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β-Adrenergic Receptor Signaling Regulates rAAV Transduction through Calcineurin in Heart Muscle Cells

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Abstract

Recombinant adeno-associated virus (rAAV)-based gene therapy represents a promising approach for the treatment of heart muscle diseases, but the molecular mechanisms that direct rAAV transduction remain unclear. Here we demonstrate that β-adrenergic receptor stimulation with isoproterenol (ISO) markedly increased cardiomyocyte transduction of rAAV *in vitro* and *in vivo*. Conversely, chronic β-adrenergic receptor downregulation significantly suppressed rAAV transduction. Pretreatment with calcium signaling cascade inhibitors including calcineurin inhibitory peptide (CNIP) strongly suppressed the positive effects of ISO on rAAV transduction. Additionally we document that ISO treatment led to a significant increase in double-stranded (ds) DNA synthesis of the rAAV genome and an increase in promoter activity. Moreover, stimulation with ISO did not affect rAAV transduction in calcineurin nullizygous mice. Collectively, we conclude that a calcium-dependent pathway regulates rAAV vector transduction at a number of stages that may include vector mobilization, conversion, and transcription activity. Modulating this pathway through β-adrenergic signaling enhances rAAV-mediated gene delivery to cardiomyocytes, and may be valuable when considering therapeutic approaches for heart muscle diseases.

Keywords: rAAV Vector; Transduction; Heart; β-adrenergic receptor; Calcineurin

Abbreviations: AAV: Adeno-Associated Virus; ISO: Isoproterenol; hGH: Human Growth Hormone; MHC: Myosin Heavy Chain; CREB: Cyclic AMP-dependent kinase response Element Binding Protein; PKA: Protein Kinase A; PKC: Protein Kinase C; MAPK: Mitogen-Activated Protein Kinase; PI3-K: Phosphatidylinositol 3-Kinase; CN: Calcineurin; CyA: Cyclosporin A; CNIP: Calcineurin Autoinhibitory Peptides; D.N.CN: Dominant Negative Mutant of CN; C.A.CN: Constitutively Active Mutant Of Calcineurin; ss DNA: single-strand DNA; ds DNA: double-strand DNA; CM: Cardiac Myocytes

Introduction

While other vector systems have suffered setbacks, recombinant adeno-associated virus (rAAV) has continued to show promise as a gene transfer vector for muscle diseases [1,2]. The physical stability of rAAV makes these vectors attractive for *in vivo* use, and transgene expression can persist long-term in a wide range of tissues including heart muscle [3-5]. Three phase I/II clinical trials have been completed in which rAAV vectors were administered systemically or intramuscularly for hemophilia [6,7]. Although encouraging results from animal studies have prompted many clinical studies, surprisingly little is known about the specific molecular mechanisms of rAAV transduction. Barriers to gene delivery are reported to exist at the steps of cellular attachment, cellular uptake, subcellular processing, nuclear targeting, and stabilization of the transgene [2]. It has been demonstrated that the efficiency of many of these steps can be improved through the use of pharmacological agents.

We explored whether isoproterenol (ISO), a non-selective β-adrenergic receptor agonist, could be exploited to increase tranduction of rAAV in heart muscle tissue. ISO activates adenylate cyclase via the stimulatory G protein, Gαs, to trigger cyclic AMP formation [8], and has a number of downstream effects that may benefit transgene delivery and expression. Cyclic AMP production leads to activation of PKA [9], with subsequent phosphorylation of several key calcium cycling and contractile proteins. The β-adrenergic signaling cascade is centrally involved in the positive chronotropic and inotropic response of the heart to physiological stimuli [8,9]. ISO regulates the opening of L-type calcium channels by activating adenylate cyclase [10,11]. Elevation of cytosolic Ca^{2+} in turn induces the activation of the Ca^{2+}-dependent effectors, calmodulin and calcineurin [12]. Administration of ISO strongly upregulates the expression of various genes and induces cardiac hypertrophy mediated the via calcineurin isoform Aβ [13]. Calcineurin induces the desphosphorylation and nuclear translocation of the transcriptional factor NFAT in hearts [12]. Moreover, ISO also targets and phosphorylates the cyclic AMP-dependent kinase response element binding protein (CREB) [14]. Previously, Flotte et al. reported that CREB is an important regulator of the AAV p5 promoter [15]. In this light, we initiated these experiments in an attempt to use ISO stimulation to enhance transduction of a rAAV vector that includes a CMV promoter, since the CMV promoter contains five CREB binding sites.

Here we show that ISO markedly increased rAAV gene expression and transduction through activation of the calcium/calcineurin cascade in cardiomyocytes. Using combinatorial drug studies,

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we demonstrate ISO positively influences multiple steps of rAAV transduction, potentially affecting vector trafficking, stabilization of the transgene, and promoter activity. In addition, ISO regulated rAAV expression *in vivo*, and calcineurin nullizygous mice exhibited delayed rAAV expression compared to wild-type mice. These results highlight the role of calcium/calcineurin pathways on AAV biology and provide opportunities to enhance rAAV-mediated gene delivery in heart muscle tissue.

Materials and Methods

Materials

EGTA, Calphostin C, Wortmannin, BAPTA-AM, and Ionomycin were obtained from Sigma, and Isoproterenol, cell permeable PKI, Genistein, PD98059, SB203580, W7, Cyclosporin A, and Calcineurin autoinhibitory peptides were from Calbiochem.

rAAV vector production

rAAV vectors were prepared as described [3]. Briefly, vector plasmids were cotransfected into subconfluent 293 cells with the pLTAAV (serotype 2) or pXR1 (serotype 1) helper plasmid using the calcium phosphate method. Cells were then infected with adenovirus Ad5dl312 (an E1A-null mutant) at a multiplicity of infection of 2 and after 72h the cells were harvested, lysed by three freeze/thaw cycles, and the virions were isolated by cesium chloride gradient. The gradient fractions containing rAAV were dialyzed against sterile PBS, heated for 30 min at 56°C, and stored at –80°C. rAAV vectors with titers of 5 x10^{11} to 2 x 10^{12} vector genomes (vg)/ml were prepared as described previously [3].

Cell culture and rAAV transduction assays

H9C2 cardiac cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin in a humidified atmosphere at 37°C with 5% CO_2. Primary neonatal rat cardiomyocytes were isolated and prepared as described [3]. The cells were incubated in PBS or 10^{-5} M ISO for 2 h, which was replaced to fresh medium after PBS washes. Transduction was achieved by adding 1000 vector genomes/cell of rAAV-CMV-lacZ. After 24 h incubation, the cells were fixed and stained for β-gal activity. To discriminate neonatal rat cardiomyocytes, the cells were fixed and stained with an anti α-tropomyosin antibody (Sigma Chemical Co. #2780) and goat anti-mouse IgG Alexa 594 (Molecular Probes) following β-gal staining [3].

Animal studies

The Tufts University Institutional Animal Care and Use Committee approved all animal studies. FVB mice (7～8 week-old) were purchased from Charles River Laboratories and CNAβ$^{-/-}$ mice were provided from Cincinnati Children's Hospital Medical Center. Mice were anesthetized with an intraperitoneal injection of ketamine (90-120 mg/kg) and xylazine (5-10 mg/kg). To inject rAAV vectors directly into mouse myocardium the respiration of anesthetized mouse was controlled using a dedicated ventilator. The heart was exposed following thoracotomy to enable injection of 25μl containing 5x10^{10} genomes of rAAV-hGH vectors using a 30-gauge needle. The injection was made under direct visualization to one site of the myocardium on the left ventricular wall around the anterior descending coronary artery. Ten minutes after rAAV injection, we administered ISO (2μg/g) or PBS (equivalent volume) into intraperitoneal portion. To induce β-adrenergic receptor downregulation, we inserted an osmotic pump in the subcutaneous space on the back of mice. Four days after pump insertion, we injected rAAV into heart muscle as above and continued chronic ISO stimulation (30μg/g/day) for a total of 28 days. After infection, blood samples were obtained periodically from the retro-orbital vein of anesthetized mice, and the plasma hGH concentrations were determined by ELISA as described [3].

mRNA expression

Total RNA was isolated by cesium chloride ultracentrifugation. Total RNA was copied using the Superscript First Strand Synthesis System (Invitrogen, *cat. #11904-018*), and then a 268 bp fragment of the β-galactosidase gene was amplified using sense 5'-TCAATCCGCCGTTTGTTCCC-3' and antisense 5'-TCCAGATAACTGCCGTCACTCC-3' lacZ-specific primers. Control RT-PCR reactions using mouse β-actin primers were carried out in parallel reactions.

Southern blotting

Two hours after incubating with 10^{-5} M ISO, rAAV-CMV-lacZ vectors were infected into H9C2 cells. Total DNA that had been isolated using Puregene DNA Isolation Kit [3] 24 h later, was digested with *Eco*RI, separated by agarose gel electrophoresis and transferred onto nylon membrane. The membrane was hybridized with a [^{32}P]-labeled *Eco*RI pAAV-CMV-lacZ fragment and exposed to Kodak BioMAX film. For Alkaline-gel Southern blots, total DNA was digested with *Sac*I and separated by alkaline agarose gel electrophoresis, using a protocol derived from http://www.bio.brandeis.edu/haberlab/jehsite/protocol.html, and blotted onto nylon membrane. A [^{32}P]-labeled *Sac*I-*Xho*I pAAV-CMV-lacZ fragment was used as probe.

Promoter activity assay

In transient transfection assays, AAV-lacZ vector plasmids were transfected into H9C2 cells using Polyfect reagent with a dominant negative mutant of calcineurin (D.N.CN) or control plasmid (Qiagen, *cat. #301107*). Following incubation with 10^{-5} M ISO for 24 h the cells were harvested, and the β-gal activity was measured using a luminometer (Pierce, Mammalian β-gal assay kit *cat. #75707*). A constitutively active mutant of calcineurin (C.A.CN) was trasnfected with the reporter gene plasmid without ISO. To control for transfection efficiency we normalized luminescence values to total protein concentration measured by the BCA protein assay reagent (Pierce, *cat. #23223*).

Statistical analysis

The mean and standard error (S.E.) were determined for replicate samples. For multiple treatment groups, a factorial analysis of variance was applied followed by Fisher's least significant difference test as computed by the Statview computer program. A p value of less than 0.05 was considered significant.

Results

Isoproterenol (ISO) increases rAAV transduction of muscle cells

To determine whether β-adrenergic receptor stimulation enhances rAAV (serotype 2) transduction, we pretreated rat cardiac myoblasts (H9C2) with a β-adrenergic receptor agonist, isoproterenol (ISO). Pretreatment with ISO between 10^{-8} M and 10^{-5} M for 2 h markedly increased the transduction by rAAV-CMV-lacZ vectors in H9C2 cells (Figures 1A and 1B). We reported previously that rAAV vectors containing the cardiac muscle specific alpha myosin heavy chain (MHC) gene promoter selectively express in cardiomyocytes

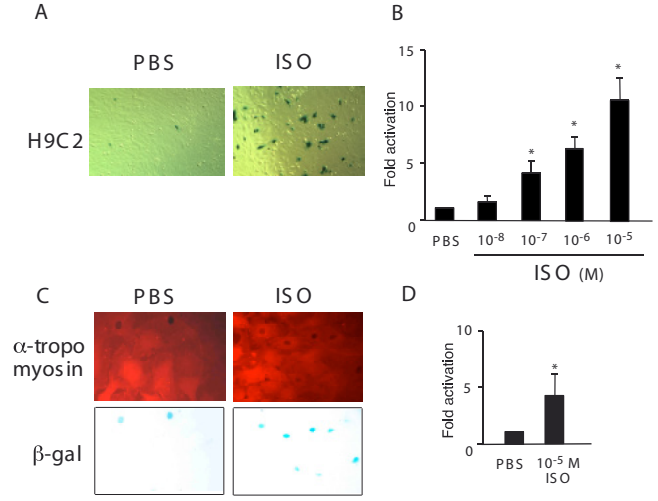

Figure 1: rAAV transduction was increased by isoproterenol (ISO) *in vitro*. (A) H9C2 cells were transduced by 1000 vector genomes/cell of rAAV with or without preincubation using ISO. Twenty-four hours after infection, the cells were stained for β-gal activity. The scale bar shows 200μM. (B) After incubation with indicated concentrations of ISO, the number of β-gal positive cells were counted. (C) Rat neonatal cardiomyocytes (CM) were transduced with rAAV-MHC-lacZ vector with or without preincubation of using ISO. Twenty-four hours after infection, the CM were immunostained using an anti tropomyosin antibody following β-gal staining. The scale bar shows 40μM. (D) The number of β-gal positive CM were enumerated. Data are mean±SEM, *, $p<0.05$ ($n=5$) vs. PBS control.

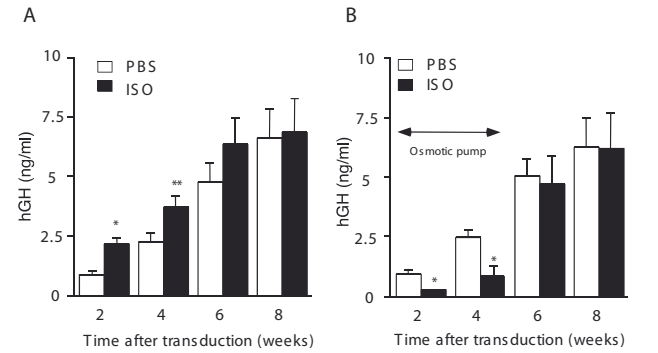

Figure 2: β-receptor signaling regulates rAAV transduction *in vivo*. (A) rAAV-CMV-hGH vector was directly injected into the heart muscle of adult FVB mice, and then ISO was administered into the peritoneum 5 min later. Blood was obtained every two weeks, and the concentration of human growth hormone (hGH) in serum was measured by ELISA. Data are mean±SEM, *$p<0.05$, **$p<0.01$ ($n=6$) vs PBS control. (B) β-adrenergic receptor down regulation by chronic stimulation of ISO using an osmotic pump. rAAV-CMV-hGH vector was directly injected into the hearts of adult FVB mice 4 days after the pump was inserted. Data are mean±SEM, *$p<0.01$ ($n=6$) vs. PBS control.

[3]. We therefore used rAAV-MHC-lacZ to transduce neonatal rat cardiomyocytes (CM) to evaluate whether ISO increases the transduction of cardiac specific rAAV. CM were incubated with 10^{-5} M ISO vs. PBS for 2 h before application of rAAV-MHC-lacZ vector. The cells were stained for both β-galactosidase activity and the cardiac muscle sarcomeric protein α-tropomyosin. The number of β-gal positive cells was augmented following ISO treatment compared to PBS incubation in cardiomyocytes (Figures 1C and 1D). In addition, we examined another time point (96 h) after rAAV infection, and 10^{-5} M ISO similarly increased rAAV transduction in H9C2 (7.6±1.1 fold) cells compared to control. These results demonstrate that β-adrenergic receptor stimulation increased transduction by rAAV-CMV-lacZ or rAAV-MHC-lacZ.

β-receptor signaling regulates rAAV transduction *in vivo*

To determine whether ISO would increase rAAV transduction *in vivo,* we used human growth hormone (hGH) as a secreted, highly-quantitative marker of rAAV expression [3], and tested transduction in a variety of experiments. We injected rAAV-CMV-hGH vector (5×10^{10} particles) into the hearts of FVB mice, with or without a single intraperitoneal injection of ISO (2μg/g) followed by ELISA assay for serum hGH [3]. In the ISO-treatment group, circulating hGH levels were significantly higher than in the PBS control group at 2 and 4 weeks after injection (Figure 2A). To determine whether the β-adrenergic receptor is involved in regulating rAAV-mediated hGH expression, we implanted osmotic mini-pumps to achieve continuous ISO administration. Chronic ISO exposure in this model was previously reported to cause β-adrenergic receptor desensitization, including β-adrenergic receptor down-regulation [16]. Four days prior to rAAV-CMV-hGH injection into the myocardium, the mini-pump was inserted subcutaneously in the dorsum of mice. Four weeks of continuous ISO infusion (30μg/g /day), led to inhibition of hGH expression by rAAV, and the hGH level gradually increased after ISO exposure was discontinued (Figure 2B). Theses results indicate that β-adrenergic receptor activation can positively influence rAAV-mediated hGH expression in cardiac muscle *in vivo*.

Calcineurin (CN) mediates ISO-increased rAAV transduction and gene expression

Many signaling cascades are activated in response to β-adrenergic stimulation with ISO [9,17-20] including protein kinase A (PKA), calcium, protein kinase C (PKC), the mitogen-activated protein kinase family (ERK and p38MAPK), phosphatidylinositol 3-kinase (PI3-K) and tyrosine kinase, and specific antagonists of these pathways have been identified [18,21-26]. Pretreatment of H9C2 cells with a PKC inhibitor (1μM Calphostin C [21,23]); ERK1/2 inhibitor (50 μM PD98059 [25]); or p38MAPK inhibitor (10μM SB203580 [27]) did not affect rAAV transduction without (Figure 3A) or with ISO (Figure 3B). In contrast, pretreatment with an extracellular Ca^{2+} chelator (1 mM EGTA [21,23]) or a PI3-K inhibitor (0.1μM wortmannin [25]) mildly suppressed rAAV transduction in the cells without ISO (Figure 3A). Moreover, incubation with EGTA completely suppressed the increase in rAAV transduction mediated by ISO and wortmannin significantly inhibited the ISO effect (Figure 3B). Although a PKA inhibitor (100μM cell permeable PKI [26]) did not influence rAAV transduction rates, it significantly suppressed ISO-augmented rAAV transduction (Figures 3A and 3B). Qing et al have already reported that a tyrosine kinase inhibitor, genistein augments rAAV transduction in Hela cells [28]. In H9C2 cells, 20μM genistein [21] augmented the number of positive cells under basal conditions and further enhanced rAAV transduction in the presence of ISO (Figures 3A and 3B).

EGTA pretreatment [21,23] (0.01 to 1 mM) inhibited rAAV transduction by ISO in a concentration-dependent manner (Figure 3C). It has been reported that increased intracellular Ca^{2+} binds to Ca^{2+}-binding proteins including calmodulin [23] and activates effectors such as calcineurin [22]. To further examine downstream components of the Ca^{2+} cascade in ISO-stimulated rAAV transduction, we used an intracellular Ca^{2+} chelator (10μM BAPTA-AM [21,23]), a calmodulin inhibitor (W7 (0.1μM) [23]), and calcineurin inhibitors (500 ng/ml cyclosporin A (CyA) [22] or calcineurin autoinhibitory peptides (500 mM CNIP) [24]). Each of these inhibitors potently suppressed the ISO-induced increase in rAAV transduction (Figure 3C). In addition, incubation with calcium ionophore (1μM ionomycin) [29] markedly increased rAAV transduction (Figure 3C). These results

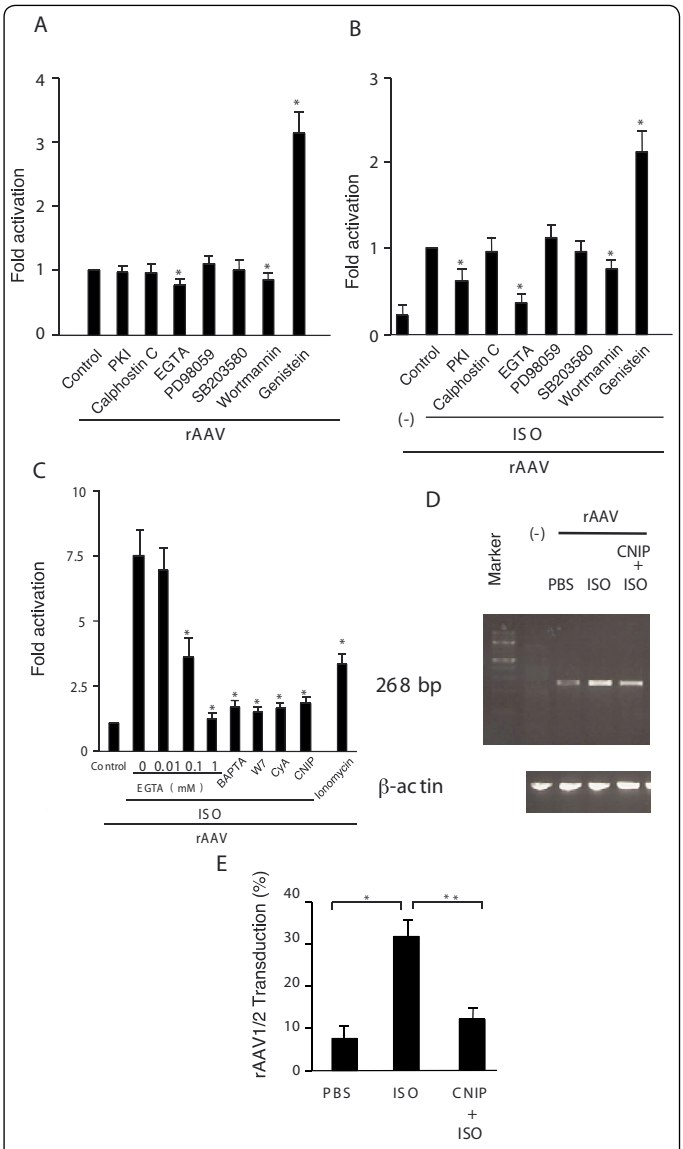

Figure 3: The calcium signalling cascade mediates the ISO-increased rAAV transduction. The fold activation of rAAV transduction is indicated following preincubation with: 100μM PKI, 1μM calphostin C, 1μM EGTA, 50μM PD98059 (PD), 10μM SB203580 (SB), 0.1μM wortmannin or 20μM genistein with (A) or without 10^{-5} M ISO (B) in H9C2 cells cells compared with control. Data are mean±SEM, *p<0.05 (n=4). (C) The fold activation of rAAV transduction is indicated following preincubation with the indicated concentration of EGTA, 10μM BAPTA-AM, 0.1μM W7, 500 ng/ml cyclosporin A (CyA), 500μM calcineurin autoinhibitory peptides (CNIP), 1μM ionomycin in H9C2 cells compared with ISO alone or control. Data are mean±SEM, *p<0.01 (n=4) vs. ISO alone, **p<0.01 (n=4) vs. PBS control. (D) Inhibition of calcineurin by CNIP suppresses rAAV transgene expression as shown by RT-PCR analysis of *lacZ* gene expression. β-actin control expression in the cells is shown. (E) Abrogation of ISO-increased transduction of pseudotype rAAV1/2 vectors following CNIP incubation in H9C2 cells. Data are mean±SEM, *p<0.05 (n=4) vs. PBS control, **p<0.05 (n=4) vs. ISO alone.

suggest that the Calcium/Calmodulin/Calcineurin cascade mediates ISO-stimulated rAAV transduction. To verify that calcineurin was important for ISO-induced rAAV gene expression, total RNA was isolated and RT-PCR was performed using lacZ-specific primers. As shown in Figure 3D, pretreatment with ISO significantly enhanced gene expression compared with PBS control, and preincubation with CNIP markedly blocked the ISO effect in the cells. These results indicate that calcineurin is involved in rAAV transduction and gene expression induced by ISO. Moreover, we examined whether calcineurin was involved in the transduction of a different serotype of rAAV. ISO pretreatment elevated transduction using pseudotyped rAAV1 in the H9C2 cells, pretreatment with ISO similarly increased the transduction and CNIP significantly suppressed this effect (Figure 3E).

Pretreatment with CNIP suppresses ISO-enhanced accumulation of double-stranded rAAV

As an rAAV vector is packaged with a single-strand (ss) DNA genome, for gene delivery to be successful, this genome must be converted to a double-strand form [30]. This process is not well understood, but thought to occur by either host cell synthesis of the second strand, or through re-annealing to a complementary genome carried by a vector of opposite polarity. To evaluate the role of ISO and the calcium pathway in rAAV ds DNA accumulation we performed Southern blot analysis and tracked conversion of ss DNA to ds DNA. Total DNA isolated from rAAV-infected H9C2 cells was digested with *Eco*RI, which flanks *lacZ* transgene (3.0 kb) in Figure 4A. Compared to rAAV infection only, following rAAV infection with ISO pretreatment, a significant amount of the viral genome was recovered, (Figure 4B). This ISO-induced enhancement of ds DNA was markedly reduced by CNIP pretreatment (Figure 4B). Next, we performed alkaline-gel Southern blot analysis and used a different probe to further clarify if ISO promotes ds DNA rAAV genome accumulation. Similar to what was described earlier, total DNA isolated from infected cultured muscle cells was digested with *Sac*I following rAAV infection alone or with ISO pretreatment (Figure 4A). An increase in ds DNA was significantly observed with *Sac*I digestion (Figure 4C). Following CNIP pretreatment we again recovered less ds DNA (Figure 4C), which may indicate decreased ss DNA to ds DNA conversion. These results support the concept that a calcium/calcineurin pathway influences rAAV transduction through the accumulation of ds DNA.

ISO increases rAAV expression through enhanced transcription

We further examined whether β-adrenergic receptor activation could modulate rAAV transduction in a chronic state. An osmotic pump delivering ISO was inserted into mice 20 weeks after intramyocardial injection of rAAV-hGH. Expression of hGH by rAAV was stable 20 weeks after infection (Figure 5A). β-adrenergic receptor down-regulation by chronic ISO exposure (30μg/g/day) significantly reduced the hGH expression in rAAV-infected mice. Additionally

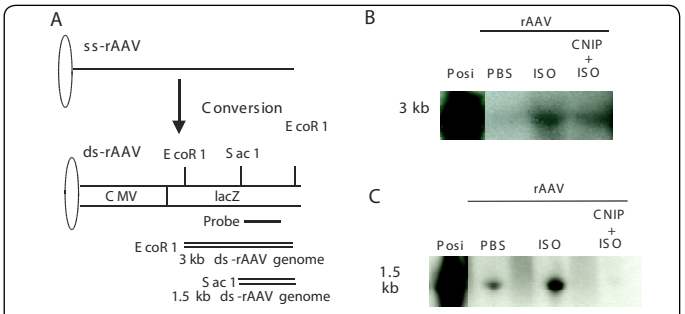

Figure 4: Inhibition of calcineurin suppressed rAAV ds DNA formation. (A) Map of rAAV vector identifying *Eco*RI and *Sac*I restriction enzyme digestion sites and the predicted sizes of ds DNA following digestion probed as shown. (B) Southern blot following *Eco*RI digestion (C) Alkaline-gel Southern blot following *Sac*I digestion. ISO increased the relative amount of ds DNA compared with PBS control, and pretreatment with CNIP blocked this increase in H9C2 cells. The lane labelled 'Posi' in (B) and (C) was pasted from another region of the blot.

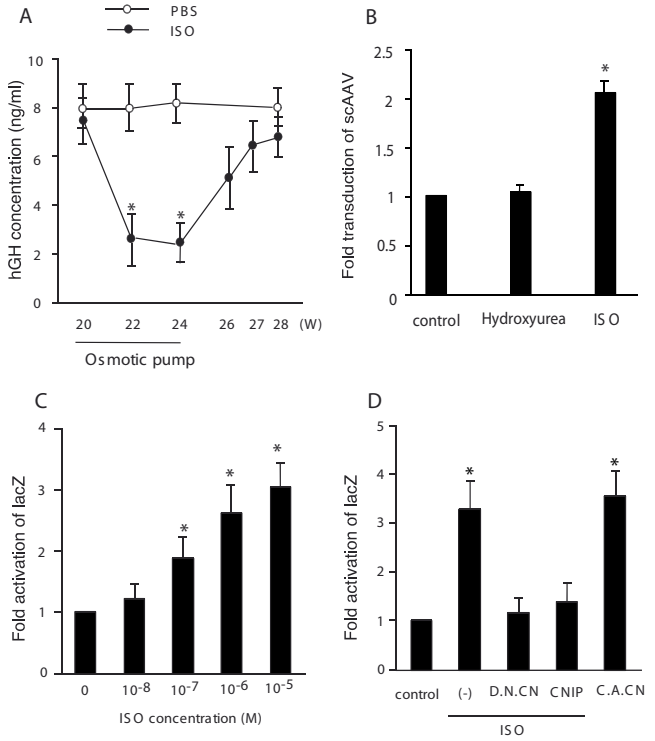

Figure 5: Inhibition of calcineurin suppressed ISO-induced AAV promoter activity. (A) Reduced hGH expression in response to chronic stimulation by ISO. An ISO-containing mini pump was inserted into mice expressing hGH following rAAV transduction. Data are mean±SEM, *p<0.05 (n=6) vs. PBS control. (B) ISO increased self-complementary (scAAV) transduction. H9C2 cells under pretreatment with PBS, hydroxyurea or ISO, and the number of GFP positive cells was counted. Data are mean±SEM, *P<0.05 (n=5) vs. PBS control. (C) AAV-CMV-lacZ vector plasmid was transfected into the cells, and indicated concentration of ISO was added. (D) Some samples were further cotransfected with the dominant negative calcineurin plasmid (D.N.CN) or constitutively active calcineurin (C.A.CN) plasmid, or pretreated with CNIP. Twenty-four hours later, cells were lysed and β-galactosidase activity was determined. Data are mean±SEM, *p<0.05 (n=5) vs. PBS control, **p<0.05 (n=5) vs. ISO alone.

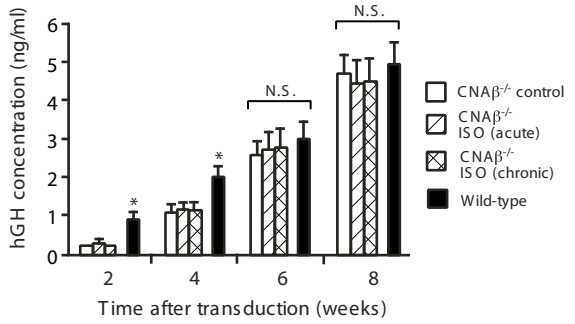

Figure 6: Loss of the ISO effect on rAAV expression in calcineurin Aβ-nullizygous mice. rAAV-CMV-hGH vector was injected into the heart muscle of calcineurin Aβ nullizygous mice with β-receptor stimulation (ISO acute) or β-receptor down regulation (ISO chronic), or wild type control mice. The mean±SEM serum hGH concentration is shown. *p<0.05 (n=6) vs. CNAβ$^{-/-}$ mice, N.S.: not significant.

hGH expression gradually recovered to the pre-stimulation level (Figure 5A) following withdrawal of the mini-pump. Since stable hGH expression by rAAV were suppressed by β-adrenergic receptor down-regulation, we examined whether the ISO-induced effects was regulating the activation of the rAAV CMV promoter. First, to discriminate an ISO effect on rAAV double-strand formation, we used a self-complementary (sc) AAV-GFP vector, which has a complementary double-strand DNA, which bypasses the need for double-strand conversion in traditional rAAV vectors [31]. Hydroxyurea (HU), has been reported to increase transduction of both rAAV and scAAV through a number of mechanisms that include enhanced nuclear transport [32], mobilization of vector to nucleoplasmic sites [33], and promotion of double-strand DNA formation of rAAV [31]. In our studies 50 mM HU did not affect scAAV transduction, however ISO significantly increased scAAV transduction in H9C2 cells (Figure 5B), indicating that ISO and HU likely operate through different mechanisms to improve transduction. To investigate whether ISO regulated rAAV promoter activity, we performed transient transfection assays in the H9C2 cells using the original vector plasmid to produce rAAV-CMV-lacZ. Subsequent incubation with ISO induced lacZ activity of the reporter plasmid in a concentration dependent manner at the range from 10^{-7} to 10^{-5} (Figure 5C). After co-transfection with a dominant negative mutant of calcineurin (D.N.CN) or control plasmid, we stimulated the cells with 10^{-5} ISO and measured lac Z activity. Overexpression of D.N.CN significantly suppressed ISO-induced lacZ activity. In contrast, a constitutively active mutant of calcineurin (C.A.CN) induced significant activation in the absence of ISO (Figure 5D). These results suggest that ISO regulates rAAV expression by controlling promoter activity through a calcineurin pathway.

Abrogation of the ISO effect in *CANβ*$^{-/-}$ mice

There are two subunits of calcineurin, Aα and Aβ in the heart of vertebrate species, and mRNA and protein levels of CNAβ, but not CNAα, are significantly increased by ISO stimulation [13]. The pronounced cardiac hypertrophy observed following ISO is suppressed in mice nullizygous for CN Aβ (CNAβ$^{-/-}$) [13]. We therefore investigated whether CNAβ$^{-/-}$ mice exhibited altered rAAV transduction following ISO stimulation. Nullizygous and their wild-type littermates were transduced using rAAV-CMV-hGH in conjunction with direct I.P. injection of ISO, osmotic minipump infusion of ISO, or PBS control. As expected, hGH levels gradually increased in wild-type mice between 2 and 8 weeks after rAAV infection. In CNAβ$^{-/-}$ mice, there was no significant difference in hGH levels between the three groups over 8 weeks (Figure 6). In addition, the rise in hGH levels in CNAβ$^{-/-}$ mice was delayed compared with wild type mice, but reached comparable levels by 6 weeks (Figure 6). Importantly, in contrast to wild-type mice, neither acute nor chronic stimulation with ISO altered hGH levels in CNAβ$^{-/-}$ mice. These results suggest that the absence of CNAβ produces a slow progression of rAAV transduction and abrogates the effects of ISO on rAAV transduction in heart muscle tissue.

Discussion

We have previously demonstrated that rAAV containing a cardiac-specific myosin heavy chain (MHC) gene promoter can transduce normal mouse hearts with expression for over 16 weeks [3]. In addition, rAAV containing a CMV promoter transduced injured rat hearts for over 20 weeks [34], supporting the idea that long-term transgene expression can be attained in the heart. We have continued to explore the biology of rAAV vectors in the heart muscle and here demonstrates that ISO can be exploited to modulate rAAV transduction. ISO adminstration was found to increase transgene expression in cells with a β-adrenergic receptor such as H9C2 cells and neonatal cardiomyocytes infected with either rAAV-CMV-lacZ or rAAV-MHC-lacZ (Figures 1A to 1D). Moreover, in vivo study, a short-term ISO stimulation promoted transduction by rAAV in heart muscle, and β-adrenergic receptor downregulation induced by chronic ISO

stimulation suppressed rAAV transduction (Figures 2A and 2B). These results suggest that β-adrenergic receptor signaling is important for rAAV transduction in heart muscle tissue.

In this study, rAAV vectors were directly injected into heart muscle tissues and ISO was administered after rAAV injection. We observed no significant differences in expression levels between ISO and control groups 6 or 8 weeks after transduction (Figures 2A and 2B) which indicates ISO affects the initial rate of transduction levels *in vivo,* and suggests that the same number of viral particles were present in the tissues of the two groups. Certain pharmacological treatments are reported to improve transduction by increasing vascular permeability and augment entry of rAAV particles into tissues. Such is the case with pretreatment with histamine [35] or VEGF [36], which increases rAAV transduction following systemic administration of rAAV *in vivo*. Since ISO does not increase vascular permeability [37], other mechanisms likely mediate the effects ISO administration has on rAAV transduction.

As a β-adrenergic agonist, ISO is well recognized to induce Ca^{2+} influx by increasing L-type Ca^{2+} channel opening through PKA, and to activate the Ca^{2+} cascade including calmodulin and calcineurin in H9C2 cells [10,11,29]. Pretreatment with chelators of Ca^{2+}, a calmodulin inhibitor, and calcineurin inhibitors significantly suppressed the increase in rAAV transduction and gene expression following ISO stimulation (Figures 3B, 3C, and 3D), indicating that the calcium signaling cascade plays a vital role in mediating the effects of ISO. In addition, pretreatment with PKI inhibited rAAV transduction augmented by ISO (Figure 3B). DiPasquale et al., have demonstrated that Rep proteins Rep78 and Rep52, which are essential for the AAV2 life cycle, directly interact and inhibit PKA in infected cells and regulate replication fitness in wild type AAV2 [38]. As recombinant AAV vectors do not possess any Rep proteins and cannot replicate themselves, it is plausible that a PKA-mediated signaling cascade is capable of being activated in cells after infection of rAAV vectors. Accordingly, ISO may induce the opening of L-type Ca^{2+} channels through PKA signaling. Consistent with a stimulating effect in muscle cell lines in this study, Zhang et al. reported a strong contribution by the calcium pathway on rAAV transduction in T-cells [39]. In contrast, EGTA mildly increased rAAV transduction in polarized airway epithelial cells [40]. Therefore, it is important to note that calcium-mediated effects on rAAV transduction appear to be cell-type specific.

In studying the effects of ISO in combination with calcium chelators and other inhibitors in muscle cells, we observed a significant increase in rAAV transduction following exposure to genistein (Figure 3A). Genistein, a tyrosine kinase inhibitor, has been shown to increase the efficiency of stable transduction by rAAV in Hela cells [28]. Moreover, we demonstrated additional stimulation with ISO further enhanced the effect of genistein (Figure 3B). These results suggest that these two compounds may mediate distinct pathways with synergistic action. Genistein is reported to inhibit the tyrosine phosphorylation status of a chaperone protein able to bind the D region of rAAV *cis*-acting sequences (145-nucleotide terminal repeats) [28]. The phosphorylated form of the protein is thought to inhibit ds DNA formation of rAAV in some cell types, and thus genistein relieves this inhibition. Accordingly, it is possible that ISO may cooperate with genistein via another mechanism to enhance rAAV transduction.

Since conversion of ss rAAV genomes to ds DNA is a critical step in transduction that can sometimes take several days to occur [30], we examined whether ISO modulation of the β-receptor/ calcineurin cascade affected ds DNA accumulation. We observed that addition of ISO increased rAAV ds DNA accumulation and demonstrated that this increase could be significantly attenuated by pretreatment with a calcineurin specific inhibitor (Figures 4A, 4B and 4C), indicating that ISO augments ds DNA accumulation through calcineurin activation. However, it still remains how calcineurin promotes accumulation of ds rAAV genome. In addition to effects on ds DNA accumulation, it is clear that ISO stimulation leads to enhanced transgene expression by increasing promoter activity. Transduction of rAAV in heart muscle was not only inhibited by β-adrenergic receptor down-regulation, but stable transgene expression was also reduced (Figure 5A). In addition, scAAV transduction was augmented by ISO stimulation (Figure 5B). These results suggest that decreased rAAV promoter activity may result from desensitization of β-adrenergic receptors. Transient transfection assays revealed that ISO significantly increased CMV promoter activity and that ISO-induced promoter activity was strongly regulated by calcineurin (Figures 5C and 5D). In the stable phase after rAAV transduction, the β-receptor/ calcineurin signaling cascade may also play an important role in rAAV transduction through a transcriptional mechanism.

In terms of intracellular trafficking, phosphatidylinositol 3-kinase (PI3-K) is necessary to initiate the intracellular movement of rAAV vector to the nucleus via both microfilaments and microtubules with activation of the GTPase Rac1 [41]. Since ISO has been shown to activate Rac1 [42], it is conceivable that ISO may induce a PI3-K signaling cascade through Rac activation and promote rAAV transduction by regulating nuclear trafficking of vector in cardiac cells.

In addition to effects on vector trafficking, ISO may promote transduction through initiating a DNA damage response. Recent studies indicate that double-strand conversion of rAAV genome can be detected with accumulation of DNA damage checkpoint protein 1 (MDC1) [43]. Genotoxic stressors such as hydroxyurea have been shown to increase rAAV efficiency through mobilizing vector to regions of the nucleus that promote transduction [33]. Since ISO is known to induce cardiomyocyte apoptosis through calcium/ calcineurin [29], a subsequent DNA damage response may serve to increase rAAV transduction by sequestering inhibitory proteins away from the vector or transgene.

To elucidate the involvement of calcineurin (CN) in rAAV transduction, we examined $CNA\beta^{-/-}$ mice after rAAV infection. ISO stimulation (acute or chronic) did not alter rAAV transduction in $CNA\beta^{-/-}$ mice, and interestingly the transduction level of all $CNA\beta^{-/-}$ mice gradually reached a similar level as in wild type controls (Figure 6). Taken together, the results suggest that calcineurin may play a central role in the enhanced rAAV transduction induced by ISO specifically by facilitating rapid transduction, but not by increasing transduction overall. It is possible that transduction of rAAV vectors are regulated slowly and compensated by other molecules in the absence of calcineurin.

In concert with other studies on rAAV, our results point to the β-adrenergic receptor pathway as a major determinant of transduction. As previously reported [44], when animals are subjected to general hypothermia by external cooling, rAAV transduction is augmented in rodent models. It has already been demonstrated that cooling produces increased epinephrine levels in serum [45]. Possibly cold stimulation may promote rAAV ds DNA formation and transduction through elevation of β-receptor agonists including norepinephrine and epinephrine. In conclusion, these findings have implications

both for our understanding of the fundamental mechanisms that govern rAAV transduction and for the practical use of rAAV vectors for skeletal muscle and cardiac muscle gene therapy.

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