Augmentation of ouabain sensitivity of rat liver Na/K-ATPase by in vivo adenovirus-mediated expression of the Na/K-ATPase α2 subunit

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Abstract These are the first experiments to study the effect of in vivo expression of the Na/K-ATPase α2 subunit which serves as a receptor for cardiac glycosides. The α2 subunit is not normally expressed in rat liver, so hepatocytes which lack endogenous α2 protein are a logical first target to study the effects of α2 expression on membrane Na/K-ATPase activity. At 3 days after α2 adenovirus vector infusion, Wistar rat livers contained α2 DNA, α2 mRNA, and α2 protein. Rat liver membrane ouabain binding activity and the sensitivity of Na/K-ATPase activity to ouabain significantly increased. Total membrane Na/K-ATPase was regulated at a constant level while expressed α2 activity represented 10% of the total active Na/K-ATPase sites in α2 transduced rat liver. These studies are the first to establish a paradigm in which an endogenous drug receptor is expressed to alter cellular pharmacologic sensitivity.
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Key words: Gene therapy; Cell membrane; DNA; Recombinant; Cardiac glycosides; Adenoviridae

1. Introduction

The Na/K-ATPase or sodium pump is an intrinsic plasma membrane enzyme which hydrolyzes ATP to maintain the transmembrane gradients of Na^+ and K^+ found in mammalian cells [1–6]. This enzyme is the functional drug receptor for cardiac glycosides. The enzyme consists of two noncovalently linked subunits. The α subunit (about 112 kDa) contains all of the ligand binding sites of the enzyme, and the β subunit (about 55 kDa) is a glycoprotein that is essential for normal assembly and function of the enzyme [4–6]. Three α subunits have been identified and functionally characterized. The most striking differences between α1, α2 and α3 are their different sensitivities to cardiac glycosides and oxygen free radicals, and their tissue distribution patterns [4–9]. In vitro, the α2 and α3 isoforms are known to be highly sensitive to ouabain and oxidants, while α1 is relatively insensitive to ouabain and oxidants. The α1 subunit serves a housekeeping function maintaining the transmembrane electrical potential for most cells, while the α2 and α3 isoforms are expressed in a tissue-specific distribution. α2 and α3 are mainly expressed in the brain and heart.

In the heart, Na/K-ATPase serves as a receptor for cardiac glycosides, and a partial inhibition of the enzyme by these drugs increases cardiac contractility [1–3]. Recent studies have demonstrated that endogenous expression of α2 and α3 isoforms of Na/K-ATPase is down-regulated by hypertrophy, hypertension, and congestive heart failure in intact hearts of several species [10–16]. Ouabain induces cardiac immediate early gene expression at doses which only inhibit α2 and α3 isoforms of Na/K-ATPase [17], demonstrating that ouabain binding to α2 and α3 isoforms plays an important role in ouabain-induced cardiac gene regulation in addition to its ionotropic effects.

To further address the role of the α2 Na/K-ATPase isoform as a drug receptor and glycoside-regulated membrane pump, it was interesting to develop an approach to express this isoform in vivo. Adenovirus-mediated gene therapy is under development for the treatment of a variety of inherited and acquired diseases [18–24]. Replication-deficient adenoviruses are one of the highest efficacy gene transfer vectors for expressing recombinant genes in vivo by somatic gene transfer [25]. Adenoviral vectors were selected to transfer and express the α2 cDNA as they effectively deliver genes in vivo to muscle and liver. Rat liver does not normally express the α2 isoform of Na/K-ATPase [26], so the absence of a background endogenous signal makes hepatocytes a logical initial target to study the effects of α2 expression on membrane Na/K-ATPase activity.

Rapid advances are being made in our understanding of Na/K-ATPase function and the development of adenovirus vectors for in vivo gene transfer. The current study was performed to explore the utility of adenovirus-mediated gene transfer to express the rat α2 subunit in vivo and determine the pharmacologic effects of α2 expression to alter the ouabain sensitivity of an endogenous drug receptor, the Na/K-ATPase pump.

2. Materials and methods

2.1. Vector specifications, amplification and purification

The in vivo Na/K-ATPase activity conferred by an α2 adenoviral vector (H5.010CMVα2) and a negative control β-galactosidase expression vector (H5.010CMV*lacZ*) were compared in this study (routine vector nomenclature [27]). These vectors are replication-defective adenoviral vectors based on a human Ad5 sub360 serotype viral genomic backbone in which sequences spanning the E1a, E1b, and E3b regions are deleted, with the E1 deletions resulting in the impaired ability of this virus to replicate in non-permissive cells [20]. The cytomegalovirus immediate early promoter and enhancer were cloned into the parent plasmid pAdBgl II to generate the proviral plasmid which was then used to make the α2 adenovirus vector used in this study (pAdCMVα2) [20,28]. The identity of recombinant clones was verified by restriction analysis of viral DNA minipreps and Southern blot analysis, and all vectors were purified through two rounds of plaque purification. Virus was purified from 293 cell lysates

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Abbreviations: CMV, cytomegalovirus; EDRE, endogenous drug receptor expression; α2, α2 subunit of Na/K ATPase enzyme; *LacZ*, *E. coli* β-galactosidase enzyme; H5.010CMVα2, α2 adenoviral vector; H5.010CMV*lacZ*, β-galactosidase expression vector

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by cesium chloride gradient ultracentrifugation then de-salted by dialysis to HBS [22]. Purified virus was used immediately for in vivo injections via the portal vein. Titers were determined by OD_{260} and standard plaque assay, and were approximately 1×10^{13} particles/ml ($\approx 1\times10^{11}$ plaque-forming units (pfu)/ml). All adenoviral preparations were shown to be free of El function by absence of replication on HeLa cells, and PCR amplification of the El region was performed to exclude reconstitution of the El genome from 293 cells in the prepared vectors (data not shown).

2.2. Intraportal vein injections

Animal experiments were performed in accordance with institutional guidelines at the University of Michigan. Adult 200 g Wistar rats were anesthetized with ketamine/rompun, and the abdominal area was scrubbed with povidone/iodine. Using sterile technique and a midline approach, 5×10^9 pfu of adenoviral vector suspension in HBS was infused into the portal vein. Four animals were infused with H5.010CMV*lacZ* and four animals were infused with H5.010CMVα2. Pressure was applied for 5 min to achieve hemostasis. At 3 days post infusion animals from each group were anesthetized and livers were divided with samples from each lobe frozen on liquid nitrogen and stored at −80°C until analysis. Liver samples were analyzed for the presence of vector DNA and transgene expression.

2.3. Southern blot hybridization

Samples were restricted with *Bam*HI and probed with a random primer labeled 2.7 kb *Bam*HI α2 cDNA fragment of pAdCMVα2. Southern blot hybridization was performed as previously described [29].

2.4. Northern blot hybridization

RNA was extracted, electrophoresed, and hybridized with the α2 probe described above [29]. The blot was subsequently stripped and reprobed using an 18S ribosomal RNA probe to demonstrate uniform lane loading and RNA integrity.

2.5. Membrane preparations

Liver membrane was prepared by a modification of previously described methods [30]. Frozen rat liver samples (2 g) were homogenized in 2 volumes each of ice-cold 1 mM $NaHCO_3$, pH 7.50, using 20 strokes of a loose-fitting Dounce homogenizer. The homogenates were diluted and filtered through 3 layers of surgical gauze. After centrifugation at $1500\times g$ for 10 min, the pellets were resuspended in buffer and 5.5 volumes of 70.7% sucrose were added, mixed, and then distributed into centrifuge tubes. 8 ml of 48.2% and 4 ml of 42.5% sucrose were layered over this suspension. After centrifuging for 60 min at $66\,000\times g$, the material which accumulated around the interface of 42.5% and 48.2% sucrose was collected, diluted, and washed. The final pellet was resuspended in 0.25 M sucrose, 30 mM histidine, 1 mM EDTA (pH 6.8), and stored at −80°C. Enzyme activities were highly reproducible using this technique.

2.6. Immunotransblot analysis

The antibodies used in this study were rabbit isoform-specific anti-rat α1 or α2 polyclonal antibodies obtained from UBI (Waltham, MA). Immunotransblots were performed on liver membrane protein preparations as described [8,15].

2.7. ATPase activity assay

Na/K-ATPase activity was assayed at 37°C by measuring the initial rate of release of ^{32}P from [γ-^{32}P]ATP in a solution containing 100 mM NaCl, 25 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 5 mM NaN_3, and 50 mM Tris-HCl (pH 7.4) [31,32]. Each ATPase assay was done with a 15 min pre-incubation in the presence and absence of 1 mM ouabain to assess the ouabain-sensitive component of the activity.

2.8. [γ-^{32}P]ATP phosphorylation assay

To estimate the total active Na/K-ATPase sites in the membrane preparation, the maximal level of the enzyme's phosphointermediate was determined [31,32]. Phosphorylation by [γ-^{32}P]ATP was done at 0°C by incubating the membrane preparation with 2 mM $MgCl_2$, 100 mM NaCl, 10 μM ATP, and 50 mM Tris-HCl (pH 7.4) for 30 s. Phosphorylation reactions performed under these conditions were noted to be sufficient to achieve the maximal level of phosphointermediate formation. Labeling reactions performed in the presence of KCl instead of NaCl were used to determine the Na^+-dependent component of phosphorylation. After denaturation with perchloric acid, the samples were filtered through a 0.45 mm membrane filter, washed, and counted [31].

2.9. Ouabain binding assay

The maximal level of bound ouabain was assayed as described previously using a saturating concentration (0.4 μM) of [^{3}H]ouabain in the presence of 3 mM $MgCl_2$, 3 mM Pi (added as Tris phosphate, 25 mM Mes, 25 mM Tris, pH 7.0). After incubation at 37°C for 15 min, the samples were cooled on ice, and 50 μg of membrane protein was placed on each 0.45 mm membrane filter which was washed, air-dried, and counted [31,32]. Non-specific binding was corrected by determining binding in the presence of 1 mM unlabeled ouabain. Protein concentration was determined with BSA used as a standard [33].

3. Results

3.1. Adenoviral vector DNA and RNA were detected 3 days post injection

Fig. 1 is a representative Southern hybridization which demonstrated nonrearranged α2 adenoviral vector sequences in Wistar rat livers infused via the portal vein with 5×10^9 pfu of H5.010CMVα2. Transduction efficiency was approximately 5 copy number at 3 days post infusion, consistent with the

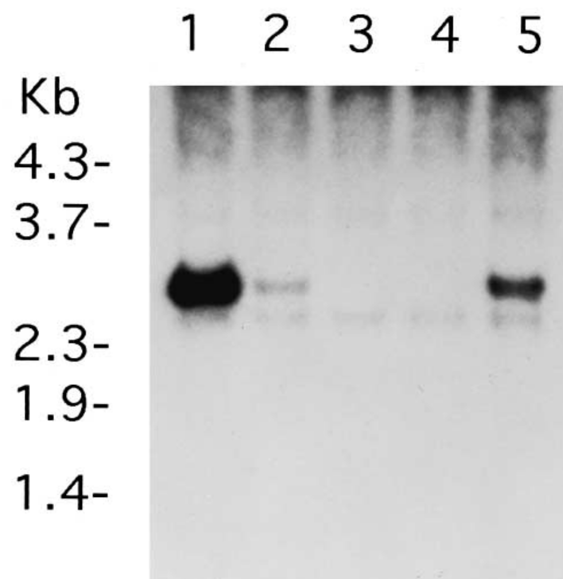

Fig. 1. Representative Southern analysis of transduced Wistar rat livers for α2 DNA. Wistar rat livers were transfected with H5.010CMV*lacZ* (control) or H5.010CMVα2 and analyzed for the presence of nonrearranged vector derived α2 sequences in preparations of total cellular DNA probed for α2 as described in Section 2. The first three lanes contain DNA (10 μg) from mock infused livers supplemented with copy number controls: (1) 10 copy number (75 pg of pAdCMVα2), (2) 1 copy number (7.5 pg of pAdCMVα2), (3) 0 copy number. The next lane (4) contains genomic DNA (10 μg) from Wistar rat liver transduced with H5.010CMV*lacZ* 3 days post infusion as labeled. Finally, the last lane (5) contains genomic DNA (10 μg) from Wistar rat liver transduced with H5.010CMVα2 3 days post infusion, as labeled. The sequence specific bands appeared at the predicted MW, 2700 bp. Faint hybridization noted with a band of lower molecular weight sequences in control and experimental animals likely reflects cross hybridization with genomic DNA. Note the absence of a 2700 bp band in the lanes containing 0 copy number and H5.010CMV*lacZ* transduced genomic DNA preparations.

hepatocyte transfection capability of adenoviral vectors. No 2700 bp bands, the predicted size of an α2 *Bam*HI fragment, were ever detected hybridizing with the α2 probe in Wistar rat liver at 3 days post infusion with H5.010CMV*lacZ*. Control infusions with the H5.010CMV*lacZ* adenovirus vector using equal doses (pfu) to the α2 adenovirus vector demonstrated comparable transduction efficiencies to H5.010CMVα2 infused livers as determined by β-galactosidase staining of cells (data not shown).

Livers infused via the portal vein with the specified vectors were analyzed for transgene expression by RNA blot hybridization of total cellular RNA (Fig. 2A). RNA hybridization of total cellular RNA from Wistar rat liver demonstrated abundant α2 mRNA in H5.010CMVα2 infused livers. Transfected Wistar rat livers at 3 days post infusion showed clear α2 mRNA sequence-specific hybridization. The distinct band detected in α2 transduced livers was approximately 3.1 kb, the anticipated band size for transcripts initiated at the internal CMV promoter-enhancer of the adenovirus vector. Hybridization to an 18S ribosomal probe demonstrated uniform content of RNA in the designated lanes (Fig. 2B).

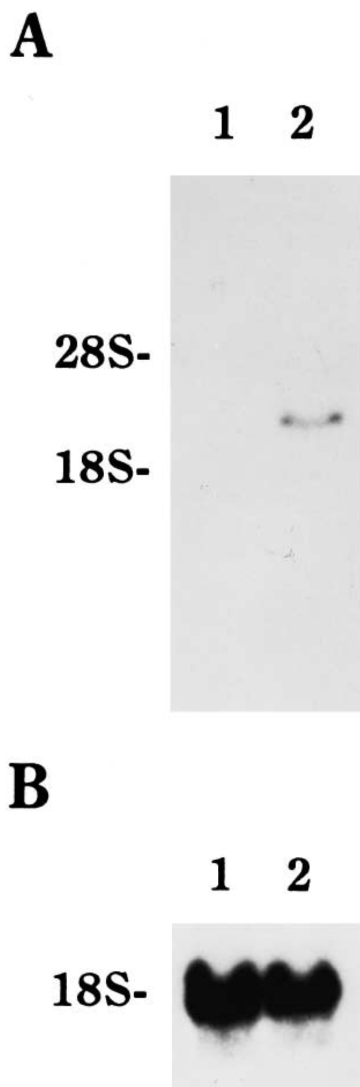

Fig. 2. Representative Northern blot hybridization of total cellular RNA extracted from Wistar rat livers following transfection with α2 and *lacZ* adenoviral vectors. Wistar rat livers were control transfected or transfected with H5.010CMVα2. Both lanes contain total cellular RNA, 15 μg per lane. The left lane (1) contains RNA from a liver 3 days post transduction with H5.010CMV*lacZ*, while the right lane (2) contains total cellular RNA derived from Wistar rat livers 3 days post transduction with H5.010CMVα2. Note the presence of sequence specific hybridization to α2 transduced liver (A, 4 h exposure). The sequence specific hybridization appeared at the appropriate size for α2 message in α2 transduced liver. The migration of ribosomal RNAs is indicated along the lefthand border. The blot was stripped and reprobed with an 18S ribosomal RNA probe to document uniformity of lane loading (B).

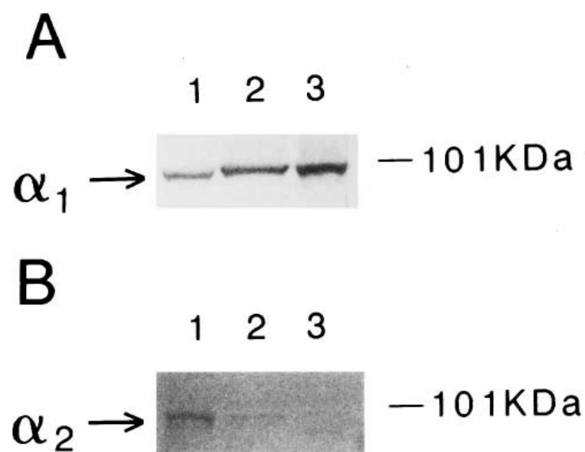

Fig. 3. Na/K-ATPase α1 and α2 subunit immunotransblot analysis of rat liver membranes following transfection with adenoviral vectors with heart membrane proteins included as a positive control. Aliquots of membrane proteins (liver, 60 μg/lane; heart, 30 μg/lane) were separated by SDS/PAGE (10% gel) and transferred to nitrocellulose. Lane 1, rat heart; lane 2, H5.010CMVα2 transduced liver; lane 3, control H5.010CMV*lacZ* transduced liver. A: Probed with an α1 specific antibody. B: Probed with an α2 specific antibody. Molecular weights as determined by pre-stained molecular weight markers are shown on the right. Note the presence of α2 protein in the α2 transduced rat liver membranes and its absence in control *lacZ* transduced liver. Note the presence of endogenous α1 signal in all three samples demonstrating the integrity of the preparations.

3.2. Immunotransblot analysis demonstrates α2 protein in rat liver membranes following infusion of H5.010CMVα2

Expression of α2 protein was assessed by immunotransblot analysis (Fig. 3B). The antibody utilized in this assay is isoform-specific for the α2 subunit of rat Na/K-ATPase [11]. Specific reactivity to the α2 isoform antibody was observed in H5.010CMVα2 transfected Wistar rat livers at 3 days post infusion. Negative control membranes which differed only in the substitution of the β-galactosidase vector for the α2 vector demonstrated no reactivity to this antibody consistent with α2 specificity of the detected signal. Rat heart was utilized as a source of α2 protein for a positive control since rat heart normally expresses the α2 isoform of Na/K-ATPase. The α2 isoform represents about 20% of total Na/K-ATPase in the heart [6,14]. The level of α2 expression in α2 transduced liver in comparison to β-galactosidase transduced liver and control rat heart is consistent with the ouabain binding activity shown in Table 1. As a further control to demonstrate the isoform specificity of the immunotransblot results, an antibody specific for the α1 subunit of rat Na/K-ATPase was utilized to similarly probe the samples for α1 protein (Fig. 3A).

Table 1
Comparison of ouabain binding activity between Ad CMV*lacZ* and Ad CMVα2 transduced liver membrane preparations

Experiment	Ad CMV*lacZ* (pmol/mg)	Ad CMVα2 (pmol/mg)
1	N.D.	0.62 ± 0.08
2	0.08 ± 0.03	1.40 ± 0.06
3	0.06 ± 0.04	0.92 ± 0.09
4	N.D.	0.66 ± 0.09

Ouabain binding assays were done in four independent membrane preparations as described in Section 2. Values are mean ± standard error. N.D., not detectable.

3.3. Ouabain binding assay demonstrates functional α2 protein in Wistar rat livers following gene transfer via the portal vein with H5.010CMVα2

As a first assay to determine if expressed α2 protein exhibits similar functional properties to native α2 protein, ouabain binding activity was measured. In agreement with the fact that rat liver expresses only the ouabain-insensitive α1 isoform [26], high affinity ouabain binding was negligible in liver membranes from rats transduced with the negative control vector, H5.010CMV*lacZ* (Table 1). In four separate experiments, H5.010CMVα2 transduction resulted in a significant increase in ouabain binding activity in rat liver membrane preparations. In agreement with the Western blot data, H5.010CMVα2-induced ouabain binding activity in the liver is lower than that seen in the rat heart preparation (data not shown).

3.4. Sensitivity of Na/K-ATPase activity to ouabain increases following transduction of rat liver with H5.010CMVα2

Liver membranes from Wistar rats transduced with H5.010CMVα2 and Wistar rats transduced with H5.010CMV*lacZ* were prepared and assayed for total Na/K-ATPase activity. While H5.010CMVα2 transduction did not increase the total enzyme activity (Table 2), an ouabain-sensitive portion of the enzyme was found in H5.010CMVα2 transduced rat liver (Fig. 4). The dose-response relationship of ouabain and total Na/K-ATPase activity clearly demonstrates a shift in ouabain sensitivity in α2 transduced liver (Fig. 4).

To determine the ratio of the expressed α2 to endogenous α1 in transduced rat liver, both ouabain binding assays and ATP phosphorylation assays were performed and compared. Since the ATP phosphorylation assay measures the total active enzyme sites in the membrane preparation while ouabain binding represents the expressed active α2 sites, the ratio of expressed α2 to the total active enzyme sites was calculated. Expressed α2 activity represented approximately 10% of the total active Na/K-ATPase sites in α2 transduced rat liver, while *lacZ* transduced rat liver had no detectable α2 activity in accord with the known tissue distribution of this enzyme [26]. These results were consistent with the ouabain binding data (Table 1).

4. Discussion

These are the first studies to apply adenovirus-mediated gene transfer to a new paradigm in which endogenous drug receptor expression (EDRE) is employed to alter the pharmacologic sensitivity to a classical drug like digitalis. It was of interest to express the α2 cDNA Na/K-ATPase isoform and examine its in vivo function. Although active Na/K-ATPase has been successfully expressed in vitro in *Xenopus* oocytes, several mammalian cell lines, yeast, and insect cells using a variety of expression vectors [5,16,17], in vivo expression with these gene transfer systems has not been successful. This is the first study to demonstrate the feasibility of using recombinant adenovirus-mediated gene transfer to express a functional Na/K-ATPase isoform in vivo and study its effects on Na/K-ATPase membrane activity.

Wistar rat liver does not express any α2 isoform of Na/K-ATPase normally [26]. Hence, detection of α2 protein and function in liver is uncomplicated by background endogenous α2 protein expression as it would be in cardiac muscle. The ability of the α2 isoform to augment membrane Na/K-ATPase sensitivity to ouabain following gene transfer in vivo had not been previously demonstrated.

DNA blot analysis demonstrated transfer of the α2 cDNA following in vivo infusion of the α2 adenoviral expression vector H5.010CMVα2. RNA blot analysis demonstrated that α2 mRNA is expressed in rat liver at 3 days post H5.010CMVα2 vector infusion. Western blot analysis of protein derived from liver membranes demonstrated immunoreactivity corroborating expression of α2 protein. DNA, RNA and protein blots were consistent. As controls, transductions performed with H5.010CMV*lacZ* did not result in expression of α2 mRNA or α2 protein excluding adenovirus induced changes in the expression of the endogenous α2 gene as the mechanism of protein production. It was established that the expressed α2 was a functional protein, the demonstration of protein function was important as the expression of largely dysfunctional, misfolded proteins has typified some prokaryotic and in vitro expression systems.

Ouabain binding assays showed that expressed α2 binds ouabain with high affinity, and its Na/K-ATPase activity is highly ouabain sensitive in comparison to that of the endogenous α1 isoform. The efficacy of the expressed α2 isoform to augment ouabain binding activity in vivo represents a further validation of the putative function of the α2 isoform as a high affinity receptor for cardiac glycosides. These findings are consistent with previous in vitro studies which suggested the α2 isoform as a major high affinity ouabain sensitive isoform [5,16,17]. It was interesting that total cellular Na/K-ATPase activity remained constant even when α2 activity was expressed using a constitutive promoter. This suggests that transgene derived Na/K-ATPase activity down regulates endogenous Na/K-ATPase activity to maintain total cellular activity constant. These observations had not been previously

Table 2
Specific Na/K-ATPase activity and estimation of the ratio of expressed α2 to the total active enzyme sites

	Na/K-ATPase activity (μmol Pi/mg/h)	α2 activity (% of total active enzyme site)
Ad CMV*lacZ*	4.4 ± 0.28	N.D.
Ad CMVα2	4.6 ± 0.27	9.8 ± 0.4

Na/K-ATPase activity was measured from six independent membrane preparations, and the values are mean ± standard error. α2 active sites were determined by ouabain binding as in Table 1, and total active enzyme sites were estimated by ATP phosphorylation as described in Section 2.

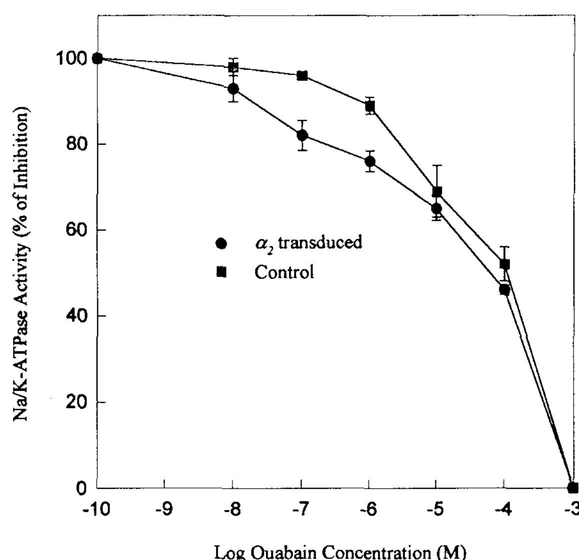

Fig. 4. Comparison of Na/K-ATPase activity as a function of ouabain concentration in α2 transduced rat liver membranes and control *lacZ* transduced liver. Membrane preparations were pre-incubated with different concentrations of ouabain for 15 min, and assayed for Na/K-ATPase activity as described in Section 2. Values are expressed as mean ± S.E. of five independent determinations. Note the increased sensitivity of liver membrane Na/K-ATPase activity to ouabain following transduction with α2.

made in vivo; the regulation of Na/K-ATPase activity has important implications for cell function and pathophysiology.

These expression studies were conducted over a 3 day time course with *LacZ* controls to avoid an immune response to vector proteins complicating interpretation of the results. Initial applications of adenoviral vectors have been confounded by a lack of true persistence attributed to immune reactions to the gene transfer vectors detectable one week after vector infusion. Modified vectors or delivery strategies with immunosuppression should lead to an effective long-term gene transfer moiety [19–25].

These studies support the potential utility of EDRE strategies in conjunction with traditional pharmacological therapy (e.g. cardiac glycosides) to alter pathophysiology. Expression of the α2 and/or α3 isoforms of Na/K-ATPase is lower in heart failure [10–16]. Since these are the ouabain sensitive Na/K-ATPase isoforms, their expression may be useful to treat heart failure. Escalating doses of ouabain lead to toxicity, primarily in conductive cardiac tissue, so gene therapy strategies directed at increasing the relative sensitivity of myocytes to ouabain by expressing the α2 isoform in contractile regions of the myocardium in preference to the Purkinje system should be of benefit.

In summary, data reported here document the utility of adenovirus vectors to express the α2 isoform in vivo in Wistar rat liver; this is significant because other α2 expression strategies have not proved useful in vivo. Following in vivo expression, the α2 isoform dramatically increases membrane ouabain sensitivity. It was interesting that transgene derived Na/K-ATPase activity down regulates endogenous membrane activity to maintain total activity constant. The levels of protein expression achieved with in vivo adenovirus infusion suggest that delivery of the α2 gene with an adenovirus vector might improve the efficacy and therapeutic index of cardiac glycosides.

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References

[1] Braunwald, E. (1985) J. Am. Coll. Cardiol. 5, 51A–59A.
[2] Akera, T. and Ng, Y.C. (1991) Life Sci. 48, 97–106.
[3] Schwartz, A., Grupp, G., Wallick, E., Grupp, I.L. and Ball Jr., W.J. (1988) Prog. Clin. Biol. Res. 268B, 321–338.
[4] Mercer, R.W. (1993) Int. Rev. Cytol. 137C, 139–168.
[5] Lingrel, J.B. and Kuntzweiler, T. (1994) J. Biol. Chem. 269, 19659–19662.
[6] Sweadner, K.J. (1989) Biochim. Biophys. Acta 988, 185–220.
[7] Xie, Z., Wang, Y., Askari, A., Huang, W.-H., Klaunig, J.E. and Askari, A. (1990) J. Cell. Mol. Cardiol. 22, 911–920.
[8] Huang, W.-H., Wang, Y., Askari, A., Zolotarijova, N. and Ganjeizadeh, M. (1994) Biochim. Biophys. Acta 1190, 108–114.
[9] Xie, Z., Jack-Hays, M., Wang, Y., Periyasamy, S.M., Blanco, G., Huang, W.-H. and Askari, A. (1995) Biochem. Biophys. Res. Commun. 207, 155–159.
[10] Kim, C.H., Fan, T.M., Kelly, P.F., Himura, Y., Debhanty, J.M., Hang, C.L. and Liang, C. (1994) Circulation 89, 313–320.
[11] Sweadner, K.J., Herrera, V.L.M., Anato, S., Moellmann, A., Gibbons, D.K. and Repke, K.R.H. (1994) Circ. Res. 74, 669–678.
[12] Book, C.B.S., Moore, R.L., Semanchik, A. and Ng, Y.C. (1994) J. Mol. Cell. Cardiol. 26, 591–600.
[13] Herrera, V.L.M., Chobanian, A.V. and Ruiz-Opazo, N. (1988) Science 241, 221–223.
[14] Charlemagne, D., Orlowski, J., Oliviera, P., Rannout, F., Beuve, C.S., Sarynghedauw, B. and Lane, L.K. (1994) J. Biol. Chem. 269, 1541–1547.
[15] Huang, W.H., Lai, C.C., Wang, Y., Askari, A., Klevay, L.M., Askari, A. and Chiu, T.H. (1995) Cardiovasc. Res. 29, 563–568.
[16] Zahler, R., Gilmore-Hebert, M., Baldwin, J.C., Franco, K. and Benz Jr., E.J. (1993) Biochim. Biophys. Acta 1149, 189–194.
[17] Peng, M., Huang, L., Xie, Z., Huang, W.-H. and Askari, A. (1996) J. Biol. Chem. 271, 17, 10372–10378.
[18] Jaffe, H.A., Danel, C., Longendcler, G., Metzger, M., Setoguchi, Y., Rosenfeld, M.A., Gant, T.W., Thorgeirsson, S.S., Stratford-Perricaudet, L.S., Perricaudet, M., Pavirani, A., Lecocq, J.-P. and Crystal, R.G. (1992) Nature Genet. 1, 372–378.
[19] Qiuntang, L., Kay, M.A., Finegold, M., Stratford-Perricaudet, L.D. and Woo, S.L.C. (1993) Hum. Gene Ther. 4, 403–409.
[20] Kozarsky, K.F. and Wilson, J.M. (1993) Curr. Opin. Genes Dev. 3, 499–503.
[21] Engelhardt, J.F., Xuehai, Y., Doranz, F. and Wilson, J.M. (1994) Proc. Natl. Acad. Sci. USA 91, 6196–6200.
[22] Askari, F.K., Hitomi, Y., Mao, M. and Wilson, J.M. (1996) Gene Ther. 3, 381–388.
[23] Yang, Y., Nunes, F.A., Benecsi, K., Gonczol, E., Engelhardt, J.F. and Wilson, J.M. (1994) Nature Genet. 7, 362–369.
[24] Yang, Y., Nunes, F.A., Berencsi, K., Furth, E.E., Gonczol, E. and Wilson, J.M. (1994) Proc. Natl. Acad. Sci. USA 91, 4407–4411.
[25] Kozarsky, K.F., McKinley, D.R., Austin, L.L., Raper, S.E., Stratford-Perricaudet, L.D. and Wilson, J.M. (1994) J. Biol. Chem. 269, 18, 13695–13702.
[26] Simon, F.R., Leffert, H.L., Ellisman, M., Iwahashi, M., Deerinck, T., Fortune, J., Morales, D., Dahl, R. and Sutherland, E. (1995) Am. J. Physiol. 269, C69–C84.
[27] Engelhardt, J.F., Litzky, L. and Wilson, J.M. (1994) Hum. Gene Ther. 5, 1217–1229.
[28] Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B. and Schaffner, W. (1985) Cell 41, 521–530.

[29] Askari, F.K., Hitomi, E., Thiny, M. and Wilson, J.M. (1995) Gene Ther. 2, 203–208.
[30] Song, C.S., Rubin, W., Rifkind, A.B. and Kappas, A. (1969) J. Cell Biol. 41, 124–132.
[31] Askari, A., Huang, W.-H. and McCormick, P.W. (1983) J. Biol. Chem. 258, 3453–3460.
[32] Askari, A., Kakar, S.S. and Huang, W.-H. (1988) J. Biol. Chem. 263, 235–242.
[33] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.