Biochimica et Biophysica Acta, 1149 (1993) 189–194

BBAMEM 76005

Expression of α isoforms of the Na,K-ATPase in human heart

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(Received 5 January 1993)

Key words: Ventricle; mRNA; Sodium ion pump; ATPase, Na^+/K^+-; Cardiomyopathy; Pulmonary hypertension; (Human); (Heart)

We studied expression of isoforms of Na,K-ATPase in normal and diseased human hearts. Na,K-ATPase α-isoform mRNA in samples from normal human left ventricle (LV) was composed of 62.5% α1, 15% α2 and 22.5% α3 on average. There was an increase in expression of the α3 isoform in samples from failing hearts, but expression of all three isoforms decreased in pressure-overloaded right ventricle (RV).

Introduction

Na,K-ATPase is an enzyme that extrudes sodium from cells in exchange for potassium, powered by ATP hydrolysis. In heart, the Na,K-ATPase is important for regulation of cell volume, maintenance of transmembrane voltage gradients, and generation of ion gradients for transport of calcium and other solutes; it is also the target of action of digitalis glycosides. This enzyme is a heterodimer whose two subunits are designated α and β; the α (catalytic) subunit, which in rodents determines ouabain sensitivity, has three isoforms. Although the Na,K-ATPase itself is ubiquitous, the α3 isoform in adult rat is found principally in neural tissue. Recently, however, we demonstrated that expression of α2 is markedly increased at all levels of the rat cardiac conduction system, and that α3 in rat heart is only found in the conduction system. There are also significant differences in α isoform expression in the heart regionally, developmentally, and in response to stress (reviewed in Ref. 1). This suggests that important functional differences exist between isoforms.

Because the Na,K-ATPase is the target of cardiac glycosides, it is possible that the low therapeutic-to-toxic ratio of these agents may be related to Na,K-ATPase isoform differences between Purkinje fibers and ventricular cells. In support of this hypothesis, Purkinje fibers contain pumps which are more ouabain-sensitive than those of ventricular muscle [2–4]. Furthermore, high levels of α3 expression are found in neonatal heart tissue [5], but α3 decreases after birth to very low levels in rodent ventricle. Such developmental isoform changes in rats and other species correspond to changes in ouabain sensitivity [6,7]. Thus, we studied Na,K-ATPase isoform expression in regions of human heart by Northern analysis.

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Materials and Methods

47 specimens from eight hearts were studied: two post-mortem hearts from patients without evidence of heart disease, three hearts from patients with RV failure undergoing heart-lung transplantation, and three hearts from patients undergoing heart transplantation (two with ischemic cardiomyopathy and one with idiopathic dilated cardiomyopathy). Myocardial specimens (approx. 10 g) were taken from RV free wall, the endo- and epicardial thirds of grossly normal LV free wall, and atrial tissue (in one ischemic heart, specimens were also taken from normal and ischemic regions of the LV free wall). In some hearts, specimens of the right and left one-third of the interventricular septum, and of the RV free wall endo- and epicardial layers, were also obtained. Samples were minced and snap-frozen, then stored at −70°C.

Total RNA was prepared by guanidinium extraction [8]. For slot blots, RNA samples were denatured in 50% formamide/6% formaldehyde at 50°C for 30 min,

serially diluted, and adsorbed to membranes using a manifold (Schleicher and Schuell). RNA samples were also separated on Northern gels, which were blotted onto nylon membranes. Most samples were run in duplicate or triplicate. Membranes were then hybridized to random-primed ^{32}P-labeled probes for the three human α isoforms of Na,K-ATPase and the human $\beta 1$ isoform. The cDNA probes represent 2.0–2.5 kb fragments of the coding regions of the three α genes ($\alpha 1$, approximate coordinates 1000–3055; $\alpha 2$, 1000–3400; $\alpha 3$, 1000–3455); they have been shown [9] to be isoform-specific under stringency conditions identical to those used in this study (washing 1 h in 2 × SSC/1% SDS at 50°C, 1 h in 0.2 × SSC/0.1% SDS at 65°C). To further verify probe isoform-specificity under our stringency conditions, RNA specimens from human brain, muscle, and kidney were included on the membranes (Fig. 1, left panel). These tissues were chosen because the major form in kidney is $\alpha 1$, the major form in skeletal muscle is $\alpha 2$, and brain expresses all three isoforms. As shown in Fig. 1, the expected patterns of tissue-specific isoform expression were reproduced. In addition, only the $\alpha 2$ probe yielded two bands on Northern blots, as found by previous investigators [1].

Autoradiography of filters was performed at −70°C on Kodak XAR-70 film; care was taken to adjust exposure times to remain within the linearity range of the film (< 1.5 O.D.). After autoradiography, membranes were stripped (completeness of stripping was checked by autoradiography) and reprobed. Digital densitometry of the autoradiograms (BioImage) was performed without knowledge of the identity of the samples.

We first calculated the proportion of total α isoform mRNA attributable to each of the three isoforms by dividing the signal for each isoform by the sum of the signals for all three (Table I). For these calculations, densitometry data were corrected for film exposure, probe activity, and probe length by multiplying by the quantity

$$\frac{24}{t} \cdot \frac{10^6}{A} \cdot \frac{1000}{L} \qquad (1)$$

(where t = exposure time in hours, A = probe activity in cpm and L = probe length in kb) and compared to previously determined isoform ratios for brain [1]. Secondly, we normalized the raw densitometry values to correct for gel loading (Table II) via probes for two

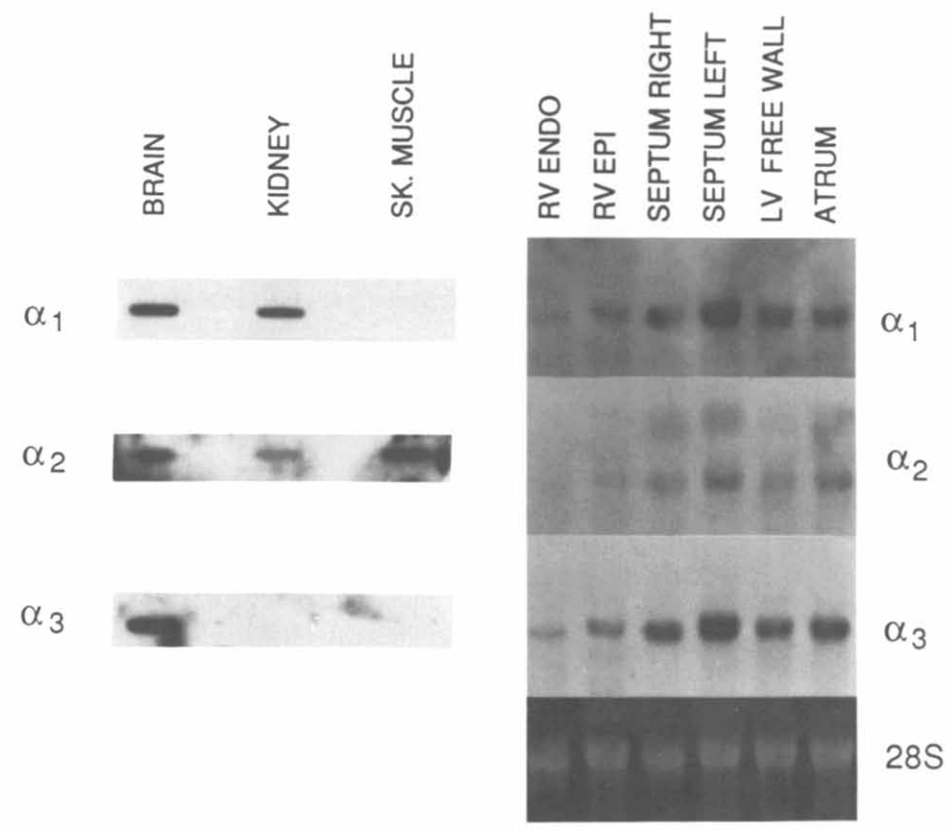

Fig. 1. Left panel: duplicate slot blots demonstrating isoform-specificity of probes under stringency conditions defined in text. Equal amounts of total RNA from tissues indicated in column labels were fixed to membranes with slot blotter and hybridized with probes indicated in row labels. Right panel: Northern blot of RNA from various regions of heart 4, probed for the three α isoforms of the Na,K-ATPase. Washing conditions same as for left panel. Note that two transcripts of the $\alpha 2$ form are detected, as previously noted [1]. Ethidium bromide fluorescence of 28S rRNA indicates equality of loading of total RNA, but signal for pump isoform RNAs varies substantially by region in this heart.

TABLE I

Proportions of the three α isoforms (fraction α1 + fraction α2 + fraction α3 = 1) in individual specimens studied

Total RNA extracted from myocardial tissue specimens was analyzed by slot blot hybridization to ^{32}P-labeled human isoform-specific probes (see text). The proportion of total α isoform mRNA attributable to each of the three isoforms was calculated by dividing the signal for each isoform by the sum of the signals for all three. For these calculations, densitometry data were corrected for film exposure, probe activity and probe length and compared to previously determined isoform ratios for brain. CMP: cardiomyopathy. n.d., specimen not obtained from this region.

	Case number	RV	LV free wall	Septum	Atria
Fraction α1					
Autopsy	5	0.39	0.60	0.66	0.59
Autopsy	1	0.84	0.76	0.43	0.52
RV overload	4	0.73	0.65	0.76	0.66
RV overload	6	0.61	0.55	n.d.	0.58
RV overload	9	0.61	0.71	n.d.	0.88
Idiopathic CMP	3	0.51	0.50	0.58	0.41
Ischemic CMP	2	0.77	0.70	0.72	n.d.
Ischemic CMP	8	0.24	0.23	n.d.	0.41
Fraction α2					
Autopsy	5	0.10	0.14	0.21	0.08
Autopsy	1	0.04	0.13	0.37	0.35
RV overload	4	0.14	0.14	0.08	0.14
RV overload	6	0.09	0.10	n.d.	0.06
RV overload	9	0.21	0.15	n.d.	0.03
Idiopathic CMP	3	0.05	0.07	0.08	0.11
Ischemic CMP	2	0.17	0.13	0.14	n.d.
Ischemic CMP	8	0.11	0.11	n.d.	0.08
Fraction α3					
Autopsy	5	0.51	0.26	0.13	0.32
Autopsy	1	0.12	0.11	0.20	0.13
RV overload	4	0.14	0.21	0.17	0.20
RV overload	6	0.31	0.35	n.d.	0.36
RV overload	9	0.16	0.14	n.d.	0.08
Idiopathic CMP	3	0.44	0.43	0.33	0.48
Ischemic CMP	2	0.06	0.17	0.14	n.d.
Ischemic CMP	8	0.66	0.66	n.d.	0.51

'housekeeping' proteins: GAPDH (glyceraldehyde-6-phosphate dehydrogenase) [10] and β-tubulin (ATCC; [11]). There was a close correlation across specimens ($r = 0.80$, $P < 0.001$) between GAPDH and tubulin expression; thus, only results normalized to GAPDH will be presented.

We found in seven normal dog hearts that there were no significant differences in isoform expression between RV and LV (unpublished data); the situation appears to be similar in normal rat heart [5,12]. Therefore, we divided the specimens into three groups: normal ventricle, pressure-overloaded (right) ventricle, and failing ventricle. These groups were compared with one-way analysis of variance and with Student's *t*-test (SYSTAT); all *P* values are two-sided.

Results

Na,K-ATPase α isoform mRNA in 13 samples from five normal LVs was composed of 62.5% α1, 15% α2, and 22.5% α3 on average (Table I). Isoform composition did not vary materially among (normal) RV, LV endocardium, LV epicardium, and atria. Similarly, total Na,K-ATPase α gene expression did not show any consistent differences between chambers, except that total α gene expression was less in LV endocardium than LV epicardium of all six hearts in which this was evaluated. This was mainly due to the α1 isoform: the ratio (α1 expression in LV endocardium/α1 expression in LV epicardium) ranged from 0.27 to 0.79 in the six hearts evaluated. Expression of β_1 subunit mRNA was approximately equal to the total expression of the three α subunit mRNAs (ratio $\Sigma\alpha/\beta_1 = 0.96 \pm 0.15$); this is similar to the value $\Sigma\alpha/\beta_1 = 0.80$ in rat heart [1].

When data from specimens of diseased ventricle were compared to normal specimens, expression of the α3 isoform (normalized to the internal standard GAPDH) was increased in failing ventricle ($P = 0.059$ versus normal ventricle by *t*-test); fraction α3 was also increased, with borderline statistical significance (Table II). Conversely, specimens from failing ventricle showed decreased expression of α2, with the decrease in fraction α2 being significant. In pressure-overloaded RV there was a two-to-threefold decrease in the ex-

TABLE II

Expression level of individual isoforms and fractional expression by type of specimen

To calculate expression levels, densitometry data from RNA slot blots was normalized to expression of RNA for GAPDH (see Materials and Methods). Differences between hearts and regions for the same isoform are meaningful with this normalization, but because of variable autoradiographic exposure, probe specific activity and probe length, expression of different isoforms cannot be compared to each other. Fractional expression was calculated as in Table I. Data are given as mean ± S.E.

	Normal ventricle ($n = 13$)	Pressure overload ($n = 5$)	Failing ($n = 13$)
α1 Expression	3.84 ± 1.16	1.72 ± 0.54	3.91 ± 0.96
α2 Expression	1.91 ± 0.52	1.10 ± 0.41	1.18 ± 0.22
α3 Expression	1.57 ± 0.51	0.52 ± 0.12	3.62 ± 0.94 *
β1 Expression	3.57 ± 1.01	1.60 ± 0.44	1.92 ± 0.41
total α isoform expression	2.63 ± 0.79	1.06 ± 0.34	3.45 ± 0.81
Fraction α1	0.62 ± 0.04	0.66 ± 0.03	0.55 ± 0.05
Fractiop α2	0.15 ± 0.02	0.16 ± 0.03	0.10 ± 0.01 **
Fraction α3	0.22 ± 0.04	0.19 ± 0.03	0.35 ± 0.06 ***

* $P = 0.059$ vs. normal ventricle.
** $P = 0.056$ vs. normal ventricle.
*** $P = 0.072$ vs. normal ventricle.

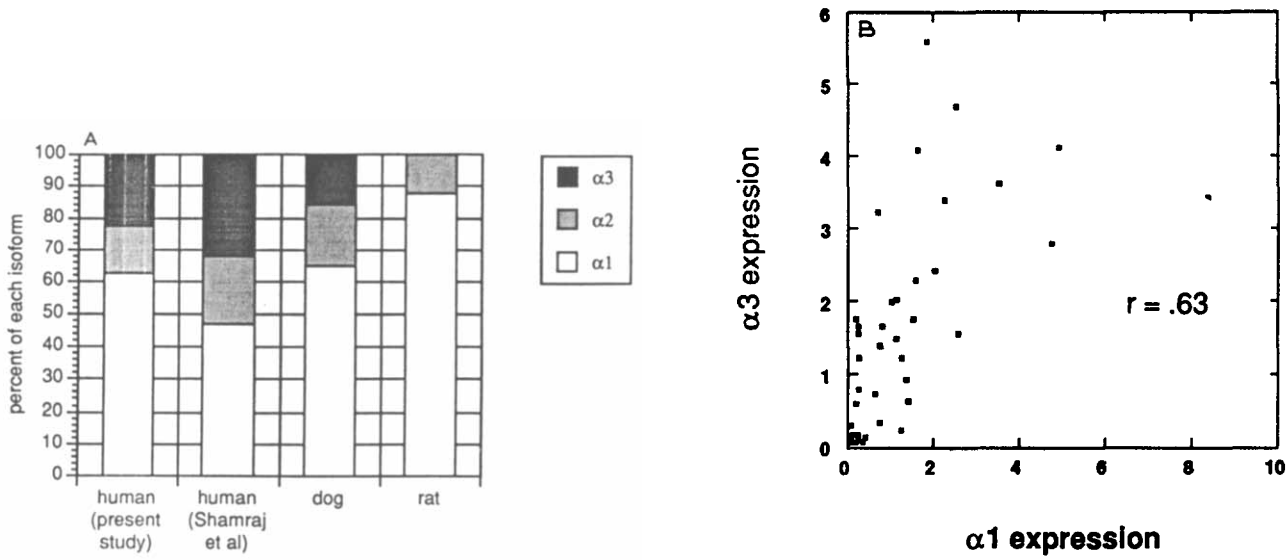

Fig. 2. Panel A: proportion of total α isoform mRNA attributable to each of the three isoforms in LV samples; comparison of data from present study, three human LVs studied by Shamraj et al. [16] and animal data from Ref. 1 and Zahler, R., unpublished data. Panel B – correlation between normalized expression of $\alpha 1$ and $\alpha 3$ in all 47 heart specimens analyzed.

pression of all three isoforms, with the proportions remaining similar; but none of the changes reached statistical significance. In the two hearts with RV overload which were subdivided into RV endocardium and RV epicardium samples, RV epicardium expression was 2–3-fold lower than that in RV endocardium for all three isoforms. There were no statistically significant changes in expression of the β_1 subunit in diseased myocardium.

Normal-appearing regions from LV of both hearts with ischemic cardiomyopathy had decreased levels of $\alpha 1$ mRNA (30% of that found in normal ventricle; data not shown). One of these hearts (heart 2) also had decreased expression of $\alpha 2$ and $\alpha 3$ in each of 5 samples from RV, septum, and LV ($\alpha 3$, 15% of normal; $\alpha 2$, 27%). The other ischemic heart had 1.7-fold increased $\alpha 1$ expression and 2.4-fold increased $\alpha 3$ expression in the ischemic zone of the LV. The heart with idiopathic cardiomyopathy, however, had 2–4-fold increases in expression of each of the α isoforms in all seven regions studied (data not shown).

In both normal and diseased hearts, isoforms tended to move in parallel (Fig. 2B): for all 47 specimens studied, the correlations between expression levels (normalized to GAPDH) of each pair of α isoforms were highly significant by the F test ($\alpha 1$ vs. $\alpha 2$: $r = 0.41$, $P = 0.007$; $\alpha 1$ vs. $\alpha 3$: $r = 0.63$, $P < 0.001$; $\alpha 2$ vs. $\alpha 3$: $r = 0.60$, $P < 0.001$). Normalized β_1 expression also correlated strongly with that of each α ($r = 0.71$, 0.75 and 0.68 for β vs. $\alpha 1$, $\alpha 2$ and $\alpha 3$, respectively; all $P < 0.001$). This suggests that similar regulatory elements may affect the expression of the Na,K-ATPase isoforms.

Discussion

Recent research has defined the patterns of expression of the Na,K-ATPase α subunit isoforms in rat heart [1]. Rodents, however, differ from larger animals in the uniquely high ouabain resistance of their $\alpha 1$ form; in addition, adult dog, ferret and human cardiac ventricle express $\alpha 3$ at high levels, unlike rat (Refs. 13–16; and Zahler, R., unpublished data). It is therefore of interest to study Na,K-ATPase isoform expression in normal and diseased human heart.

There is general agreement that normal adult rat heart contains both $\alpha 1$ and $\alpha 2$ mRNA, with more $\alpha 1$ than $\alpha 2$, and little or no $\alpha 3$ detectable by Northern analysis [1]. In human heart, however, we found that all three isoforms are expressed, with approximately equal amounts of $\alpha 2$ and $\alpha 3$. Similarly, normal dog LV expresses 65% $\alpha 1$, 19% $\alpha 2$, and 16% $\alpha 3$ on average (Zahler, R., et al., unpublished data). In the only previous publications addressing the expression of Na,K-ATPase isoforms in human heart, Gilmore-Hebert et al. found $\alpha 1$ and $\alpha 3$ in a specimen of human fetal heart [9], and Shamraj et al. [16] analyzed α subunit gene expression in LV and RV of three normal human hearts. Like Shamraj et al., we found expression of all three α isoforms; our data indicate a somewhat higher proportion of $\alpha 1$ and lower proportion of $\alpha 2$ and $\alpha 3$ than [16]. Comparisons between our data

and those of Ref. 16 are limited because of the difficulty of obtaining fresh normal human heart tissue; the hearts studied by Shamraj et al. were rejected for transplantation, while our reference samples consisted of two autopsy specimens, which were without morphologic evidence of heart disease but which had variable amounts of post-mortem RNA degradation, and LV free wall from three surgical heart-lung explants. The latter were relatively fresh, morphologically normal, and not under abnormal hemodynamic stress during life; however, ventricular interaction and/or the disease process causing pulmonary hypertension could conceivably have affected the LV free wall.

There appear to be no previous reports on Na,K-ATPase isoform expression in diseased human hearts; however, Norgaard et al. [17,18] and Kjeldsen et al. [19] found a decrease in total sodium pump concentration in LV biopsies of human cardiomyopathic hearts as compared to normals. Preliminary data by DePover et al. indicate that both high-affinity and low-affinity ouabain-binding sites (which may represent differing isoforms) decrease in the same proportion in failing human hearts (see Fig. 6 of Ref. 20), which is similar to our finding that all three α isoforms decrease in pressure-overloaded RV. Another report, however, found no decrease in total Na,K-ATPase in human dilated cardiomyopathy [21]. Our finding that $\alpha 3$ increases and $\alpha 2$ decreases in failing heart suggests a reversion to the fetal cardiac isoform pattern [5], a phenomenon seen with other protein isoforms in heart failure.

Na,K-ATPase isoform expression has been studied in several animal models of heart disease (reviewed in Refs. 1 and 15). Several groups have demonstrated decreases in $\alpha 2$ and $\alpha 3$ in hypertrophied animal heart (Refs. 22–24 and Zahler, R., et al., unpublished data), as we found in pressure-overloaded human RV. Cardiomyopathic hamsters also had a 33% reduction in cardiac Na,K-ATPase concentration [19]. Maixent and Lelievre subjected dog ventricular muscle to regional ischemia and found that within 60 min there was a marked decrease in the number of high-affinity ouabain binding sites and high-affinity Na,K-ATPase activity, but no change in site number or activity of the low-affinity form [25]. We found similar decreases in $\alpha 1$ (which may correspond to the low-affinity form) in two chronically ischemic human hearts, but the large variability observed makes the generalizability of the results uncertain.

Our finding of altered patterns of Na,K-ATPase isoform expression in diseased human hearts is thus concordant with multiple previous studies showing decreased Na,K-ATPase pump number, activity and/or gene expression in overloaded and failing hearts. It is possible that our data reflect partial replacement of myocardium by other cell types which express less Na,K-ATPase; this is unlikely, however, because other cell types would probably not have the identical proportions of isoforms as myocytes; also, histopathologic examination of the hearts we studied showed no evidence of such changes. Alternatively, remodeling of hypertrophied myocytes could have the effect of decreasing the cell surface-to-volume ratio and thus the number of pump sites. Changes in tissue water seen in heart failure would not be expected to influence our results, since we normalized to GAPDH or tubulin expression, and not to tissue weight.

The isoform changes are unlikely to be due to cross-reactivity of isoform probes, since control samples of brain, kidney, and muscle (Fig. 1) indicated correct discrimination among isoforms under the conditions we used. Our data, as well as those of Shamraj et al. [16], show considerable variability among apparently normal specimens. This may be due to variable stability of RNAs or to regional differences; in support of this, we found some consistent LV epicardial : endocardial differences in expression. In-situ hybridization studies [26], however, have so far not found striking differences in isoform expression in cardiac structures other than vessel wall and conduction system. In addition, since isoform mRNA levels may not be proportional to amounts of pump isoform proteins nor to numbers of functional pump units, further work will be needed to study these aspects of isoform physiology in human heart.

Acknowledgements

We thank Philippe Male for performing densitometry, Dr. M. Brines for providing human brain specimens, and JoSan Chung, Fabian Chen, Diego Fornasari, Prasad Devarajan and Paul Graham for their support and advice. Grant support: NHLBI Clinical Investigator Award HL-01686, BSRG Fluid Research Grant (R.Z.)

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