Expanded methods

Reagents and Adenovirus vectors:

Adenovirus harboring antisense ICER-IIγ (Ad-ICER-AS) was a kind gift from Dr. Sadoshima [1]. Although there are four isoforms of ICER (ICER-I, ICER-Iγ, ICER-II, ICER-IIγ), the function of each isoform is indistinguishable and Ad-ICER-AS inhibits expression of all ICER isoforms [1]. cDNA encoding wild type PDE3A1 and dominant negative form of MEF2C were kind gifts from Dr. V. C. Manganiello (NHLBI, National Institute of Health) and Dr. J. D. Molkentin (Cincinnati Children's Hospital Medical Center), respectively. Adenovirus expressing constitutively active form of MEK5α (Ad-CA-MEK5α), dominant negative form of ERK5 (Ad-DN-ERK5) and MEF2C (Ad-DN-MEF2C) were generated using ViraPower Adenoviral Expression System (Invitrogen). Adenovirus containing β galactosidase (Ad-LacZ) was used as a control virus.

Rat Neonatal Cardiomyocytes:

Primary cultures of neonatal rat cardiomyocytes were performed as described previously [2]. Briefly, neonatal cardiac myocytes were obtained by enzymatic dissociation of cardiac ventricles from 1-2 day old Sprague-Dawley rat neonates. The ventricular tissue parts were subjected to multiple rounds of enzymatic digestion by collagenase II (Worthington). Cells were then collected by centrifugation at 800 rpm for 5 min at 4 ºC. Non-myocytes were removed via two rounds of pre-plating on culture dishes. The enriched cardiomyocytes were cultured in DMEM with 10% BCS and 10% horse serum. The following day after cells adhered to the dish, 10μM cytosine 1-β-D-arabinofuranoside (Sigma) was added to inhibit the growth of

contaminating non-myocytes. More than 90% of cells were cardiomyocytes (positive for α-actinin). Adenovirus-mediated transfection efficiency in cardiomyocytes is 90-95%.

Western blot analysis:

Cell lysates were prepared in RIPA buffer as previously described [3]. Human and animal heart samples were homogenized in the buffer (containing 40 mM Tris-Hcl (pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 mM Na_3VO_4, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 20 μg/ml leupeptin, 1 mM bezamidine), followed by centrifugation at 800g for 10 minutes at 4°C. Supernatants were subjected for Western blot analysis. Antibodies against actin (Santa Cruz) and cleaved caspase-3 (Asp175) (Cell Signaling), phospho and non-phospho ERK5, ERK1/2, Akt and MEF2C (Cell Signaling) were used. Immunoblotting with anti-ICER and anti-PDE3A antibody was performed as described previously [4]. ICER protein corresponds to a group of four proteins of approximately 16-18 kDa and 13-14 kDa [5].

Analysis of apoptosis:

Cardiomyocyte apoptosis was measured by the terminal deoxyribonucleotide transferase(TdT)-mediated dUTP nick-end labeling (TUNEL) detecting in situ DNA fragmentation. TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche) as described previously [6]. For TUNEL method, cells were also stained for cardiomyocyte-specific sarcomeric α-actinin with EF-53 to distinguish cardiomyocytes from contaminating fibroblasts and only EF-53 positive cells were counted. An average of total 1000 EF-53 positive cells from random fields were analyzed. All measurements were performed blinded. At least three independent experiments were performed.

***Mouse Model of Thoracic Aorta Constriction (TAC)*:**

The chronic pressure overload mouse model was created by performing TAC as described previously [6]. Briefly, 10-12 week-old FVB male mice (≈20g) were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (2.5 mg/kg) and placed on a ventilator. TAC was created via a left thoracotomy by placing a ligature securely around the ascending aorta and a 26-gauge needle and then removing the needle as described previously [6]. Animals in the sham group (as control) were undergone a similar procedure without constriction. The survival rate of this surgery was about 90%.

In vivo hemodynamic measurements with cardiac catheterization:

Briefly, mice were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (2.5 mg/kg). After endotracheal intubation, mice were connected to a rodent ventilator. Following bilateral vagotomy, the chest was opened and a 1.8-French high fidelity micronanometer cather (Millar Instruments) was inserted into the left atrium, advanced through the mitral valve, and secured in the LV as described previously [6]. Bursts of pressure and ECG tracings were recorded and analyzed. LV +dP/dt and -dP/dt were calculated. After examination, hearts were excised and subjected to the following histological and biochemical studies.

Protein extract from heart tissue:

Mouse hearts were washed with 10 ml of cold PBS. Isolated mice hearts were frozen in liquid nitrogen and homogenized with 0.5 mL of lysis buffer (10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% Triton X-100, 0.05% NP-40) containing 2 mmol/L sodium orthovanadate, and protease inhibitor cocktail (Sigma St Lueis MO). Protein concentration was determined with the

Bradford protein assay (Bio-Rad). Protein (30µg) was separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes.

Echocardiographic analysis:

Echocardiographic analysis with M-mode was performed using Acuson Sequoia C236 echocardiography machine equipped with a 15 MHz frequency probe (Siemens Medical Solutions, Malvern, PA). Echocardiography (M-mode) was obtained in un-anesthetized mice. LV function was measured in the short axis view at midlevel. %FS was assessed by measurement of the end diastolic and end- systolic diameter (end diastolic diameter – end systolic diameter)/end-diastolic diameter x100%). We collected and averaged the data from 5 beats from one trace, and three traces from each animal. The pooled data were analyzed for statistical significance.

Animal Models:

Mouse constitutively active form of MEK5α (CA-MEK5α, S311D/T315D) cDNA was subcloned into a pBluescript-based Tg vector between the 5.5-kb murine-αMHC promoter and 250-bp SV-40 polyadenylation sequences, and we generated three different lines of CA-MEK5α-Tg and all three lines showed a similar phenotype and MEK5α expression as we reported previously [7]. Both non-transgenic littermate control (NLC) and CA-MEK5α transgenic mice were randomly assigned to either the non-treated group or Doxorubicin HCl (Dox, Sigma)-treated group. Dox was reconstituted fresh with 0.9% NaCl to a final concentration of 2 mg/ml following the manufacturer's instruction. Mice were treated with a single intraperitoneal injection of doxorubicin at a dose of 30 mg/kg. Control mice received

injections of 0.9% NaCl of comparable volume.

Histology:

Organs were removed, fixed in 4% paraformaldehyde, and embedded in paraffin for further histological analysis. The rate of apoptosis was assessed by terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick-end labeling (Roche). The percentage of apoptotic nuclei was analyzed and expressed as percentage of total nuclei as described previously [6].

ERK5 transcriptional activity (Mammalian one-hybrid analysis) **[8]:**

Cardiomyocytes were plated in 12-well dishes at 2 x 10^5 cells/well and 24 h later transfected with lipofectamine2000 in Opti-MEN (Invitrogen) with the pG5-luc vector and pBIND plasmids (Promega). The pG5-luc vector contains five Gal4 binding sites upstream of a minimal TATA box which, in turn, is upstream of the firefly luciferase gene. pBIND contains Gal4, and were fused with ERK5. Since pBIND also contains the Renilla luciferase gene, the expression and transfection efficiencies were normalized with the Renilla luciferase activity. Cells were collected 24 h after IGF-1 stimulation, and the luciferase activity was determined. Luciferase activity was assayed with a luciferase kit (Promega). Transfections were performed in triplicate, and each experiment was repeated at least two times.

Transfection of the erk5 siRNAs:

The *erk5* siRNAs was purchased from Dharmacon. (Lafayette, CO). The mouse and rat specific *erk5* target sequence was 5 –AAAGGGTGCGAGCCTATAT-3. A non-specific control siRNA from Invitrogen (Carlsbad, CA) was used as a negative control. The cells were

transiently transfected with 40 nM of medium control siRNA or *erk5* siRNA using Lipofectamine transfection reagent (Invitrogen) following protocols provided by the manufacturer.

Statistical Analysis:

All data are expressed as mean ± S.D. To compare western blotting data, luciferase activity, TUNEL assay, echocardiography, and hemodynamic data, data were analyzed by one-way ANOVA with post hoc analysis. Statistical significance was accepted at a value of $P < 0.05$.

Expanded discussion

ERK5 and PDE3A-ICER feedback loop

It is possible that ERK5 may also regulate cardiomyocyte apoptosis independent of the PDE3A-ICER autoregulatory positive feedback loop. However, we found that the anti-apoptotic effect of CA-MEK5α was lost by ICER overexpression (Fig. 5A), thus the regulation of PDE3A-ICER feedback loop by ERK5 activation has at least a partial effect on ERK5-mediated anti-apoptosis in cardiomyocytes. The precise mechanism by which ERK5 activation–regulated ICER protein destabilizationy (Fig.4) will require further investigation. Since it has been reported that ERK1/2 targets ICER to ubiquitin-mediated destruction [4], a similar mechanism may be involved. We cannot exclude the possibility that ERK5 and MEF2 activation can directly increase PDE3A expression and inhibit ICER induction, because several putative MEF2 binding sites exist in PDE3A the promoter region. Further investigation will be required to clarify the role of ERK5/MEF2 activation on regulation of the PDE3A-ICER feedback loop.

Another possible role of PPDE3A-ICER feedback loop in heart failure

Since both Ang II and ISO can induce PDE3A-ICER feedback loop [9], which was observed in the pathological progression of heart failure with various etiologies, this feedback loop may represent a common mechanism of cAMP-related signaling. Of note, 24 hrs after Ang II stimulation, Ang II receptor blocker was unable to reverse Ang II-induced PDE3A reduction and ICER induction, as well as subsequent apoptosis [9]. Therefore, in addition to Ang II receptor and β-blockers, inhibiting PDE3A-ICER feedback loop using agents that can restore PDE3A expression and inhibit ICER induction may be useful for slowing the development of heart failure. Indeed, in the current study we found that the IGF-1-mediated ERK5 activation could inhibit the PDE3A-ICER feedback loop, apoptosis, and subsequent cardiac dysfunction by regulating PDE3A-ICER feedback loop. These data provide a novel possible therapeutic intervention for the treatment of heart failure. Since it has been reported that induction of ICER can also down-regulate β1-adrenergic receptor expression [10], it is intriguing to hypothesize that the PDE3A-ICER feedback loop regulates cardiac function through not only inducing apoptosis but also decreasing cardiac function by inhibiting β1-adrenergic receptor expression in various heart failure models. Further studies will be required to clarify the exact mechanism and role of PDE3A-ICER positive feedback loop not only for apoptosis but also on the other cardiac function regulating molecules.

The difference between MEK5α and MEK5β

The importance of ERK5 activation in pressure overload- and Dox-induced cardiomyocyte apoptosis was also provided *in vivo* by using CA-MEK5α-Tg mice. Nicol *et al*. [11] reported that cardiac-specific expression of activated MEK5β in transgenic mice resulted in

eccentric cardiac hypertrophy that progressed to dilated cardiomyopathy and sudden death. As we described previously [7], the differences between the two transgenic models were apparent: 1) MEK5 isoforms: we used MEK5α, but Nicol et al. used MEK5β, and 2) Mouse strain: we used FVB, but Nicol et al. used C3HB6. MEK5β is 89 amino acids shorter than MEK5α at the N-terminus (which does not contain PB-1 domain), and we recently reported a novel functional difference in the way full-length MEK5α and the shorter MEK5β splice variant regulate ERK5 activity [12]. In fact only MEK5α activated ERK5 while MEK5β exhibited a dominant-negative phenotype inhibiting CA-MEK5α or growth factor-induced activation of ERK5[12]. Seyfried et al. reported that MEK5β, which does not contain PB-1 domain, could activate ERK5 via its weak interaction with ERK5 [13]. In contrast, Nakamura et al. have recently reported that deletion of the MEK5 PB1 domain cause essentially complete loss of MEK5-ERK5 interaction and ERK5 phosphorylation [14], which is significantly different from the report by Seyfriend et al. [13], and is consistent with our previous report [12]. Further investigation may be necessary to define why the two CA-MEK5 transgenic models exhibited different phenotypes.

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Online
Table 1
Cardiac parameters in NLC and CA-MEK5 α-Tg mice with vehicle or Dox treatment (conscious echocardiography).

	vehicle	Dox	
	NLC (n = 10)	NLC (n = 8)	CA-MEK5 α-Tg (n = 8)
Heart rate, bpm	574.6 ± 24.9	538.0 ± 39.5	591.2 ± 20.4
LVEDd, mm	2.61 ± 0.08	2.87 ± 0.15	2.74 ± 0.11
LVESd, mm	0.79 ± 0.06	1.57 ± 0.09	1.14 ± 0.08**
%FS	70.6 ± 1.5	44.3 ± 2.9	58.0 ± 2.7**
IVSWDd, mm	0.68 ± 0.02	0.87 ± 0.05	0.82 ± 0.03
LVPWDd, mm	0.73 ± 0.02	0.82 ± 0.03	0.81 ± 0.03

NLC; age-mached nontransgenic littermate control mice, LVEDd indicates left ventricle end-diastolic dimemsion; LVESd, left ventricle end-systolic dimension, %FS, percentfractional shortening; IVSWDd, interventricular septum wall diastolic dimension, PWDd, left ventricle posterior wall diastolic dimension. mean ± SEM, **P<0.01 vs NLC, Dox-treated mice.

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Table 2
Cardiac function after Dox or vehicle treatment in NLC and CA-MEK5 α-Tg mice based on in vivo hemodynamic analysis (anesthetized)

	NLC		CA-MEK5 α-Tg	
	vehicle	Dox	vehicle	Dox
Body weight, g	25.9 ± 2.4	26.5 ± 1.9	26.7 ± 2.3	27.3 ± 1.6
LV weight, mg	73.7 ± 5.8	71.9 ± 3.8	75.2 ± 4.3	73.3 ± 6.1
LV/body weight, mg/g	3.4 ± 0.5	3.7 ± 0.2	3.5 ± 0.3	3.7 ± 0.1
LV systolic pressure, mmHg	76.1 ± 6.1	54.3 ± 5.4*	82.3 ± 4.8	76.9 ± 4.5
LV diastolic pressure, mmHg	2.8 ± 0.6	17.8 ± 1.0**	3.2 ± 0.9	4.2 ± 1.5
LV developed pressure, mmHg/g	997 ± 64	509 ± 71**	1,053 ± 66	995 ± 51
Peak +dp/dt, +mmHg/sec	8,350 ± 766	3,321 ± 825 **	8,958 ± 667	7,391 ± 1,330
Peak -dp/dt, -mmHg/sec	7,416 ± 954	2,518 ± 740**	7,900 ± 867	6,120 ± 1,173
Heart rate, bpm	345 ± 22	355 ± 32	364 ± 58	365 ± 37

NLC; age-mached nontransgenic littermate control mice, Dox; Doxorubicin treated group, LV; left ventricle, LV developed pressure; LV systolic developed pressure/g LV mass, n = 8 per group, All data are shown as mean ± SEM. *p<0.05 vs. age-matched vehicle treated NLC mice, **p<0.01 vs. age-matched vehicle treated NLC mice.

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Table 3
Cardiac function after TAC operation in NLC and CA-MEK5 α-Tg mice based on in vivo hemodynamic analysis (anesthetized)

	4 weeks after operation			8 weeks after operation		
	sham	TAC		sham	TAC	
	NLC	NLC	CA-MEK5 α-Tg	NLC	NLC	CA-MEK5 α-Tg
Body weight, g	27.8 ± 2.9	28.3 ± 1.6	27.2 ± 1.2	29.4 ± 1.8	28.5 ± 3.2	27.8 ± 1.7
LV weight, mg	82 ± 7	113 ± 15	117 ± 5**	80 ± 10	151 ± 18 **	140 ± 3 **
LV/body weight, mg/g	2.9 ± 0.3	3.9 ± 0.8	3.1 ± 0.4	2.7 ± 0.5	5.1 ± 0.9 *	5.1 ± 0.1 *
LV systolic pressure, mmHg	69 ± 14	107 ± 10 *	110 ± 9 *	72 ± 9	82 ± 15	137 ± 8 **
LV diastolic pressure, mmHg	3.2 ± 0.5	7.6 ± 1.5 *	8.5 ± 0.9 **	2.9 ± 0.7	11.9 ± 2.8 *	7.7 ± 0.7 **
LV developed pressure, mmHg/g	946 ± 63	985 ± 97	876 ± 66	946 ± 12	427 ± 30 **	922 ± 68
Peak +dp/dt, +mmHg/sec	6,850 ± 490	12,563 ± 604 **	12,333 ± 1626 *	7,181 ± 497	10,266 ± 430 **	16,110 ± 975**
Peak -dp/dt, -mmHg/sec	5,150 ± 890	7,188 ± 969	6,700 ± 948	4,950 ± 748	6,850 ± 854	8,880 ± 942 **
Heart rate, bpm	395 ± 40	410 ± 37	383 ± 13	386 ± 46	388 ± 30	390 ± 37

NLC; age-mached nontransgenic littermate control mice, TAC; thoracic aortic constriction, LV; left ventricle, LV developed pressure; LV systolic developed pressure/g LV mass, n = 8 per group, All data are shown as mean ± SEM. *p<0.05 vs. age-matched sham-operated NLC mice, **p<0.01 vs. age-matched sham-operated NLC mice , p<0.01 vs. age-matched TAC-operated NLC mice.