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

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Abstract

This study examined whether endogenous extracellular adenosine acts to facilitate the adaptive response of the heart to chronic systolic overload. To examine whether endogenous extracellular adenosine can protect the heart against pressure overload induced heart failure, transverse aortic constriction (TAC) was performed on mice deficient in extracellular adenosine production as the result of genetic deletion of CD73. While there was no difference in left ventricular (LV) size or function between CD73 deficient mice (KO mice) and wild type (WT) mice under unstressed conditions, aortic constriction for 2 or 4 weeks induced significantly more myocardial hypertrophy, LV dilation and LV dysfunction in KO mice compared to WT mice. Thus, after 2 weeks of TAC, LV fractional shortening decreased to 27.4±2.5% and 21.9±1.7% in WT and KO mice respectively ($p<0.05$). Consistent with a role of adenosine in reducing tissue remodeling, KO mice displayed increased myocardial fibrosis and myocyte hypertrophy compared to WT mice. Furthermore, adenosine treatment reduced phenylephrine induced cardiac myocyte hypertrophy and collagen production in cultured neonatal rat cardiac myocytes and cardiac fibroblasts, respectively. Consistent with a role for adenosine in modulating cardiomyocyte hypertrophy, KO mice demonstrated increased activation of mTOR signaling, accompanied by higher expression of the hypertrophy marker atrial natriuretic peptide (ANP). Conversely, the adenosine analogue 2-chloro-adenosine significantly reduced cell size, mTOR/p70S6K activation, and ANP expression in cultured neonatal cardiomyocytes. These data demonstrate that CD73 helps to preserve cardiac function during chronic systolic overload by preventing maladaptive tissue remodeling.

Keywords

hypertrophy; heart failure; fibrosis; 5'-nucleotidase; adenosine

Introduction

Adenosine is a nucleoside that is released in response to stresses that increase ATP catabolism. In the heart, adenosine acts on multiple cell types through interaction with four different

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adenosine receptor subtypes[1-3]. In addition to its well known role in ischemic pre-conditioning[3, 4], there is recent evidence that the adenosine analogue 2-chloroadenosine (CADO) or treatment that increases endogenous adenosine levels (e.g., inhibition of adenosine uptake with dipyridamole) can attenuate infarct-induced LV remodeling[5]. Adenosine may protect against heart failure induced by pressure overload, as administration of CADO attenuated hypertrophy, fibrosis and heart failure in mice exposed to transverse aortic constriction (TAC)[6]. Increasing interstitial adenosine levels by blocking adenosine uptake using dipyridamole was also reported to reduce hypertrophy in rats exposed to pressure overload.[7] The anti-fibrotic effects of adenosine appear to involve activation of A2b receptors[8, 9], while a role for cardiac adenosine A1 receptors has been suggested in the adenosine mediated reduction of cardiomyocyte hypertrophy[6]. Interestingly, endogenous adenosine levels rise during compensatory hypertrophy, but are diminished as hearts become decompensated [10, 11]. It has thus been suggested that modulation of adenosine levels may be a target for treatment of ventricular dysfunction in failing hearts[11]. However, whether reduction of endogenous extracellular adenosine levels can contribute to the development of heart failure in the overloaded heart is not known.

The membrane-anchored cell surface enzyme CD73 catalyzes the conversion of extracellular AMP to adenosine, thereby increasing extracellular adenosine production [12]. Darvish et. al. reported that CD73 activity accounts for approximately 46% of total adenosine production in rat heart homogenates [13], while other studies also demonstrated that CD73 mediated adenosine production is critical to ischemic pre-conditioning[14,15], implying that CD73 contributes significantly to extracellular myocardial adenosine production under stress. The actual contribution of CD73 and the role of adenosine production in the development and progression of heart failure under chronic systolic overload are not known. Here we used CD73-KO mice to investigate the role of CD73 in protection against heart failure during chronic pressure overload. Our data demonstrate that CD73-KO exacerbated systolic overload induced ventricular hypertrophy, fibrosis and dysfunction. Furthermore, collagen production, cardiomyocyte hypertrophy, and mTOR activation were directly inhibited by adenosine or CADO in isolated cultures of cardiac fibroblasts or cardiomyocytes, respectively. These results indicate that CD73 activity and endogenous extracellular adenosine play a significant role in protection against systolic overload induced ventricular hypertrophy, fibrosis and congestive heart failure.

Materials and methods

Mice and TAC procedure

CD73-KO mice (129 background) and control wild type mice were generated as previously described [16] . This study was approved by the Institutional Animal Care and Use Committee of University of Minnesota. TAC was performed using the minimally invasive suprasternal approach described[16].

Echocardiography and Western blots were performed with methods as previously described[17, 18]. For details, please see the online data supplement available at http://hyper.ahajournals.org.

Neonatal rat cardiomyocyte (NVM) isolation and culture

NVM were isolated from 2-day-old Sprague-Dawley rats by enzymatic digestion[19] and separated from non-muscle cells on a discontinuous Percoll gradient as previously described[19]. Detailed method is included in the online supplementary data section (http://hyper.ahajournals.org).

Results

CD73 Knockout exacerbates TAC-induced myocardial hypertrophy, fibrosis and dysfunction in the overloaded heart

Under basal conditions, we observed no statistical differences in LV structure or function (**Figures** 1-3, Table 1) between CD73 KO mice and their wild type control littermates. To examine the role of extracellular adenosine in modulating the response to systolic overload, we exposed KO mice and WT mice to TAC. In response to TAC for 2 weeks, KO mice developed significantly greater increases of ventricular weight and the ratio of ventricular weight to body weight or tibia length than WT mice (Table 1, Figure 1A, 1B), indicating that KO exacerbated the TAC-induced myocardial hypertrophy. In addition, the ratio of lung weight to body weight or tibia length was significantly greater in KO mice as compared to WT mice 2 weeks after TAC, indicating more pulmonary congestion in the KO mice (Figure 1C, 1D, Table 1). During the 2 weeks of study, the mortality rate following TAC was not different between KO mice and WT mice (Figure 1E).

As anticipated, myocardial CD73 activity was abolished in KO mice as compared to WT mice under control conditions or after 2 weeks TAC (Figure 1F). In addition, KO significantly attenuated 5'-AMP induced bradycardia, indicating that KO significantly disrupted extracellular adenosine production from 5'-AMP (Figure 1G, 1H). Adenosine A1 receptor KO almost totally abolished 5'-AMP induced bradycardia (Figure 1G, 1H), consistent with the concept that extracellular adenosine caused bradycardia through activation of the adenosine A1 receptor.

Histological analysis demonstrated that TAC resulted in more ventricular fibrosis (Figure 2), and a greater increase in cardiac myocyte cross-sectional area (Figure 2) in KO mice as compared with WT mice, indicating that the greater ventricular hypertrophy in the KO mice after TAC was due to both larger cardiomyocytes and an increase of fibrosis. The fibrosis after TAC in both wild type and KO mice was more apparent in the perivascular region. In comparison with WT mice, the relative increase in myocardial fibrosis following TAC in the KO mice was much greater than the relative increase in myocyte hypertrophy.

Echocardiographic imaging of the heart 2 weeks after TAC (Figure 3A) demonstrated significant increases of LV wall thickness (Table 1) and LV end-diastolic diameter (Figure 3). TAC for 2 weeks resulted in significant impairment of LV systolic function in the KO mice, as demonstrated by a greater reduction of systolic fractional shortening (Figure 3B) and a significant increase in LV end-systolic diameter as compared to WT mice (Figure 3C). TAC for 2 weeks also resulted in significant impairment of LV contractility in the KO mice, as demonstrated by a greater reduction of LV dP/dt_{max} and LV dP/dt_{min} as compared to WT mice (Table 1). After TAC for 2 weeks, mean aortic pressure and LV systolic pressure were significantly lower in KO mice than WT mice (Table 1), consistent with the finding of more ventricular dysfunction in KO mice.

Consistent with increased hypertrophy in KO mice, myocardial ANP protein was significantly higher in KO mice than WT mice after TAC (Figure 4A, 4B). Consistent with the greater increase of LV fibrosis after TAC, KO mice hearts contained higher myocardial collagen-I content than WT mice. Myocardial TNFα levels were also significantly higher in the KO than WT mice after TAC (Figure 4a, 4e), suggesting an increased inflammatory response in the KO mice. This observation is in agreement with reports that adenosine can reduce cardiac TNFα expression[20].

CD73 KO enhances Akt- mTOR-p70S6K activation

The PI-3 kinase/AKT signaling pathways target mTOR activity to increase translation of proteins important for cell growth[21]. Signaling from mTOR appears critical for cardiac hypertrophy, and also promotes the transition to heart failure during chronic pressure overload[22]. Akt can increase activation of mTOR indirectly by reducing TSC2 activity[23], and it is also a direct target of mTOR kinase activity[24]. Interestingly, Western blot analysis revealed that the mTOR effector phosphorylation sites at Akt^{Ser473} and $p70S6K^{Thr389}$ were significantly increased in the KO mice above levels found in WT mice even under basal conditions. In WT mice, TAC increased levels of $p\text{-}Akt^{Ser473}$ and $p\text{-}70S6K^{Thr389}$, and both were further elevated in the KO mice. Consistent with increased phosphorylation of mTOR targets in the KO mice, KO mice demonstrated higher levels of $p\text{-}mTOR^{Ser2488}$ as compared with WT mice 2 weeks after TAC (Figure 5). The increased levels of $p\text{-}mTOR^{Ser2488}$ in KO mice were the result of both increased mTOR expression, as well as increased phosphorylation relative to total levels. The lipid phosphatase known as Phosphatase and Tensin Homologue on Chromosome Ten (PTEN) can down regulate PI-3 kinase signaling by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate, and mutations that inhibit PTEN or cardiac specific deletion of PTEN result in constitutive Akt activity[25]. Under basal conditions, phosphorylation at serine 380 (Serine 380 phosphorylation of PTEN involved in reducing membrane association and activity) was significantly increased, while total PTEN levels were slightly, but not significantly ($p=0.06$) lower in KO mice. TAC increased expression of PTEN to similar levels in both WT and KO mice, while $phospho\text{-}PTEN^{Ser380}$ was raised to significantly higher levels in KO than WT, suggesting that PI-3 kinase signaling may be particularly enhanced in these mice via down regulation of PTEN activity. In addition, TAC for 2 weeks resulted in greater increases of p-PKCα in KO mice as compared with WT mice (Figure 5).

Adenosine or adenosine analogue attenuates cardiac myocyte hypertrophy and activation of mTOR and p70S6K

Because *in vivo* adenosine can increase blood flow[26], reduce inflammatory responses[20], inhibit norepinephrine release from nerve endings[27], and decrease ET-1 production[28], it was important to determine whether the amplified mTOR/p70S6 signaling in the KO mice was the result of indirect effects of adenosine that caused paracrine regulation of these signaling pathways, or a direct effect of adenosine on cardiomyocytes. Therefore, we examined the effect of the adenosine analogue CADO on *phenylephrine* (PE)-induced hypertrophy and activation of $p\text{-}mTOR^{Ser2488}$ and $p70S6K^{Thr389}$ in isolated neonatal cardiomyocytes. PE significantly increased the size of the cardiac myocytes and expression of the hypertrophy marker ANP, while CADO significantly attenuated the PE-induced increase in cell size and reduced ANP expression (Figure S1, http://hyper.ahajournals.org). PE treatment also significantly increased phosphorylation of $mTOR^{Ser2448}$ and $p\text{-}70S6K^{Thr389}$, and this activation was dramatically reduced by CADO (Figure 6). Similarly, adenosine attenuated the PE-induced increase of cardiac myocyte size and activation of $p\text{-}70S6K^{Thr389}$ and $p\text{-}mTOR^{Ser2488}$ (**data not shown**).

Adenosine attenuates collagen synthesis and fibroblast proliferation

We also determined the effect of adenosine on cardiac fibroblast proliferation and collagen production, and the results showed that adenosine significantly reduced cardiac fibroblast proliferation and collagen production (Figure S2, see the online data supplement available at http://hyper.ahajournals.org), which is in agreement with previous reports[9].

Discussion

The major finding in this study is that deletion of CD73 significantly exacerbates LV hypertrophy, dilation and dysfunction in the TAC model of LV pressure overload. These results suggest that conversion of extracellular AMP to adenosine plays a significant role in

modulating maladaptive tissue remodeling and hypertrophic signaling pathways activated in response to systolic overload. The greater ventricular hypertrophy in CD73 KO mice after TAC was the result of both increased fibrosis and greater cardiomyocyte hypertrophy. The more prominent hypertrophy and dysfunction in the KO hearts was associated with increased activation of the mTOR signaling pathway. Moreover, the demonstration that adenosine or the stable adenosine analogue CADO attenuated phenylephrine-induced cardiomyocyte mTOR/p70S6 kinase signaling and myocyte hypertrophy, as well as fibroblast collagen production, suggests that the *in vivo* effects of CD73 deletion can be attributed to loss of adenosine production. Taken together, these findings provide the first direct evidence that endogenous extracellular adenosine exerts a protective effect against ventricular tissue remodeling and cardiac myocyte hypertrophic signaling pathways during chronic systolic overload.

Although no previous reports have directly examined the effect of CD73 on pressure overload-induced ventricular remodeling, there is evidence that increased endogenous adenosine can attenuate the development of cardiovascular disease[29]. Adenosine is known to inhibit norepinephrine release from presynaptic vesicles[27], reduce production of ET-1[28], and reduce TNF-α production[20]. Recently we also found that while 8-SPT significantly increased myocardial oxygen consumption in dogs with failing hearts, it had no effect on oxygen consumption in normal dogs[26], suggesting that endogenous adenosine may help reduce myocardial oxygen demand particularly in the failing heart. Studies in rats in which extracellular adenosine was increased by blockade of adenosine uptake with dipyridamole[7] also reported attenuation of pressure overload induced myocardial hypertrophy. Interestingly, a mutation of the adenosine monophosphate deaminase 1 gene (which results in increased adenosine production) predicted a better clinical outcome in patients after myocardial infarction[30], implying that increased endogenous adenosine levels can exert a protective effect on the diseased human heart. Myocardial adenosine concentrations increase during the compensated phase of ventricular hypertrophy, but then decrease when there is evidence of decompensation[10, 11], suggesting that a decrease of extracellular adenosine levels might be a contributing factor in the transition to heart failure. Our finding that a genetically engineered loss of CD73 activity exacerbated TAC-induced ventricular hypertrophy and dysfunction provide further evidence that endogenous adenosine production protects against progression to heart failure under conditions of pressure overload.

Adenosine Effects on Cardiomyocyte Hypertrophic Signaling

The present data identify a major hypertrophic signaling pathway targeted by extracellular adenosine. KO mice demonstrated increased phosphorylation of p-mTOR^{Ser2488}, p-70S6K^{Thr389} and pAKT^{Ser473}, suggesting that adenosine normally acts to down-regulate these signaling pathways. Activation of mTOR and its downstream targets results in increased cell size and is commonly associated with cardiac hypertrophy. Furthermore, over-expression of p70s6 kinase resulted in cardiac hypertrophy[31], while inhibition of mTOR signaling with rapamycin attenuated the development of ventricular hypertrophy in mice exposed to ascending aortic constriction[22]. The finding that phospho-PTEN^{Ser380} was increased under basal conditions and after TAC suggests that PI-3 kinase signaling may be particularly enhanced in KO mice by the increase of phospho-PTEN^{Ser380}, and may explain increased levels of mTOR and AKT activation[25]. The direct effect of CADO on inhibition of cardiac myocyte hypertrophy and mTOR/p70S6 kinase activation in isolated cardiomyocytes confirms that adenosine regulates mTOR signaling. Interestingly, PKCα phosphorylation within the activation loop was also significantly increased in CD73 KO mice compared to WT mice under both basal conditions and during TAC. Increased PKCα activity may contribute to the reduced contractility found in CD73 KO mice during pressure overload, as PKCα deletion has been shown to increase contractility and protect against heart failure from pressure overload[32].

Extracellular Adenosine Protection against Systolic Overload

The specific roles of adenosine receptor subtypes in protection against pathologic hypertrophy have not been well defined. While an A1 receptor agonist has been shown to reduce hypertrophy and heart failure in response to pressure overload, and also reduces hypertrophy of isolated cardiomyocytes[6], transgenic over-expression of A1 or A3 receptors in the heart actually promotes cardiac hypertrophy and dilation[33, 34]. The role of the A2b receptor is slightly more well defined, as most published data suggests a role in reducing cardiac fibroblast proliferation and collagen synthesis[8, 9]. The A2b receptor also plays a role in ameliorating pathological LV tissue remodeling after infarct[15]. Activation of type 2A adenosine receptors, which are highly expressed in the coronary vasculature, can down regulate v-cam expression[35] to reduce monocyte adhesion to endothelial cells and vascular inflammation. The increased vascular inflammation and fibrosis in the CD73 KO mice after TAC suggests that A2A and A2b receptors may not be adequately activated in absence of CD73 dependent adenosine production. In addition to increased vascular inflammation, CD73 KO has been reported to cause a 15% decrease in basal coronary flow[36]. While this modest decrease in coronary flow would not likely affect cardiac function under basal conditions, abnormalities of coronary flow might impair oxygen delivery during pressure overload, when oxygen demand is increased and diffusion distances are increased by perivascular fibrosis. It is therefore probable that the protective effects of extracellular adenosine against the TAC induced ventricular hypertrophy and dysfunction are not mediated by activation of any individual adenosine receptor subtype alone, but more likely involves complimentary effects of multiple adenosine receptor subtypes on multiple cardiac cell types. Additional studies will be needed to distinguish the specific adenosine receptors and cell types which mediate the protective effects of adenosine in the pressure overloaded heart.

Limitations—Although we demonstrated that CD73-KO abolished myocardial CD73 activity, and a previous study using the same mouse strain demonstrated that KO abolished extracellular adenosine production in other tissues, we were not able to collect extracellular fluid from the mouse heart for adenosine analysis due to the small size of the heart. Secondly, as all of the adenosine receptors are expressed in the heart, future studies will be needed to determine the specific adenosine receptor(s) responsible for the protective effect against pressure overload induced ventricular hypertrophy.

Perspectives—Previous studies have demonstrated that adenosine analogues and selective adenosine A1 or A3 receptor agonists protect the heart from ischemia/reperfusion induced myocardial damage. However, the effect of endogenous extracellular adenosine on chronic pressure overload induced ventricular hypertrophy and heart failure has not been previously studied. Here we demonstrated that loss of CD73 activity exacerbates the ventricular hypertrophy, fibrosis and dysfunction that occur in the heart exposed to chronic hemodynamic overload. This study also identifies, for the first time, a specific hypertrophic signaling pathway (mTOR-p70S6K) that is targeted by adenosine and which may explain the anti-hypertrophic effects of adenosine. These findings provide the first direct evidence that endogenous extracellular adenosine plays an important role in regulating pressure overload induced ventricular remodeling, indicating that increasing extracellular adenosine production or activation of specific adenosine receptors may be a therapeutic approach for treating the pressure overloaded heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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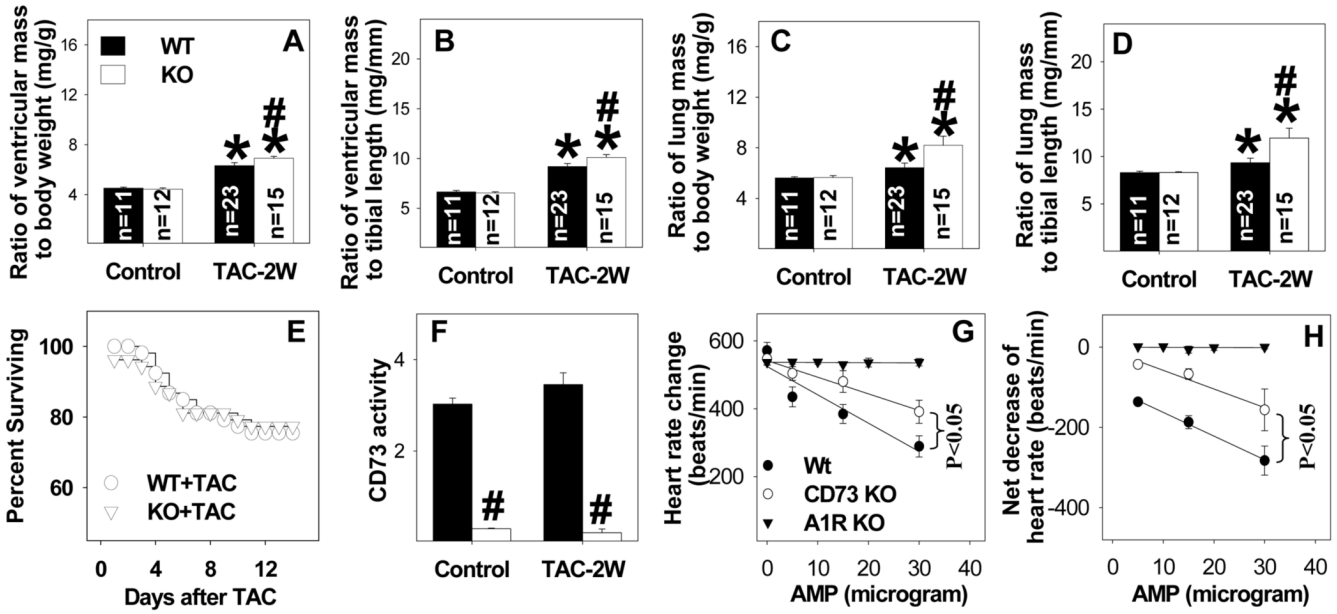

Figure 1.

CD73 KO exacerbates TAC-induced ventricular hypertrophy (A, B), and pulmonary congestion (C, D). KO had no effect on TAC induced mortality (E), but decreased CD73 activity (F). KO significantly attenuated the 5'-AMP induced decrease of heart rate (G, H), consistent with the diminished capacity of KO mice to produce extracellular adenosine from 5'-AMP. *P<0.05 compared to the corresponding control; #p<0.05 compared to wild type mice. CD73 activity was obtained from 4-5 mice per group (F). The decrease of heart rate in response to 5'-AMP infusion was obtained from KO (n=5), wild type littermates of KO mice (n=5), and adenosine A1 receptor KO mice (n=3). The rest of the data was obtained from 11- 23 mice per group as labeled.

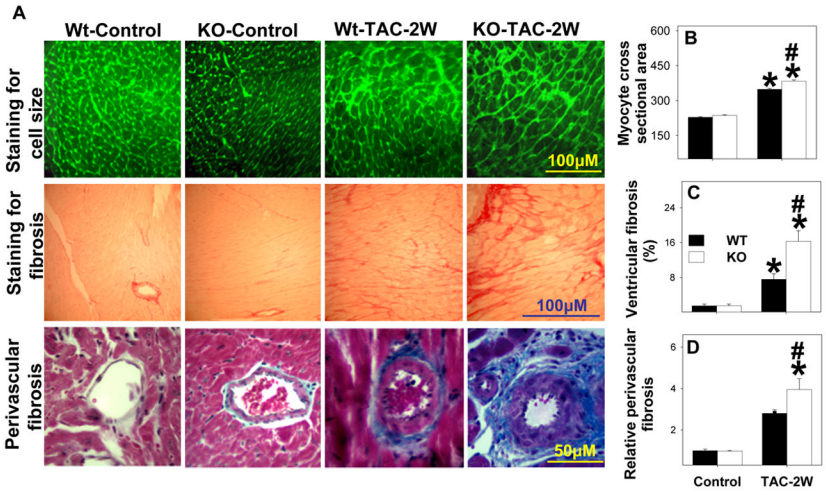

Figure 2.

CD73 KO exacerbates TAC-induced cardiac myocyte hypertrophy (A, B), ventricular fibrosis (A, C) and perivascular fibrosis (A, D). *P<0.05 compared to the corresponding control; #p<0.05 compared to WT (n=4 mice/group).

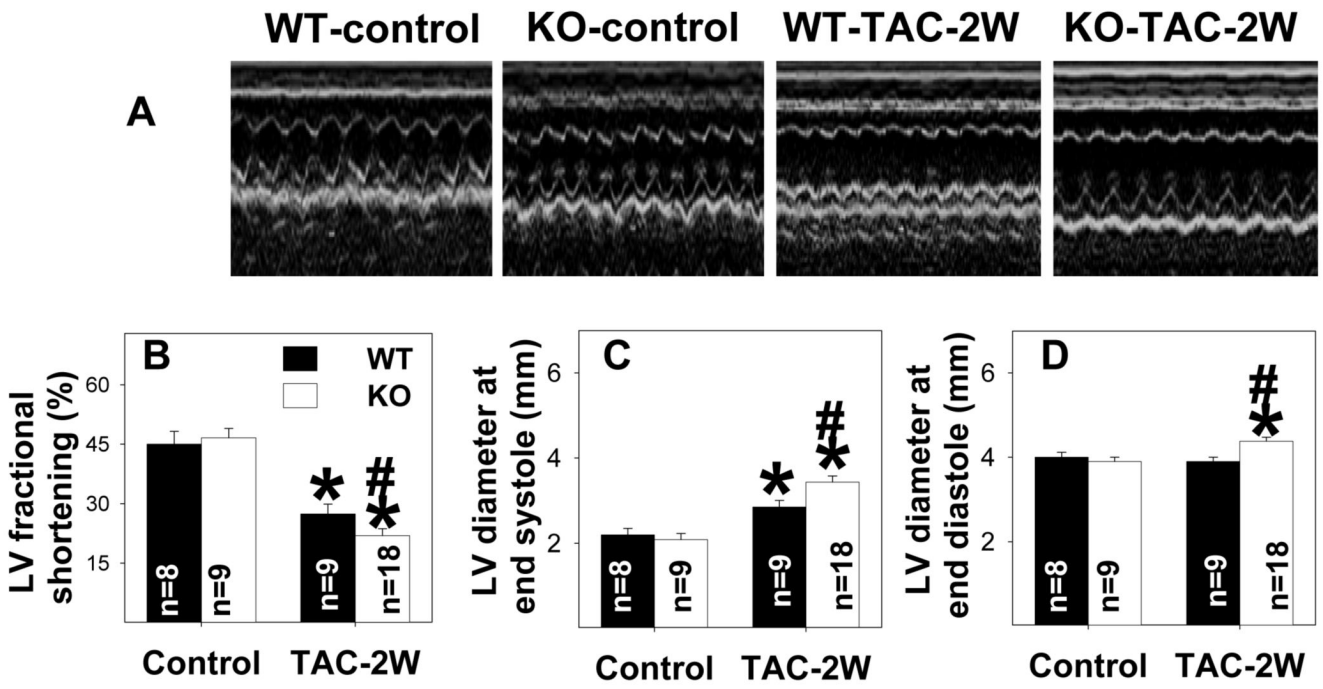

Figure 3.
CD73 KO exacerbates TAC-induced ventricular dysfunction (A, B) and dilation (C, D).
*P<0.05 compared to the corresponding control; #p<0.05 compared to WT.

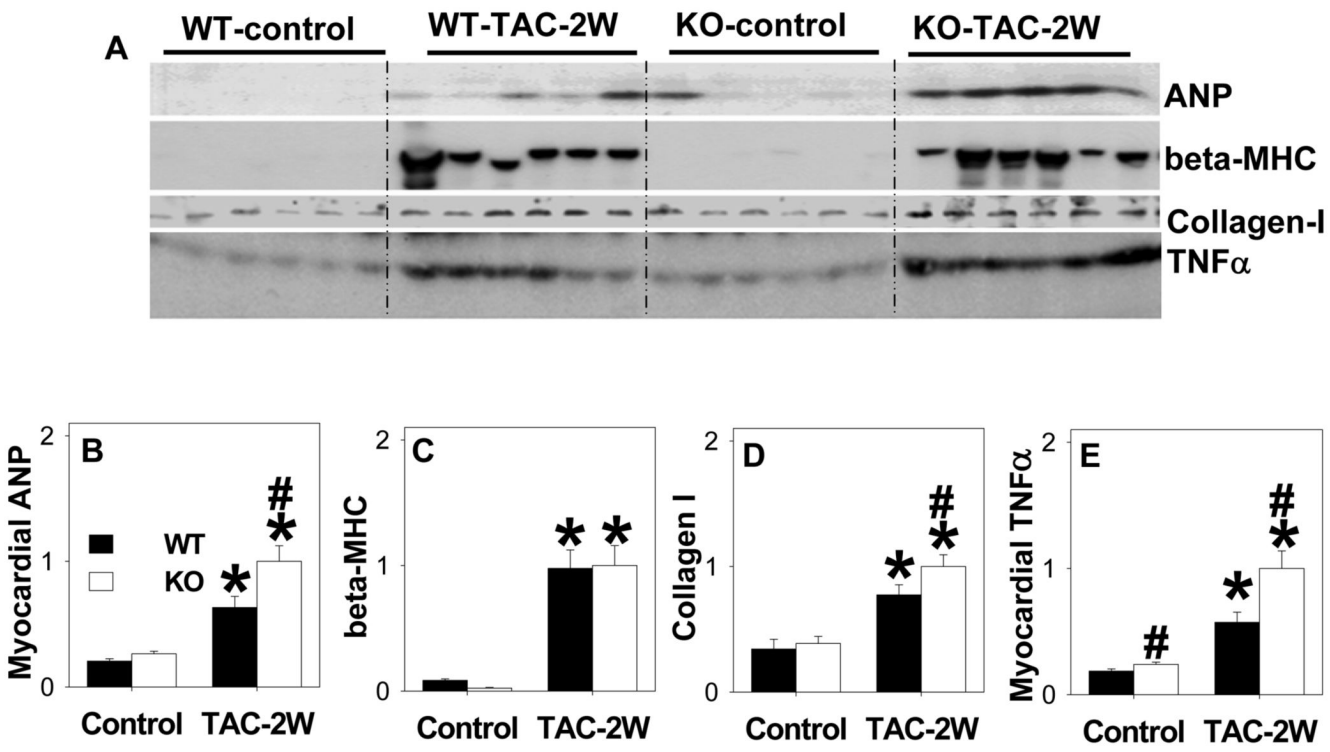

Figure 4.

CD73-KO significantly exacerbated TAC induced increase of myocardial ANP, β-MHC, Type I Collagen and TNFα *P<0.05 relative to sham; # P<0.05 compared to WT (n=6 samples/group).

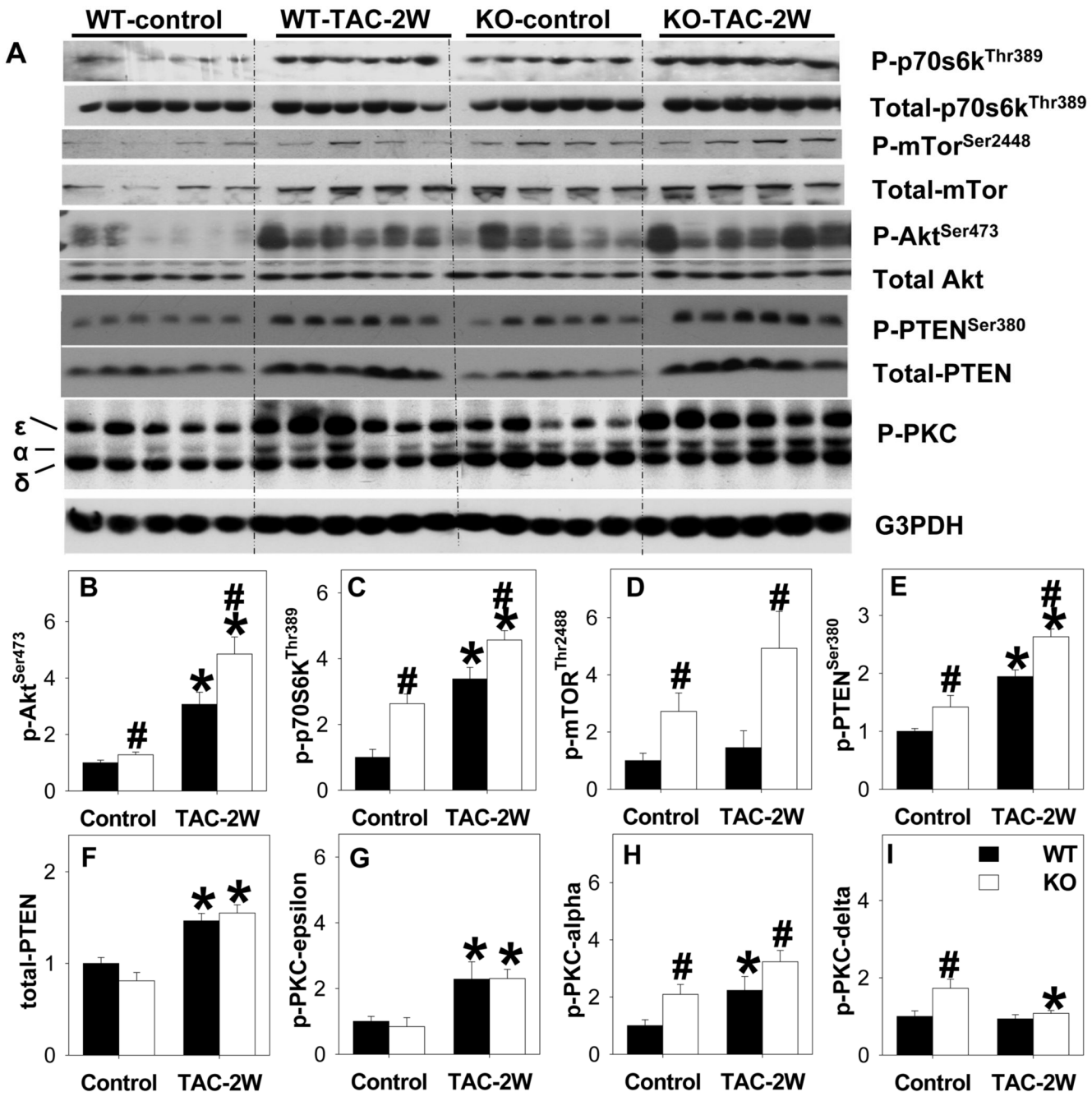

Figure 5.

Systolic overload produced by TAC for 2 weeks increased phosphorylated AKT^{ser473}, p70S6 kinase$^{thr\ 389}$ and mTOR^{ser2448} in heart lysates as measured by western blot and scanning densitometry. Phosphorylation of these sites in KO was significantly elevated above levels WT animals. The results indicate that KO increased mTOR signaling after TAC. KO also increased PKCα under both control conditions and after TAC. *P<0.05 relative to sham; # P<0.05 compared to WT (n= 4 -6 samples/group).

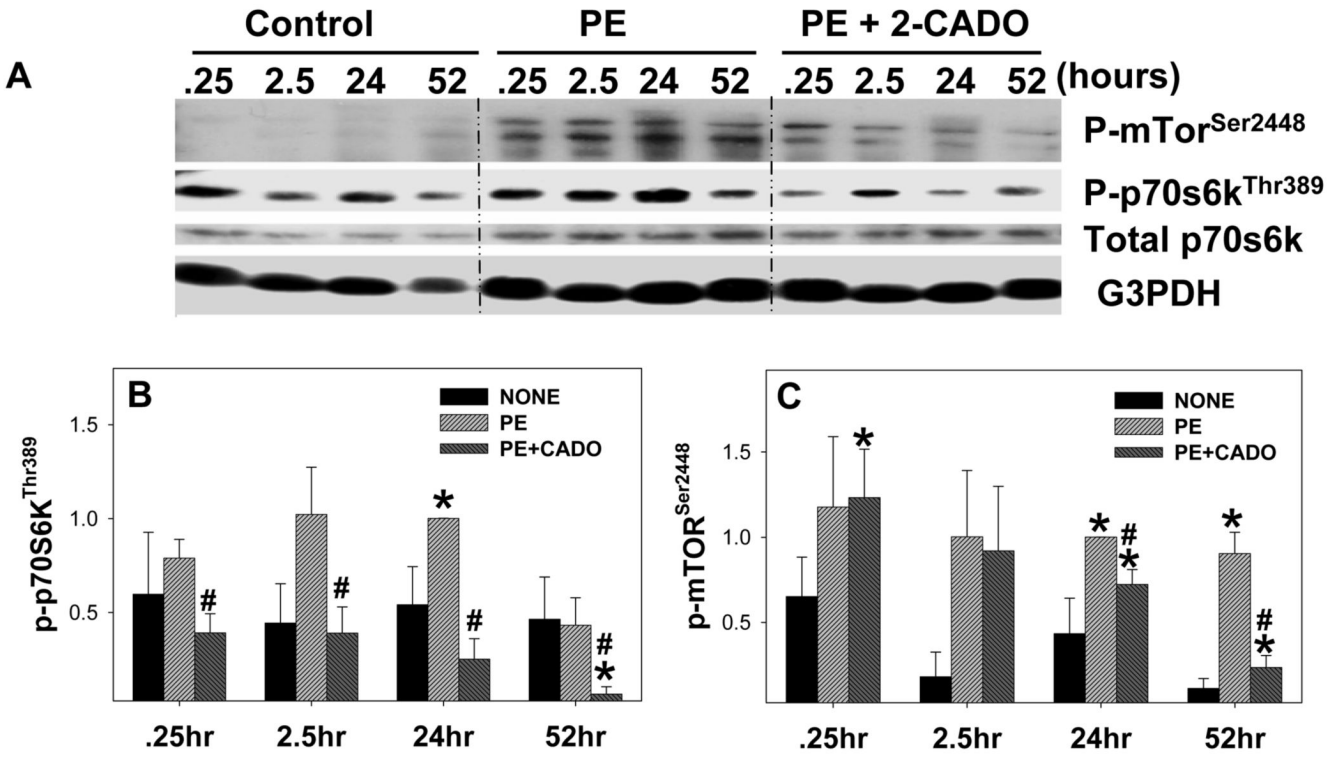

Figure 6.

2-chloroadenosine (2-CADO, 5μM) time-dependently reduced phenylephrine (PE) induced phosphorylation of mTOR and p70S6 kinase in cultured neonatal cardiomyocytes(A,B,C). Cultured neonatal cardiomyocytes were treated with PE in the presence or absence of 2-CADO. At the indicated time points, lysates were collected and analyzed by western blot for phosphorylation of p70S6 kinase$^{thr\ 389}$(B), and mTOR^{ser2448}(C). Glyceraldehyde 3-phosphate dehydrogenase and total p70S6 kinase are shown as controls. Each bar represents average from 3-7 individual experiments. *P<0.05 relative to untreated; # P<0.05 compared to PE treated.

Table 1

Anatomic and functional data for wild type and CD73-KO mice.

Parameter	Wild type control	CD73 -KO control	Wild type-TAC	CD73-KO TAC
Body weight (g)	25.8 ± 0.4	26.1 ± 0.6	25.8 ± 1.0	25.8 ± 0.7
Ventricular mass (mg)	116 ± 2.0	115 ± 2.4	161 ± 4.9*	178 ± 5.7,*†
Ratio of ventricular mass to body weight (mg/g)	4.52 ± 0.08	4.44 ± 0.10	6.33 ± 0.23†	6.91 ± 0.14*,†
Lung mass (mg)	145 ± 2.4	146 ± 1.6	164 ± 8.4*	211 ± 18.5,*†
Ratio of lung mass to body weight (mg/g)	5.63 ± 0.08	5.65 ± 0.15	6.43± 0.36*	8.20 ± 0.73*,†
Tibia length (mm)	17.5 ± 0.14	17.5 ± 0.14	17.5 ± 0.13	17.6 ± 0.12
Heart rate (beats per minute)	499 ± 37	511 ± 17	466 ± 15*	444 ± 7.8*
LV end systolic diameter (mm)	2.21 ± 0.15	2.09 ± 0.14	2.85 ± 0.16*	3.44 ± 0.14*,†
LV end diastolic diameter (mm)	4.01 ± 0.12	3.90 ± 0.12	3.91 ± 0.11*	4.38 ± 0.10*,†
LV ejection fraction (%)	82.2 ± 2.6	84.0 ± 2.2	60.6 ± 3.7*	51.3 ± 3.0,*†
LV posterior wall thickness at end diastole (mm)	0.69 ± 0.01	0.68 ± 0.03	0.97 ± 0.03*	0.94 ± 0.03*
LV posterior wall thickness at end systole (mm)	1.14 ± 0.03	1.09 ± 0.02	1.25 ± 0.02*	1.26 ± 0.23*
Mean aortic pressure (mmHg)	87.1 ± 5.9	85.5 ± 2.7	96 ± 3.3	92 ± 3.6
Systolic LV pressure (mmHg)	106 ± 4.6	103 ± 2.5	153 ± 3.5*	148 ± 4.7,*†
LV end diastolic pressure (mmHg)	9.5 ± 1.1.7	8.8 ± 1.2	20 ± 2.9*	25 ± 4.4*
LV dP/dt_{max} (mmHg/s)	8791 ± 855	7181 ± 390	7171 ± 286*	5208 ± 350*,†
LV dP/dt_{min} (mmHg/s)	−7638 ± 743	−6526 ± 284	−7984 ± 271	−6036 ± 496†

The anatomic data were obtained from all mice studied (11-23 mice/group). The hemodynamic data were obtained from 8-9 mice/group after TAC, and 5 mice/group under control conditions.

* $p<0.05$ as compared with corresponding control conditions

† $p<0.05$ as compared with WT mice.