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Original article

2-Deoxy adenosine triphosphate improves contraction in human end-stage heart failure

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ABSTRACT

We are developing a novel treatment for heart failure by increasing myocardial 2 deoxy-ATP (dATP). Our studies in rodent models have shown that substitution of dATP for adenosine triphosphate (ATP) as the energy substrate in vitro or elevation of dATP in vivo increases myocardial contraction and that small increases in the native dATP pool of heart muscle are sufficient to improve cardiac function. Here we report, for the first time, the effect of dATP on human adult cardiac muscle contraction. We measured the contractile properties of chemically-demembranated multicellular ventricular wall preparations and isolated myofibrils from human subjects with end-stage heart failure. Isometric force was increased at both saturating and physiologic Ca^{2+} concentrations with dATP compared to ATP. This resulted in an increase in the Ca^{2+} sensitivity of force (pCa_{50}) by 0.06 pCa units. The rate of force redevelopment (k_{tr}) in demembranated wall muscle was also increased, as was the rate of contractile activation (k_{ACT}) in isolated myofibrils, indicating increased cross-bridge binding and cycling compared with ATP in failing human myocardium. These data suggest that dATP could increase dP/dT and end systolic pressure in failing human myocardium. Importantly, even though the magnitude and rate of force development were increased, there was no increase in the time to 50% and 90% myofibril relaxation. These data, along with our previous studies in rodent models, show the promise of elevating myocardial dATP to enhance contraction and restore cardiac pump function. These data also support further pre-clinical evaluation of this new approach for treating heart failure.

1. Introduction

Heart failure is a growing epidemic in developed countries, with the incidence and prevalence rising each year [1]. Despite advancements in treatment, the five year mortality approaches 50% [2]. At least half of the patients suffering from heart failure have low systolic function [3]. However, currently available inotropic agents which increase contractility via altering intracellular Ca^{2+} do not improve survival in patients with heart failure [4,5]. Some of the reasons for failure of inotropic agents include tachyarrhythmias, increased myocardial oxygen consumption, decreased coronary perfusion and alteration of intracellular Ca^{2+} [5]. Hence, there is an urgent need for development of novel agents that improve contractility and systolic function. One way to avoid unwanted side effects may be to directly target myofilaments to alter contractility at the level of the sarcomere and motor proteins.

In previous animal studies we found that the use of 2-deoxy adenosine triphosphate (dATP) instead of adenosine triphosphate (ATP) as the energy source improves contractility in striated muscle by enhancing crossbridge binding and cycling kinetics and improving allosteric activation [6–9]. In fact, increasing the dATP level from the typical <0.1% of the adenosine nucleotide pool to 1% is enough to significantly increase contraction [10]. We have developed a novel approach to elevate dATP in vivo by increasing the expression of the enzyme ribonucleotide reductase (R1R2), the rate-limiting step in de novo dNTP biosynthesis. This results in increased levels of 2-deoxy ATP (dATP). We have shown that increasing dATP in intact cardiomyocytes via adenovirus mediated transfection increased contractile magnitude and kinetics [10]. In addition, transgenic mice that overexpress R1R2 have increased left ventricular systolic function compared to control animals [11]. Based on these results, overexpression of R1R2 and increased cardiomyocytes dATP constitutes an exciting and novel therapy with potential to treat heart failure. However, before clinical studies in humans are convened, a critical step is to test the efficacy of elevated dATP levels on human

Abbreviations: ATP, adenosine triphosphate; dATP, 2-deoxy adenosine triphosphate; HMM, heavy meromyosin; LVAD, left ventricular assist device; R1R2, ribonucleotide reductase; RT50, time to 50% relaxation; RT90, time to 90% relaxation; SL, sarcomere length

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cardiac myocardium to ensure that the effect of dATP is consistent across species.

Here we report for the first time that dATP improves contraction in myocardial samples isolated from human subjects with end-stage heart failure. By measuring isometric force of demembranated multicellular samples we show that dATP enhances force development at both maximal and submaximal Ca^{2+} concentrations and increases Ca^{2+} sensitivity of force. We also show that for isolated myofibrils there is an increase in activated force and rate of activation without alteration of relaxation. This study represents an important next logical step in the progression toward using dATP therapy in a clinical setting. We conclude that elevation of myocardial dATP has merit as an approach worth further investigation for the treatment of heart failure and in particular patients with low systolic function.

2. Methods

2.1. Human left ventricular tissue collection

Adult heart tissue was obtained following written informed consent from subjects who were undergoing cardiac placement of left ventricular-assist device or cardiac transplantation for end stage heart failure under a study protocol approved by the University of Washington Institutional Review Board. For samples from transplanted patients, a piece of the left ventricular free wall was obtained. Samples were transported to the laboratory in cold phosphate buffered saline solution and immediately used with preparations as detailed in sections below. The time from harvest in the operating room to arrival to the laboratory was less than 1 h.

2.2. NTPase and in vitro motility assays

Myosin was purified on the day of acquisition from human left ventricular samples as previously described [12]. Purified myosin was stored at 4 °C and used for up to three days. ATPase and dATPase (NTPase) activities of human cardiac myosin were measured in the presence of actin at 23 °C using a colorimetric method to detect the nucleotide hydrolysis rate as previously described [7]. Myosin, actin and NTP (ATP or dATP) concentrations were 0.2 μM, 10 μM and 1 mM respectively. Heavy meromyosin (HMM) was prepared by digestion of myosin with 0.05 mg/ml chymotrypsin as previously described [7,12,13] and stored for up to three days at 4 °C. In vitro *motility* assays were performed at 30 °C using unregulated Rhodamine Phalloidin-labeled F-actin in the presence of 2 mM ATP or dATP as previously described [7,12,13]. Images of filaments were recorded and digitally analyzed using custom-built software as previously described. Previously described filtering methods were used to define non-erratically moving filaments based on the ratio of standard deviation to the mean of the velocity of the filament (0.75 cutoff). Weighted mean and standard deviation for each condition were calculated using the deviation of each filament's speed and the number of filaments on each slide and in each condition, as described in our previous studies [13]. Comparison between the two groups was done on the weighted average and standard deviation using a two-sample t-test with equal variance.

2.3. Multicellular left ventricular force measurements

Left ventricular wall tissue was demembranated in relaxing solution (in mM: 100 KCl, 10 imidazole, 2 EGTA, 5 $MgCl_2$, and 4 ATP) containing 50% glycerol (vol:vol) and 1% Triton X-100 overnight at 4 °C then stored in glycerinated relaxing solution at −20 °C and used within one week. Demembranating and storage solutions contained protease inhibitor cocktail (P8340; Sigma-Aldrich). Thin left ventricular strips (189 ± 9 μm wide and 0.79 ± 0.05 mm long) were dissected out from the trabeculated layer with fibers going in a single direction. Ends of the strips were wrapped in aluminum T-clips and mounted between a motor (Aurora Scientific, Model 312B) and force transducer (Aurora Scientific, Model 403A). Sarcomere length (SL) was set to 2.3 μm either by direct measuring using fast Fourier transform analysis of the preparation image obtained via MyoCam (IonOptix Corp.) or by stretching the sample to 15% over non-strained length. Steady-state force was measured using a custom built mechanical apparatus at 15 °C during Ca^{2+} activation at various concentrations in the presence of 5 mM ATP or dATP [14] with half of the preparations measured first in ATP and half in dATP. Experimental solutions were maintained pH 7.0 at 15 °C and contained (in mM): 15 phosphocreatine, 15 EGTA, 80 MOPS, 1 free Mg^{2+}, 1 DTT, and 5 Mg_2ATP or 5 Mg_2dATP. Ca^{2+} concentration (reported as $pCa = -\log[Ca^{2+}]$) was adjusted by varying amounts of $CaCl_2$. Ionic strength was set to 0.17 M with KCl [14]. Relaxing and activating solutions were prepared using a custom software package as described previously [15]. Passive force (pCa 9.0) was subtracted from force at other pCa concentration to calculate Ca activated force. Force–pCa curves were fitted using the Hill equation to calculate pCa at half maximum force (pCa_{50}) and slope (n_H).

$$F = F_{max}/\left(1 + 10^{n_H(pCa_{50}-pCa)}\right) \quad (1)$$

Reported pCa_{50} and n_H values for force–pCa relationships are the average of individual fits for each experimental curve ± S.E.M.

The rate of isometric tension redevelopment (k_{tr}) was calculated in each activation solution following a rapid release–restretch protocol [16] and fitting the resulting tension trace with mono-exponential equation [8].

$$F = F_0\left(1-e^{-k_{tr}t}\right) \quad (2)$$

For additional demonstration of the ability of dATP to increase contraction, force was measured at submaximal (pCa = 5.6) and maximal (pCa = 4.5) Ca^{2+} activation first in ATP, then dATP and back to ATP. In order to eliminate the effect of the order of activation, for one half of the samples the first ATP activation force was used and for the other half the force of the second ATP activation was used.

2.4. Myofibril mechanical measurements

Small myofibril bundles were prepared from demembranated left ventricular wall tissue as previously described [13]. Briefly, bundles were rinsed twice in Rigor solution containing 2 mM DTT and 1:200 dilution of protease inhibitor (Sigma-Aldrich, St. Louis, MO) before being homogenized for 1 or 2 pulses of 30 s at high speed, stored at 4 °C, and used for up to three days. Experiments were performed on a custom set up as previously described [13,17]. In brief, myofibrils were mounted between two needles; one acted as a cantilever force transducer and the other as an inflexible mount attached to a piezo-electric computer controlled motor. A duel diode system was used to measure needle displacement and developed force was measured based on this displacement and the known stiffness of the needle. Needle stiffness was 2–7 nN/μm for this study. Relaxing (pCa = 9.0) and activating (pCa = 5.6) solutions were delivered to the mounted myofibril using a double-barreled glass pipette. Activation and relaxation data were collected at 15 °C and fitted with either single-exponential curves, linear coefficients, or 50% times as previously described [13,17,18].

2.5. Statistics

Comparison between groups of data was performed using paired or unpaired Student's t test as appropriate. All the data passed the normality test (Shapiro–Wilk method). Data are expressed as mean ± standard error of the mean and "n" represents the number of experimental samples in each group, and "N" represents the number of patients in each group. Statistical significance was accepted as $p < 0.05$ and when