ELSEVIER

European Journal of Pharmacology 327 (1997) 151–156

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Alterations in alpha subunit expression of cardiac Na^+,K^+-ATPase in spontaneously hypertensive rats: effect of antihypertensive therapy

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Received 12 December 1996; revised 12 February 1997; accepted 18 March 1997

Abstract

The alpha-2 subunit abundance of Na^+,K^+-ATPase in the rat heart has been reported to be reduced in several induced hypertensive models. To determine whether this reduction also occurs in a genetic model of hypertension, we studied expression of the alpha subunits in left ventricles of spontaneously hypertensive rats (SHR), and normotensive Wistar-Kyoto (WKY) and Sprague-Dawley rats using Western blotting and quantitative dot-blotting analysis with monoclonal antibodies. While the alpha-1 subunit was not affected in any of the strains, a significant reduction of the alpha-2 subunit expression was noted in 19-week-old SHRs, but not in age-matched WKY and Sprague-Dawley rats, supporting the hypothesis that elevated arterial pressure may differentially downregulate the alpha-2 subunit in the rat heart. To further test this hypothesis we designed experiments in which hypertensive rats were treated with the antihypertensive agents hydralazine and nifedipine. Both agents effectively normalized the blood pressure in the SHRs with no significant effect on the blood pressure in the WKY and Sprague-Dawley rats. The alpha-2 subunit in SHRs treated with hydralazine and nifedipine showed a 63.3% ($n = 6$, $P < 0.05$, analysis of variance and Fischer's test) and a 27.4% increase, respectively, over the hypertensive SHR controls, although the reversal effect of nifedipine did not quite reach significance. The alpha-1 subunit expression was not affected by any of the drug treatments. No effect of either of the drugs on the alpha-1 or alpha-2 subunit was observed in the WKY or Sprague-Dawley rat groups. These data support our hypothesis that the alpha-2 subunit may be a pressure-sensitive isoform of the cardiac Na^+,K^+-ATPase and that high blood pressure is, directly or indirectly, responsible for the reduction of the alpha-2 subunit protein expression.

Keywords: Na^+,K^+-ATPase; Na^+,K^+-ATPase, α-subunit; Hypertension; Spontaneously hypertensive rat (SHR); Ca^{2+} channel blocker; Vasodilator

1. Introduction

Na^+,K^+-ATPase is a ubiquitous transmembrane protein which is a contributing factor to the establishment and maintenance of the electrochemical gradients across the plasma membrane by using ATP as its energy source (Skou and Esmann, 1992). It is composed of a 113-kDa catalytic alpha subunit and a 35-kDa glycosylated beta subunit (Lingrel and Kuntzweiler, 1994). At least three isoforms of the alpha subunit, alpha-1, -2 and -3, and two of the beta subunit, beta-1 and -2, are expressed in a species- and tissue-specific manner in mammals (Jewell et al., 1992; Lingrel, 1992). These isoforms have some distinct biochemical and immunological properties while their true functional differences remain to be elucidated. The alpha-1, -2 and beta-1 subunits are the major isoforms in the adult rat heart (Sweadner, 1989). The alpha-3 subunit has also been detected in the conduction system of rat heart (Zahler et al., 1992).

Na^+,K^+-ATPase plays a role in tension development in both cardiac and smooth muscle cells (Blaustein, 1993). Inhibition of the enzyme, for example, by cardiac glycosides, enhances contractility of the heart. The involvement of the functional counterpart of Na^+,K^+-ATPase, the Na pump, in the pathogenesis of hypertension has been suggested and supported by numerous studies (Overbeck et al., 1976; Blaustein, 1977; Songu-Mize et al., 1982, 1983). In recent years, there has been accumulating evidence for differential regulation of the isoforms of Na^+,K^+-ATPase in cardiovascular tissues in hypertension. In adult rat heart and aorta, decreased levels of mRNA for the alpha-2 subunit were reported in deoxycorticosterone acetate (DOCA)-salt-induced hypertension as well as in angiotensin-II-induced hypertension (Herrera et al., 1988).

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PII S0014-2999(97)00062-9

Furthermore, we and others have recently shown that the protein expression of cardiac alpha-2 subunit was also suppressed in DOCA-salt hypertension (Sweadner et al., 1994; Sahin-Erdemli et al., 1995). A study of Goldblatt hypertensive rats also showed the suppression of cardiac alpha-2 subunit protein expression as compared to the normotensive controls (McDonough et al., 1993). However, the expression of cardiac alpha-1 subunit was not affected in any of the hypertensive models studied. Since it appeared that the alpha-2 subunit in the heart was a pressure-sensitive isoform, we designed experiments to determine whether the alpha-2 subunit expression was suppressed in spontaneously hypertensive rats. In addition, we studied the effect of antihypertensive drugs in this model to determine whether a reduction in blood pressure would have any influence on the protein expression of the alpha subunits of Na^+,K^+-ATPase.

2. Materials and methods

2.1. Experimental animals and blood pressure measurements

Male spontaneously hypertensive rats (SHR), Wistar-Kyoto (WKY) and Sprague-Dawley rats were used in this study. All rats were purchased from Harlan and housed in an experimental animal room at 22°C with a 12 h light-dark cycle, and were provided standard chow and tap water ad libitum. The care and use of the rats were in accordance with institutional and NIH guidelines. The systolic blood pressure was measured using tail-cuff plethysmography. The rats were acclimated to the tail cuff blood pressure apparatus (IITC Model 29 Pulse Amplifier) for about five times before the actual measurement procedures were carried out. At the age of 15 weeks, baseline systolic blood pressure and body weight measurements were taken. Then systolic blood pressure and body weight measurements were repeated every week during the following 4 weeks of antihypertensive drug treatment.

2.2. Antihypertensive drug treatment

The rats of three strains, SHR, WKY and Sprague-Dawley, were divided into nine groups with three groups for each strain: the control group, hydralazine-treated group and nifedipine-treated group. There were six rats in each group. Hydralazine was administered by dissolving the drug (25 mg/kg per day) in drinking water and nifedipine (20 mg/kg per day) was mixed with the powder chow due to the water-insoluble nature of the drug. The corresponding control groups received tap water and powder chow, respectively. The doses of the drugs were calculated according to the amounts of water and chow the animals consumed each day. The treatments continued for at least 2 weeks after the blood pressure of the hypertensive rats treated with antihypertensive drugs reached normotensive levels.

2.3. Preparation of tissue homogenates

Tissue homogenates were prepared as described before (Sahin-Erdemli et al., 1995). At the end of the fourth week of treatment, the rats were killed, the hearts removed and immediately frozen in liquid nitrogen. The frozen tissues were stored below −70°C for a maximum of a month before the homogenates were prepared. The left ventricles were dissected and minced in ice-cold Tris-sucrose buffer (Tris-HCl 1.0 mM; EDTA 1.0 mM; sucrose 0.25 mM, pH 7.4), containing a mixture of protease inhibitors (10 μg leupeptin, 10 μg aprotinin and 50 μg phenylmethylsulfonyl fluoride per ml of buffer). The minced tissue was then homogenized in 8 volumes of the Tris-sucrose buffer using a Polytron homogenizer. The homogenates were centrifuged at $10\,000 \times g$ for 10 min and the supernatants saved. The pellets were rehomogenized and recentrifuged at $10\,000 \times g$ for 30 min. The supernatants from the first and second centrifugations were then pooled and centrifuged at $100\,000 \times g$ for 90 min. The pellets containing the microsomal fraction were reconstituted (1:3, w/v) in Tris-EDTA buffer (Tris-HCl 1.0 mM, EDTA 1.0 mM). The protein concentrations were determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard. The protein concentrations of the homogenates were in the range of 5–7 mg/ml. Different treatments did not affect the total protein recovery.

2.4. Western blotting

The proteins in the microsomal homogenates were separated by electrophoresis on 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate with prestained molecular weight standards (Gibco-BRL) as a reference (Laemmli, 1970). The separated proteins were then transferred to a polyvinylidene fluoride membrane by electroblotting. Monoclonal antibodies, McK1 and McB2, were used to detect the alpha-1 and alpha-2 subunit proteins of Na^+,K^+-ATPase, respectively, as described before (Sahin-Erdemli et al., 1995). We used alkaline phosphatase-conjugated goat anti-mouse immunoglobins (Ig)G as the secondary antibody. Visualization of the bands was achieved by treating the blots with a substrate complex composed of 5-bromo-4-chloro-3-indolyl phosphate (BICP) and nitro blue tetrazolium (NBT), until proper densities were reached.

2.5. Dot-blotting and quantitation

Since our study included nine experimental groups with six rats in each group, it would have been impossible to use Western blotting to quantitate several groups of samples on one membrane. It also would have been undesir-

able to compare bands on separate membranes. For this reason, we chose dot-blotting for quantitation after qualitative results were obtained from Western blotting. This approach allowed us to analyze several groups of samples on one membrane, for quantitation and meaningful statistical analysis. As described earlier (Sahin-Erdemli et al., 1995), aliquots of the microsomal homogenates were loaded directly on the polyvinylidene fluoride membrane using Bio-Rad Dot-Blot apparatus (Bio-Rad). Each sample was applied four times on the same membrane and the average was taken as the final density of the sample. They were probed with antibodies and developed as described above, using the alkaline phosphatase-conjugated goat anti-mouse secondary antibody and the BICP/NBT substrate system. To ensure that the density reflected the protein amount, standard curves were constructed using microsomal protein in the range of 10–50 μg. The standard curves for both the alpha-1 and alpha-2 subunits showed good linear relationships (correlation coefficients were 0.994 and 0.990 for alpha-1 and alpha-2, respectively) between the microsomal protein amounts and the dot densities. We used 30 μg total protein per sample for comparison of specific subunit protein abundances in different samples. The dot densities were then quantitated with a computerized image analyzing system (M-2 Model, Imaging Research). The background was subtracted automatically from the measurements.

3. Results

Table 1 shows the blood pressure and body weight measurements before and 4 weeks after the treatment with hydralazine and nifedipine. The body weights of the Sprague-Dawley rats were greater than those of the age-matched SHRs and WKY rats. The SHRs had significantly higher systolic blood pressures compared to those of the normotensive WKY and Sprague-Dawley rats. The time course of change in blood pressure during the 4 weeks of drug treatment is shown in Fig. 1. Treatment with nifedipine and hydralazine was fully effective in lowering the systolic blood pressure to the normal range within 2 weeks in SHRs, and the rats remained normotensive thereafter. In the normotensive WKY and Sprague-Dawley rats, treatment with antihypertensive drugs produced a small and transient drop in systolic blood pressure in the first week. The blood pressure returned to baseline level in the second week and stayed at this level (~ 135 mm Hg) until the end of the treatment (Fig. 1).

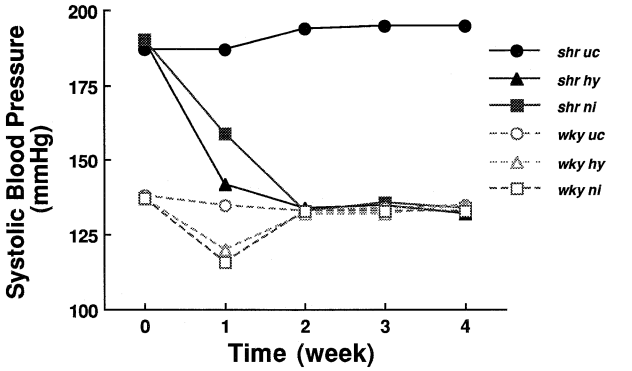

Fig. 1. The course of blood pressure changes in the SHR and WKY rats during antihypertensive drug treatment. Drug treatment was initiated at time zero. The mean systolic blood pressures and standard errors ($n = 6$ in each group) are plotted. Abbreviations: uc, untreated control; hy, hydralazine; ni, nifedipine.

To investigate whether expression of the alpha subunits of the ventricular Na^+,K^+-ATPase was regulated in genetically hypertensive SHRs, we determined the protein expression of the alpha-1 and alpha-2 subunits in 19-week-old, fully hypertensive SHRs and age-matched normotensive WKY and Sprague-Dawley rats. Fig. 2 shows the quantitation of the alpha-2 subunit expression by dot-blot analysis in the different rat strains. The alpha-2 subunit expression in SHRs was 63.2% and 51.5% of that in WKYs and Sprague-Dawley rats, respectively ($n = 6$, $P < 0.05$, analysis of variance (ANOVA) and Fischer's test), while no difference was found between the latter two groups (Fig. 2). The expression of alpha-1 subunit protein

Table 1
The systolic blood pressure and body weight measurements in normotensive and hypertensive rats: effect of antihypertensive treatment

Strain	Treatment	Body weight (g)		Blood pressure (mmHg)	
		Before	After	Before	After
SHR	Control	263 ± 8	315 ± 7	187 ± 2 [a]	195 ± 3 [b]
	Hydralazine	275 ± 6	313 ± 4	190 ± 3	132 ± 2
	Nifedipine	273 ± 9	305 ± 8	190 ± 2	134 ± 2
WKY	Control	265 ± 12	312 ± 11	138 ± 1	135 ± 1
	Hydralazine	265 ± 7	315 ± 6	137 ± 4	135 ± 1
	Nifedipine	273 ± 15	333 ± 6	137 ± 3	133 ± 2
SD	Control	335 ± 6 [a]	413 ± 8 [a]	137 ± 2	134 ± 1
	Hydralazine	336 ± 4	430 ± 5	137 ± 2	130 ± 1
	Nifedipine	335 ± 4	410 ± 7	138 ± 2	132 ± 1

Values are expressed as means ± S.E. ($n = 6$ in each group). [a] Significantly different compared to the other control groups. ($P < 0.05$). [b] Significantly different compared to drug-treated SHR groups ($P < 0.05$). ANOVA and Fisher's post-hoc test were used.

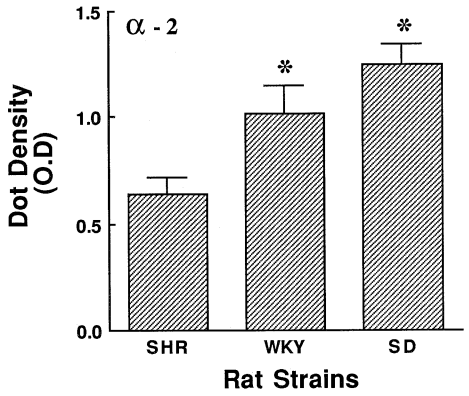

Fig. 2. Quantitation of the alpha-2 subunit protein in left ventricles of SHRs, WKY and Sprague-Dawley rats by dot-blotting. The bar graphs represent means ± S.E. for dot densities of the alpha-2 subunit protein. * $P < 0.05$ (ANOVA and Fischer's test, $n = 6$), compared to the SHRs.

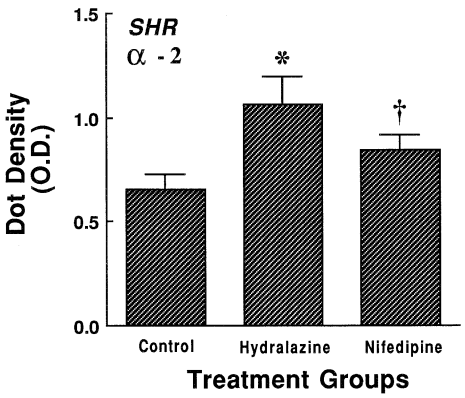

Fig. 3. Quantitation of the alpha-2 subunit protein in left ventricles of control, nifedipine-treated and hydralazine-treated SHR groups by dot-blotting. The bar graphs represent means ± S.E. for dot densities of the alpha-2 subunit protein. * $P < 0.05$. † $P = 0.0864$ (ANOVA and Fischer's test, $n = 6$), compared to the control group.

did not seem to differ among the rats of different strains (Table 2).

To investigate the effects of blood pressure on the expression of the alpha subunits, we treated the SHRs with two antihypertensive agents, hydralazine and nifedipine, and measured the alpha subunit expression. The untreated SHRs expressed less of the alpha-2 subunit than did the hydralazine- and nifedipine-treated SHRs, indicating a reversal of the suppression of the subunit expression by antihypertensive treatment (Fig. 3). Quantitation by dot-blotting showed a 63.3% increase by hydralazine ($n = 6$, $P < 0.05$, ANOVA and Fischer's test) and a 27.4% increase by nifedipine over the SHR controls (Fig. 3). The reversal effect of nifedipine did not reach significance. However, the confidence limit was 91.4% ($P = 0.0864$). Alpha-1 subunit expression was not affected by any of the drug treatments (Table 2).

We did not observe any effect of either of the drugs on the alpha-1 or alpha-2 subunit in the WKY or Sprague-Dawley rat groups. This suggests that the drugs themselves did not significantly affect the expression of these subunits (Table 2).

Table 2
The abundance of alpha isoform proteins in the left ventricle in three rat strains: effect of antihypertensive treatment

Rat group and treatment	Alpha-1 subunit (dot density)	Alpha-2 subunit (dot density)
SHR untreated	0.91 ± 0.07	
WKY untreated	0.98 ± 0.05	
SD untreated	0.97 ± 0.10	
SHR hydralazine	0.87 ± 0.09	
SHR nifedipine	0.86 ± 0.08	
SHR control	0.85 ± 0.11	
WKY hydralazine	0.91 ± 0.05	0.85 ± 0.09
WKY nifedipine	0.93 ± 0.09	0.87 ± 0.17
WKY control	0.95 ± 0.12	0.82 ± 0.14
SD hydralazine	0.94 ± 0.12	0.76 ± 0.15
SD nifedipine	0.93 ± 0.10	0.81 ± 0.13
SD control	0.88 ± 0.05	0.79 ± 0.09

Values are expressed as means ± S.E. ($n = 6$ in each group) of dot densities of the isoform proteins determined by dot-blotting.

4. Discussion

In this study, we have demonstrated that the alpha-2 subunit expression of Na^+,K^+-ATPase in the left ventricle of SHRs is suppressed compared to normotensive WKY and Sprague-Dawley rats. Furthermore, the antihypertensive agent hydralazine, which reduced the blood pressure to normal levels, reversed this suppression. Another antihypertensive agent, nifedipine, also produced a considerable reversal of the alpha-2 suppression, although its effect did not quite reach significance ($P = 0.0864$). These results from spontaneous hypertension are in good agreement with our recent findings in DOCA-salt hypertension (Sahin-Erdemli et al., 1995). In that study, a reduction in alpha-2 subunit expression was clearly demonstrated in an induced form of hypertension. Others have also reported a reduction of the myocardial alpha-2 subunit in several other models of experimental hypertension (Herrera et al., 1988; McDonough et al., 1993; Sweadner et al., 1994). Since these models have little in common other than elevated blood pressure, and since, in our model, the effect is reversed by returning blood pressure to normal, these findings suggest that blood pressure itself, or its influence on the heart and/or vasculature, regulates alpha-2 subunit expression of Na^+,K^+-ATPase in rat heart. Alpha-1 subunit expression did not differ between either hypertensive and normotensive rat strains, or untreated hypertensive and drug-treated normotensive SHRs, indicating that its expression is not regulated by changes in blood pressure. The smaller magnitude of the effect of nifedipine on cardiac alpha-2 expression in SHRs could be attributed to a possi-

ble additional effect of this drug on the enzyme. Although some direct effect of hydralazine or nifedipine is a possibility, in our study, neither of these two agents had any effect on the expression of either alpha-1 or alpha-2 subunits in normotensive WKY and Sprague-Dawley rats.

A reduction in the activity of the cardiac Na^+,K^+-ATPase has been corroborated by studies in several hypertensive models, including spontaneous hypertension. Both decreased Na^+,K^+-ATPase activity and decreased [^{3}H]ouabain binding sites in the heart have been demonstrated in SHRs (Lee et al., 1983). Clough et al. (1983) reported that the myocardial Na^+,K^+-ATPase activity in one-kidney, one-clip hypertensive rats is decreased. Our study on DOCA-salt hypertension also showed a net reduction of cardiac Na^+,K^+-ATPase activity in DOCA-salt hypertensive rats, compared to DOCA-treated normotensive controls (Sahin-Erdemli et al., 1995). The reduction of the alpha-2 subunit in the left ventricle may be interpreted as a compensatory mechanism to maintain cardiac output by increasing the intracellular Ca^{2+} level within the myocardium in face of increased peripheral vascular resistance. With the reduction of alpha-2 subunit and no change in alpha-1 subunit, the net expression of the enzyme would be decreased. Although we did not measure the Na^+,K^+-ATPase activity in this study, others have shown reduced activity of this enzyme in the heart in the same hypertensive model (Lee et al., 1983), which would result in an increase of intracellular Na^+ and enhanced cardiac contractility through Na^+-Ca^{2+} exchange. The effect would be similar to the cardiac effects of digitalis (Akera and Brody, 1982).

Although the cellular mechanisms by which pressure is transduced in the heart to regulate expression of Na^+,K^+-ATPase are unclear, they might involve transmembrane ion flow mediated by stretch-activated channels. Since stretch affects ion flow across the cell membrane by activating various types of channels (Bevan et al., 1990; Kirber et al., 1992; Ruknudin et al., 1993; Dopico et al., 1994), and some cations, such as Na^+ and Ca^{2+}, have been reported to affect Na^+,K^+-ATPase alpha subunit expression in certain tissues (Rayson, 1991; Yamamoto et al., 1994), it is possible that alpha subunit expression in the heart may also be regulated by one or more of the ions entering the cell through stretch-activated channels. Le Guennec et al. (1991) showed that stretching of cardiac myocytes increases intracellular Ca^{2+} in a few seconds. Sigurdson et al. (1992) also reported that mechanical stimuli on tissue-cultured heart cells produce a transsarcolemmal influx of Ca^{2+} which leads to waves of Ca^{2+}-induced Ca^{2+} release, and the response can be blocked by removing extracellular Ca^{2+}. Other candidate signal transduction cascade elements in cardiac cells that respond to mechanical force include Na^+ (Kent et al., 1989), cAMP (Watson et al., 1989), c-*fos* (Komuro et al., 1990), PKC (Komuro et al., 1991) and inositol phosphates (Von Harsdorf et al., 1989).

Since SHRs do not share a completely homogeneous genetic background with WKY rats (Louis and Howes, 1990), and much less with Sprague-Dawley rats, and blood pressure is just one of the differences among them, the observed difference in the alpha-2 subunit expression could be the effect of factors other than blood pressure. However, reversal of alpha-2 subunit reduction in the left ventricle of SHRs by antihypertensive treatment favors our hypothesis that increased intraventricular pressure is responsible, at least in part, for the suppression of the isoform expression. The ideal way of demonstrating the influence of blood pressure on alpha-2 subunit expression would be to monitor subunit abundance changes during the development of hypertension in SHRs. Unfortunately, the expression of the alpha subunits in rat heart is also affected by developmental stages (Lucchesi and Sweadner, 1991).

The individual alpha subunits of Na^+,K^+-ATPase are differentially expressed among various tissues in rats (Orlowski and Lingrel, 1988; Shyjan and Levenson, 1989) and are thought to serve distinct functional and regulatory roles. Although different ion affinities of the isoforms for Na^+ and K^+ have been demonstrated (Jewell et al., 1992), more extensive studies are still needed to define possible functional differences of these isoforms with regard to ion transport capacity. Numerous factors such as insulin (McGill and Guidotti, 1991), thyroid hormone (Horowitz et al., 1990) and glucocorticoids (Whorwood et al., 1994) have been shown to differentially regulate the expression of these isoforms. Our study suggests that blood pressure, or its effect mediated through mechanosensitive ion channels, might be another factor that specifically regulates the isoform expression of the Na^+,K^+-ATPase.

In summary, we have found a reduction of the alpha-2 subunit expression of Na^+,K^+-ATPase in the left ventricle of spontaneously hypertensive rats which can be reversed by lowering blood pressure. This might be a compensatory response to maintain cardiac output in the face of elevated peripheral vascular resistance.

Acknowledgements

We thank Dr. K. Sweadner (Harvard Medical School) for kindly providing the monoclonal antibodies used in this study. This study was supported by a grant (HL-32270-10 to E.S.-M.) from NIH – Heart, Lung, and Blood Institute. We are also grateful to Dr. L. Hymel for reading the manuscript and making helpful comments and suggestions.

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