Ecto-5'-nucleotidase deficiency exacerbates pressure-overload induced left ventricular hypertrophy and dysfunction

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Running Title: CD73$^{-/-}$ impairs the response to pressure overload

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Supplementary data

Detailed Materials and Methods:

Mice: Heterozygous mice were first interbred to produce homozygous ($cd73^{-/-}$, n=10) and wild type (WT) littermate (n=6) for 2 weeks TAC procedure, and results showed that TAC caused significantly more ventricular hypertrophy and dysfunction in $cd73^{-/-}$ as compared with WT littermates ($p<0.05$). We subsequently used $cd73^{-/-}$ mice and their WT littermates as breeders to generate offspring for TAC procedure $cd73^{-/-}$, n=13; WT mice, n=9). Data for heart weight and lung weight was pooled from initial experiments using $cd73^{-/-}$ and wild type littermates, as well as the offspring of $cd73^{-/-}$ and WT mice. The data of aortic pressure, ventricular pressure and dP/dt was collected from offspring of $cd73^{-/-}$ and WT mice. All of the data is expressed as Mean ± standard error.

Minimally invasive TAC procedure. Mice were anesthetized with a mixture of 80 mg/kg ketamine and 30 mg/kg xylazine intraperitoneally. A horizontal incision was made at the level of the suprasternal notch to allow direct visualization of the transverse aorta without entering the pleural space. Aortic constriction was performed by ligating the aorta between the right innominate artery and the left carotid artery over a 26 G needle using 5-0 silk suture. The needle was immediately removed after ligation, leaving the aortic constriction in place. The incision was closed and the animals were allowed to recover. Analgesia was provided before the mice awoke from anesthesia.

Evaluation of aortic pressure and LV hemodynamics. Mice were anesthetized with *1.5% isoflurane*. A 1.2 Fr. pressure catheter (Scisense Inc. Ontario Canada) was introduced into the right common carotid artery for aortic pressure measurement and then advanced into LV for measurement of LV systolic and end-diastolic pressures, and positive and negative LV dP/dt_{max}. Data represent the mean of at least 10 beats of recording during stable hemodynamic conditions.

5'-AMP induced decrease of heart rate. It is known that extracellular adenosine stimulates adenosine A1 receptors to cause dose dependent bradycardia[1]. Since CD73 uses 5'-AMP as a substrate to generate adenosine, to determine whether CD73 KO disrupts extracellular adenosine production *in vivo*, we determined the bradycardiac response to 5'-AMP in a group of CD73 KO and wild type mice as previously described[2]. Briefly, mice were anesthetized with 1.5% isoflurane and the left jugular vein cannulated for bolus injections of 5 to 30 µg of 5'-AMP while the heart rate was observed. The effect of 5'-AMP on heart rate was also assessed in a group of A1AR KO mice [1]

Histological staining and measurement of myocardial fibrosis. Tissue sections (6µm) from the central portion of the LV were stained with Sirius Red (Sigma) for detection of fibrosis, and FITC-conjugated wheat germ agglutinin (AF488, Invitrogen) to evaluate myocyte size. For mean myocyte size, the cross sectional area of at least 120 cells/sample (from 4 areas) and at least 4 samples of each group were averaged. The percent volume fibrosis was determined using the method described previously [3].

Western blots. Protein content was analyzed using Western blots as previously described [3]. Western blots were performed using primary antibodies against ANP(Penninsula Laboritories;San Carlos, CA), type I collagen and β-MHC(Sigma; St Louis MO), TNFα, total-Akt, p-Akt^{Ser473}, p-S6$^{Ser235/236}$, PTEN, p-PTEN^{ser380}, total p70S6K, p-p70S6K^{Thr389}, mTOR and p-mTOR (Cell Signaling Technology; Beverly, MA), followed by visualization using HRP-labeled secondary antibodies (Bio-Rad; Hercules, CA)

CD73 activity assay: Mouse heart tissue was pulverized under liquid nitrogen and homogenized using 30 strokes of a dounce homogenizer in 10 mmol/L HEPES-potassium hydroxide (HEPES-KOH) buffer (pH 7.4) containing 0.25 mol/L sucrose, 1 mmol/L $MgCl_2$, and 1 mmol/L mercaptoethanol. Large unbroken tissue fragments were removed by centrifugation at 500g for 5 minutes. The supernatant was centrifuged at 200,000g for 1 hour to collect the

membrane fraction. Cell membranes were dialyzed at 4°C for 4 hours against 10 mmol/L HEPES-KOH (pH 7.4) containing 1 mmol/L $MgCl_2$, 1 mmol/L mercaptoethanol, and 0.01% activated charcoal. Dialyzed samples were divided into aliquots and stored at -80°C. Ectonucleotidase activity was analyzed in a 96 well plate enzymatic assay in which cd73 induced phosphate release from AMP was measured using a colormetric assay. 10µl of 2mg/ml isolated membrane fraction from each sample was added to 40 µl of assay buffer (HEPES-KOH (pH 7.4) 2 mmol/L $MgCl_2$) containing 1mM AMP (Sigma). After 15 min incubation at 37°C, the reaction was terminated by addition of 100 µl Biomol Green Reagent (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), and phosphate release was measured 30 minutes later at OD_{620nm} and compared to a known set of phosphate standards. Duplicate wells containing samples in the absence of AMP were measured for background phosphate levels and subtracted from each sample. Activity is expressed as nmoles/mg protein/minute.

Neonatal cardiomyocyte isolation, culture, and sample collection: NRVM were isolated from 2-day-old Sprague-Dawley rats by enzymatic digestion[4] and separated from non-muscle cells on a discontinuous Percoll gradient according a modified protocol from Dr. U. Mende (Brigham and Women's Hospital, Cardiovascular Division) [4]. A total of 2-4 million viable myocytes were isolated per ventricle with very little fibroblast contamination(<2%) and plated in serum-containing DMEM (4×10^5 cells/well of a 6-well dish). Myocytes were incubated for 48-72 hrs to allow attachment and spreading, after which the medium was replaced with serum free media for 24 hours prior to treatment. Before harvesting, cells were washed once with PBS, then collected in10mM Tris HCl pH 7.4, 100mM NaCl,1mM EDTA, 1mM EGTA, 1% triton X-100, 1mM NaF, 1mM PMSF, 1 mM vanadate, and 1x protease inhibitor mix(Boehringer-Mannheim). Protein concentration was measured using BioRad Protein Assay(Biorad; Hercules, Ca) and equal amounts of soluble protein were separated on 10% polyacrylamide gels for western blot analysis. Scanning densitometry and Image J software was used to quantify band intensity.

Graphs depict phosphorylated proteins relative to GAPDH on the same blot. Multiple blots were compared by designating 24 hours of PE treatment as 100% in each experiment, and presenting each other time point value as relative to 100%. 3-5 individual experiments are represented by each bar.

***Cell area determination and cellular* ANP expression**. To accurately measure cell area, cells were plated at a lower density (0.5 x10^5 cells/cm^2). After treatment, cells were fixed with 4% paraformaldehyde/PBS pH 7.4, permeabilized with 0.1% triton x-100 for 5 minutes, and stained using Rhodamine conjugated Phalloidin (5 units/ml in PBS pH 7.4)(Invitrogen) and DAPI (300nM) (Invitrogen). Cell area was analyzed using Image J 1.34s (NIH, USA). At least 100 individual cells were measured per experiment. Graphs represent averages of 3-6 individual experiments. Cells fixed as described above were also used for immunocytochemical analysis of ANP expression, using primary rabbit anti-ANP**,** followed by Alexa-fluor 488 anti-rabbit (Molecular Probes)

Neonatal fibroblast isolation, culture, and [3H]-leucine and proline incorporation

Myocytes were separated from cardiac fibroblasts by low speed centrifugation(500 rpm).The supernate was centrifuged at 2200 rpm to collect fibroblasts. Fibroblasts were initially plated on 75 cm^2 flasks in 10% FCS in DMEM, passaged twice, and replated in 24 well plates at a density of 1x10^4/cm^2 in 0.2% FCS in DMEM. After 48 hrs, fresh serum free media was added containing 1µCi/ml [H^3]-Proline or 1µCi/ml [H^3]-Leucine(Amersham;Piscataway, NJ), to measure collagen production and total protein synthesis, respectively[5]. Cells were treated in triplicate with the indicated concentrations of adenosine for 15 minutes prior to addition of 2.5% FCS. Tritium incorporation was measured at 48 hrs by washing cells twice in PBS, precipitation in 10% TCA for ten minutes at 23°C, followed by solubilization in 0.3N NaOH, 0.1%SDS at 37ºC for 2hrs. Radioactivity was measured in a liquid scintillation cocktail (EcoScintA; National Diagnostics, Atlanta. GA)**. Cardiac fibroblast DNA synthesis** was measured in parallel to protein synthesis

in cells plated at $1x10^4/cm^2$ in triplicate in 96 well plates. At the indicated time, DNA was measured using the Cyquant assay according to the manufacturer's instructions (Invitrogen; Carlsbad, CA).

Data and statistical analyses. All values were expressed as mean ± standard error of the mean (SEM). Statistical significance was defined as $P < 0.05$. Two-way analysis of variance (ANOVA) was used to test each variable for differences among the treatment groups with StatView (SAS Institute Inc). If the ANOVA demonstrated a significant effect, post hoc pairwise comparisons were made with Fisher's least significant difference test.

References

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Figure legends:

Figure S1. 2-chloroadenosine(2-CADO, 5μM) attenuated phenylephrine (PE) induced cardiomyocyte hypertrophy (A,B) and atrial-naturietic-peptide (ANP) expression (A,C). For cell size and ANP measurements, neonatal cardiomyocytes were treated with PE for 52 hours in the presence or absence of 5μM 2-chloroadenosine (2-CADO), fixed, immunostained for ANP, and further stained for cell size using Wheat Germ Aggluttinin (WGA). Each bar represents average from 3-7 individual experiments. *P<.05 relative to untreated; # P<.05 compared to PE treated.

Figure S2. Adenosine reduces protein synthesis, collagen synthesis and DNA synthesis in cardiac fibroblasts (D). Cardiac fibroblasts were plated under confluent conditions to prevent proliferation, treated with 2.5% FCS for 48 h in the presence of the indicated concentrations of adenosine. [3H]-Leucine or [3H]-Proline incorporation were measured to indicate total protein synthesis or collagen synthesis, respectively. (E) Sub-confluent cardiac fibroblast cultures were starved for 24 hours, then stimulated with 2.5% FCS in the presence or absence of 10μM adenosine. DNA levels were quantitated at the indicated time points using a fluorescence based assay). Each bar is average of 3-6 experiments *P<.05 relative to untreated; # P<.05 compared to FCS treated.

Figure S1

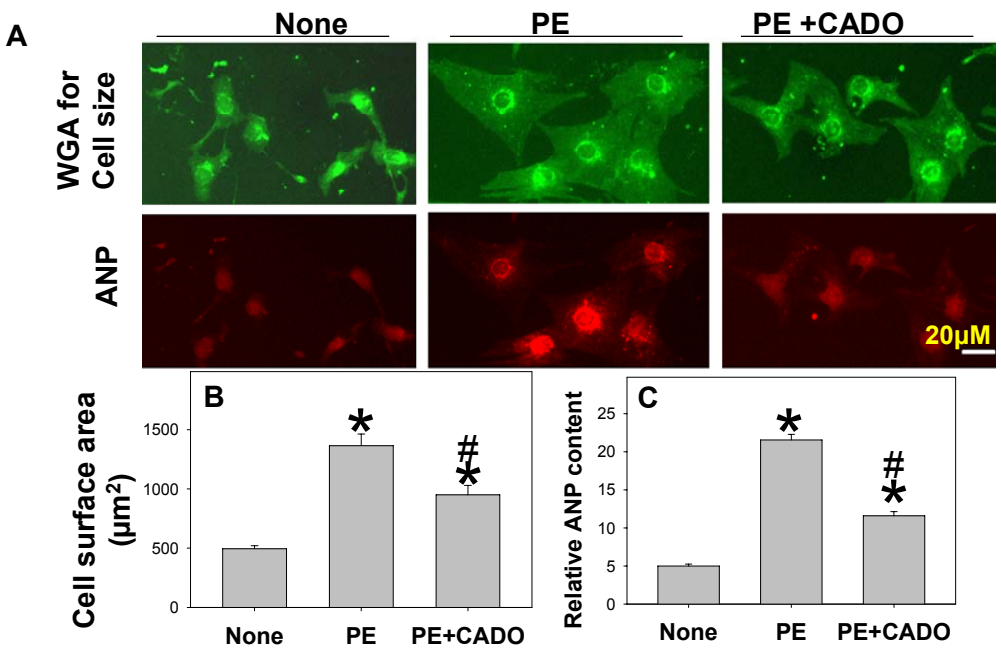
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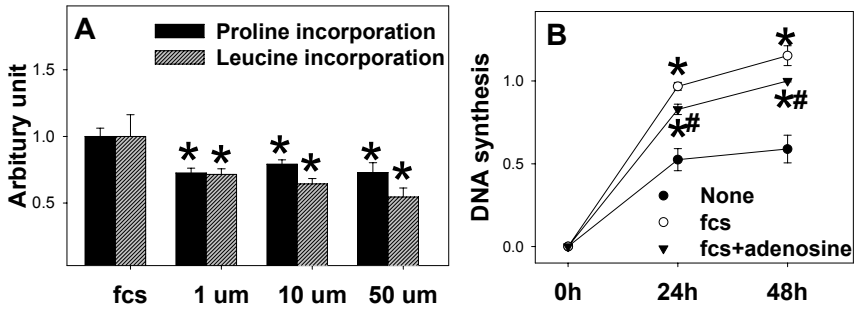
Figure S2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DNA synthesis
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0.5
0.0
None
fcs
fcs+adenosine
0h
24h
48h