic constriction and subsequent ventricular hypertrophy induced the expression of Na, K-ATPase that exhibited kinetic properties similar to that of newborn rat hearts. However, we found no expression of the neonatal type $\alpha 3$ isoform mRNA in hypertrophic SHR ventricles at 8 and 16 weeks of age.

It is very difficult to determine whether our results reflect causal, primary genetic defect in the expression of Na, K-ATPase gene or the secondary compensation of Na, K-ATPase to elevated blood pressure or other initiating events. The basal and developmental regulation of Na, K-ATPase gene expression is abnormal in SHR, suggesting that a primary defect in Na, K-ATPase gene produces hypertension in this model. Genetic linkage analysis would provide a method of investigating the above hypothesis. Indeed, the $\alpha 1$, $\alpha 2$ and $\beta 1$ isoform genes have been reported to show restriction fragment length polymorphisms (RFLP) between SHR and WKY (29) and in humans (30–33), supporting the possibility of a primary genetic defect of Na, K-ATPase gene. However, no previous reports have shown the coordinate segregation of the RFLPs and the development of hypertension. Murine chromosomal mapping studies revealed that the $\alpha 1$, $\alpha 2$ and $\alpha 3$ isoforms are encoded by separate genes, each of which is located on a different murine chromosome (34). All three α isoform mRNA expression was increased in pre-hypertensive SHR tissues, suggesting that the altered gene expression reflects a compensatory mechanism secondary to other genetic or functional abnormalities.

There are numerous reports showing an abnormal increase in intracellular Na^+ levels in SHR tissues due to either an increased Na^+ flux or a decreased Na^+ efflux that requires an increased level of Na, K-ATPase expression. Friedman *et al.* (9) reported that the tail arteries of 26- to 28-week-old SHR showed increased accumulation of Na^+ compared with that of the control rats. Pickar *et al.* (10) also described an increased Na content in skeletal muscles of 24- to 28-week-old SHR compared with that of age-matched WKY. Zidek *et al.* (11) found that the intracellular Na^+ activity of SHR erythrocytes was significantly increased compared with that of Wistar rat erythrocytes. Mendonca *et al.* (35) reported that Na^+ extrusion of erythrocytes was im-

paired in 4-week-old SHR compared with that of WKY at the same age. Alterations in intracellular Na^+ levels can induce a change in Na, K-ATPase gene expression, as in our previous study showing that Na^+ induces a transient increase in the α and β isoform mRNA expression in cultured neonatal rat cardiocytes (36).

The endogenous digitalis-like factor may also affect the expression of Na, K-ATPase gene. Goto *et al.* (37) reported that the plasma level of the digitalis-like factor was increased in patients with hypertension. Tsuda *et al.* (38) also observed that the plasma level of the digitalis-like factor was higher in hypertensive than in normotensive subjects. Inhibition of Na, K-ATPase by this digitalis-like factor could increase intracellular Na^+ levels and stimulate Na, K-ATPase gene expression (35). In addition, other hormones such as epinephrine, angiotensin II or aldosterone, which are elevated in the plasma of SHR or hypertensive subjects, may be involved in the increased expression of Na, K-ATPase gene in SHR tissues (39,40).

In the present study, we observed that the expression of Na, K-ATPase α and β isoform mRNAs was increased in the early, pre-hypertensive ventricles, aortae and kidneys of SHR. These results do not rule out an additional regulation at the translational and post-translational levels culminating in a functional Na, K-ATPase. However, our data suggest that an abnormality in the expression of Na, K-ATPase gene is an early, if not a primary, event in the development of hypertension in SHR.

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