

Activation of Extracellular Signal-Regulated Kinase 5 Reduces Cardiac Apoptosis and Dysfunction via Inhibition of a Phosphodiesterase 3A/Inducible cAMP Early Repressor Feedback Loop

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Abstract—Substantial evidence suggests that the progressive loss of cardiomyocytes caused by apoptosis significantly contributes to the development of heart failure. β -Adrenergic receptor activation and subsequent persistent phosphodiesterase 3A (PDE3A) downregulation and concomitant inducible cAMP early repressor (ICER) upregulation (PDE3A/ICER feedback loop) has been proposed to play a key role in the pathogenesis of cardiomyocyte apoptosis. In contrast, insulin-like growth factor-1 can activate cell survival pathways, providing protection against cell death and restoring muscle function. In this study, we found that insulin-like growth factor-1 activates extracellular signal-regulated kinase 5 (ERK5) and inhibits PDE3A/ICER feedback loop. Insulin-like growth factor-1 normalized isoproterenol-mediated PDE3A downregulation and ICER upregulation via ERK5/MEF2 activation, and also inhibited isoproterenol-induced myocyte apoptosis. To determine the physiological relevance of ERK5 activation in regulating PDE3A/ICER feedback loop, we investigated the PDE3A/ICER expression and cardiomyocyte apoptosis in transgenic mice with cardiac specific expression of a constitutively active form of mitogen-activated protein (MAP)/extracellular signal-regulated protein kinase (ERK) kinase 5 α (MEK5 α) (CA-MEK5 α -Tg). In wild-type mice, pressure overload– or doxorubicin-induced significant reduction of PDE3A expression and subsequent ICER induction. Cardiac specific expression of CA-MEK5 α rescued pressure overload– or doxorubicin-mediated PDE3A downregulation and ICER upregulation and inhibited myocyte apoptosis as well as subsequent cardiac dysfunction in vivo. These data suggest that preventing the feedback loop of PDE3A/ICER by ERK5 activation could inhibit progression of myocyte apoptosis as well as cardiac dysfunction. These data suggest a new therapeutic paradigm for end stage of heart failure by inhibiting the PDE3A/ICER feedback loop via activating ERK5. (*Circ Res.* 2007;100:510-519.)

Key Words: ERK5 ■ phosphodiesterase 3 ■ inducible cAMP early repressor ■ heart failure ■ insulin-like growth factor-1

Cardiac remodeling, including dysregulated myocyte apoptosis, contributes to the development and progression of pathological remodeling after cardiac ischemia/reperfusion and the transition from cardiac hypertrophy to chronic heart failure.^{1–3} A number of observations suggest that cardiac myocyte loss by apoptosis contributes to the transition from cardiac hypertrophy to heart failure.⁴ Previously, we reported a key role of phosphodiesterase 3A (PDE3A) and inducible cAMP early repressor (ICER) in cardiomyocyte apoptosis.⁵ PDEs exist as a superfamily with multiple isoforms that differ in tissue distribution, biochemical properties, and sensitivity to chemical inhibitors.⁶ At least 21 genes encoding more than 50 different PDE isoforms have been identified and grouped

into 11 broad families (PDE1 to PDE11).⁶ Each tissue/cell type expresses a distinct set of PDEs. In cardiomyocytes, PDE3, together with PDE4, accounts for >90% of the basal cAMP-hydrolyzing activity, although their relative contributions may differ among species.⁷ We have recently found that in both neonatal and adult cardiomyocytes, inhibition of PDE3 function but not PDE4 significantly increased cardiomyocyte apoptosis despite the fact that PDE4 inhibition elicited a more profound elevation of cAMP compared with PDE3 inhibition.^{5,7,8} These observations indicate that cardiomyocyte apoptosis is regulated by a subset of cAMP molecules that are preferentially coupled to PDE3, which are independent of the overall cellular cAMP levels in the cell.

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ICERs are members of the cAMP-responsive element (CRE) modulator family. The expression of ICERs is transcriptionally induced by CRE-binding protein (CREB) via a CREB sequence in the ICER promoter.⁹ ICER proteins contain DNA-binding and leucine zipper domains but not the N-terminal transactivation domain, which renders them endogenous inhibitors of gene transcription driven by its cognates such as CREB and activating transcription factor 1 (ATF-1).⁹ ICER has been shown to function as an important inducer or mediator of apoptosis in various cell types including neuronal cells¹⁰ and cardiomyocytes.^{5,11} We have recently shown that angiotensin II (Ang II) and β -adrenergic receptor activation induced a sustained downregulation of PDE3A1 expression via a “positive-feedback loop,” where ICER represses PDE3A gene expression and PDE3A reduction then feeds back, leading to increased ICER expression resulting from protein kinase A (PKA) activation.⁸ This feedback regulatory mechanism is essential for sustained ICER induction and subsequent cardiomyocyte apoptosis. Our previous findings demonstrated a key role of PDE3A in the regulation of cardiomyocyte survival and suggested that strategies to block the PDE3A/ICER feedback loop could reduce cardiomyocyte apoptosis and benefit heart failure.

Extracellular signal-regulated kinase 5/big mitogen-activated protein kinase 1 (ERK5/BMK1) is a member of the mitogen-activated protein kinase family, which is activated by oxidative and hyperosmotic stress, growth factors, and pathways involving certain G protein-coupled receptors.¹² The upstream kinase that phosphorylates ERK5 has been identified as mitogen-activated protein kinase/ERK kinase (MEK5).^{13,14} Like many mitogen-activated protein kinase family members, ERK5 plays a significant role in cell growth and differentiation via activation of its downstream substrate myocyte enhancer factor 2 (MEF2), although emerging evidence suggests unique functional characteristics. Oxidative stress-mediated activation of ERK5 is documented to have an antiapoptotic effect¹⁵ and ERK5 knockout mice have impaired cardiac and vascular development.¹⁶ Recently we, along with Kasler et al, reported that ERK5 is not only a kinase but also possesses transcriptional activity.^{17,18} We also reported that ERK5 activation was significantly decreased in human heart failure.¹⁹ Although activation of ERK5 by the constitutively active form of MEK5 α (CA-MEK5 α) has been demonstrated to have a cardioprotective effect against acute ischemia/reperfusion in an isolated heart model,²⁰ the exact role of ERK5 activation and its possible cardioprotective mechanism in chronic heart failure remains unclear.

An important cardiomyocyte survival factor is insulin-like growth factor-1 (IGF-1), but its effect on the PDE3A/ICER feedback loop remains largely unknown. IGF-1 is synthesized under the control of growth hormone by various cell types, including cardiac muscle.²¹ IGF-1 stimulates protein synthesis and cell growth and exerts antiapoptotic effects in many organs including cardiac muscle.^{22–24} Mehrhof et al have reported that IGF-1 activates Akt and ERK1/2 and inhibits apoptosis.²⁵ Interestingly, although the importance of IGF-1/phosphatidylinositol 3-kinase activation in cell survival is well established,^{26–28} the specific role of Akt in heart failure has recently become more complicated.^{29–31}

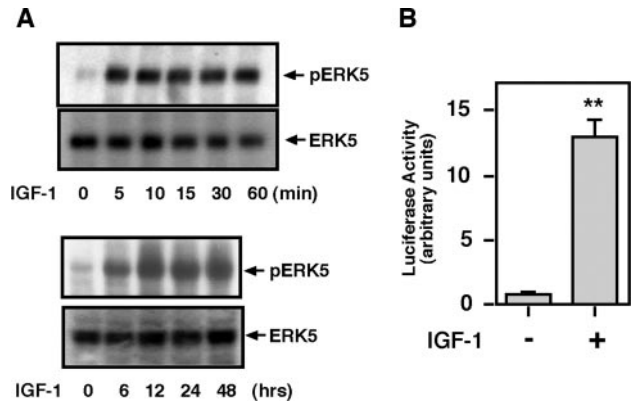


Figure 1. IGF-1 stimulated both ERK5 kinase and transcriptional activity. A, Cardiomyocytes were stimulated with IGF-1 (20 ng/mL), and phosphorylated (top) and total (bottom) ERK5 were measured by Western blot analysis at the indicated times. B, ERK5 transcriptional activity was measured by mammalian 1-hybrid assay with Gal4-ERK5 construct transfection, as we described previously.¹⁷

In this study, we found that ERK5 activation induced by IGF-1 significantly inhibited isoproterenol (ISO)-mediated apoptosis via regulating PDE3A/ICER feedback loop. In addition, activation of ERK5 in CA-MEK5 α -Tg mice prevents pressure overload- and doxorubicin (Dox)-mediated induction of PDE3A/ICER feedback loop, cardiac apoptosis, as well as subsequent cardiac dysfunction in vivo. These data suggest the critical role of ERK5 activation on cardiac apoptosis and subsequent dysfunction via preventing the development of PDE3A/ICER feedback loop.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at <http://circres.ahajournals.org>.

Details on reagents and adenovirus vectors, cultured rat neonatal cardiomyocytes, Western blot analysis, analysis of apoptosis, mouse model of thoracic aorta constriction (TAC), in vivo hemodynamic measurements with cardiac catheterization, protein extract from heart tissue, echocardiographic analysis, histology, ERK5 transcriptional activity (mammalian one-hybrid analysis), animal models, and statistical analysis are also provided in the online data supplement.

Results

ERK5 Activation by IGF-1 Inhibits the ISO-Induced Sustained Reduction of PDE3A and Concomitant Induction of ICER

Previously, we demonstrated that activation of ERK5 inhibits apoptosis in endothelial cells.³² There is increasing evidence that IGF-1 can be protective in cardiac injury, but the exact mechanism remains unclear. Therefore, we first investigated whether IGF-1 can increase ERK5 activation in cardiomyocytes. As shown in Figure 1A, we found that IGF-1 could significantly increase ERK5 kinase activation until at least 48 hours after stimulation. Because ERK5 is not just a “kinase” but also possesses strong transcriptional activity and increases MEF2 activity,^{17,18} we investigated whether IGF-1 can stimulate ERK5 transcriptional activity using a Gal4-ERK5 construct. As shown in Figure 1B, we found that IGF-1 significantly increased ERK5 transcriptional activity.

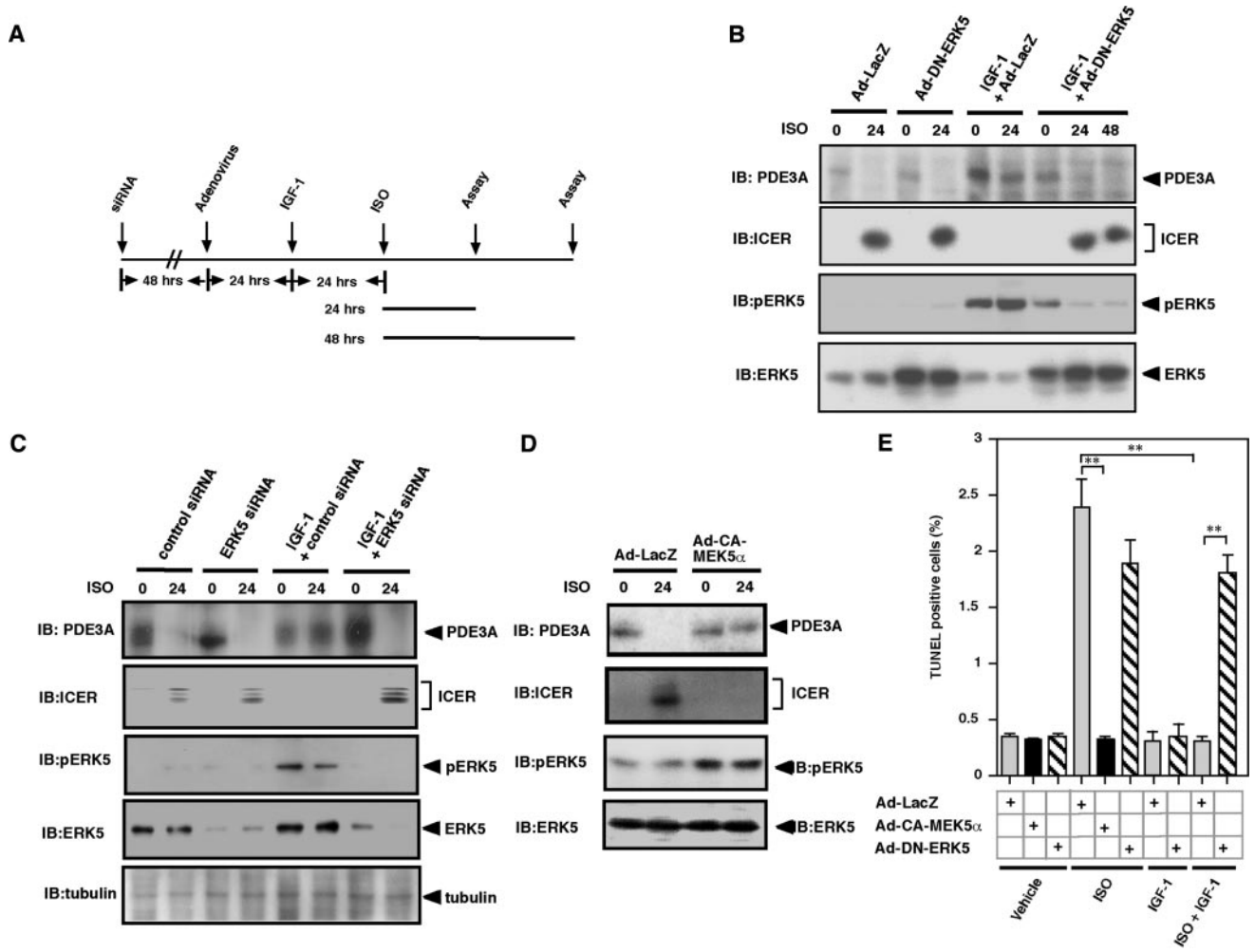


Figure 2. Role of ERK5 activation on IGF-1–mediated inhibition of PDE3A reduction and ICER induction (PDE3A/ICER feedback loop). A, Schematic diagram showing experimental protocol. Cardiomyocytes were transduced with Ad-LacZ, Ad-DN-ERK5, or Ad-DN-MEK1 at 50 multiplicities of infection for 24 hours (B) or ERK5 or control siRNA for 72 hours (C), followed by treatment with or without IGF-1 (20 ng/mL) for 24 hours and then stimulated with vehicle or ISO for 24 to 48 hours as indicated. Expression of PDE3A, ICER, and ERK5 activation were detected by Western blotting. B through D, Critical role of ERK5 activation on IGF-1–mediated inhibition on PDE3A/ICER feedback loop. B and C, Cardiomyocytes were transduced with Ad-LacZ or Ad-DN-ERK5, or ERK5 or control siRNA, followed by IGF-1 and ISO treatment, as described for A. D, Cardiomyocytes were transduced with Ad-LacZ or Ad-CA-MEK5 α , followed by ISO treatment for 24 hours. E, Role of ERK5 activation on IGF-1–mediated inhibition on ISO-induced apoptosis. Cardiomyocytes were transduced with Ad-LacZ, Ad-CA-MEK5 α , or Ad-DN-ERK5 at 50 multiplicities of infection for 24 hours, followed by treatment with or without IGF-1 (20 ng/mL) for 24 hours, and then stimulated with vehicle or ISO for 48 hours. IGF-1–induced ERK5 activation was critical for inhibiting ISO-mediated apoptosis. Data represent mean of 3 repeats (mean \pm SD). Similar results were obtained from at least 2 independent experiments. IB indicates immunoblot.

We have reported that ISO induced a sustained downregulation of PDE3A and upregulation of ICER, which are regulated by the PDE3A/ICER positive-feedback loop.⁸ The PDE3A/ICER feedback loop is essential for Ang II and ISO-induced apoptosis.⁵ Because IGF-1 stimulates ERK5 activation and inhibits cardiac apoptosis,²¹ we determined whether IGF-1–mediated ERK5 activation can inhibit both sustained reduction of PDE3A expression and induction of ICER following ISO treatment of cardiac myocytes. As shown in Figure 2A and 2B, ISO inhibited PDE3A expression and induced ICER expression after 24 hours of stimulation, and pretreatment with IGF-1 before 24 hours of ISO stimulation abolished ISO-mediated feedback loop of PDE3A reduction and ICER induction. Interestingly, transduction of dominant negative ERK5 (Ad-DN-ERK5) completely abol-

ished the inhibitory effect of IGF-1 on ISO-mediated PDE3A reduction and ICER induction (Figure 2B). To confirm the role of endogenous ERK5 activation, ERK5 expression was reduced by ERK5 small interfering RNA (siRNA) (Figure 2C). We found that the reduction of ERK5 by ERK5 siRNA but not control siRNA abolished the inhibitory effect of IGF-1 on ISO-mediated reduction of PDE3A, indicating the importance of endogenous ERK5 in regulation of the PDE3A/ICER feedback loop.

To detect whether activation of ERK5 can inhibit the ISO-mediated PDE3A/ICER feedback loop, we transduced cardiomyocytes with an adenovirus containing CA-MEK5 α . As shown in Figure 2D, ERK5 activation by CA-MEK5 α significantly inhibited ISO-mediated PDE3A reduction and ICER induction. These data indicate that ERK5 plays a

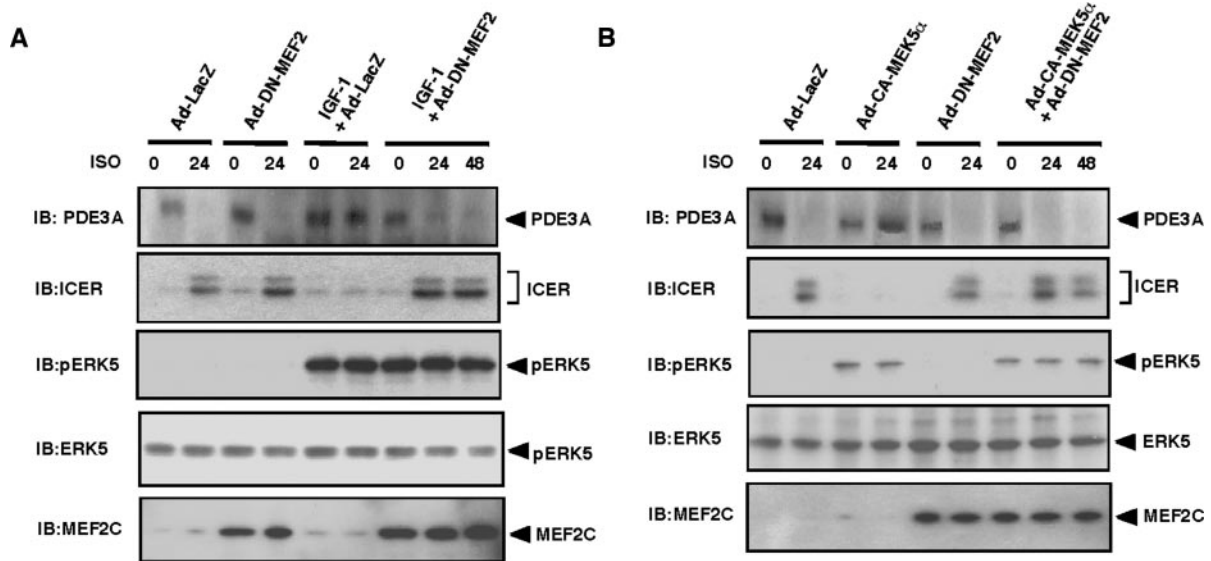


Figure 3. Critical role of MEF2 activation on IGF-1-mediated inhibition on PDE3A/ICER feedback loop. Cardiomyocytes were transduced with Ad-LacZ, Ad-DN-MEF2 (A, B), or Ad-CA-MEK5 α (B), followed by treatment with or without IGF-1 (20 ng/mL) for 24 hours (A), as described in Figure 2A, and then stimulated with vehicle or ISO for 24 to 48 hours as indicated. Expression of PDE3A, ICER, and ERK5 activation were detected by Western blotting. IB indicates immunoblot.

critical role in the inhibitory effect of IGF-1 on the ISO-mediated PDE3A/ICER positive-feedback loop.

Role of ERK5 Activation in Mediating the IGF-1 Antiapoptotic Effect

Because persistent expression of ICER causes myocyte apoptosis and ERK5 activation inhibits ISO-mediated PDE3A reduction and ICER induction, we determined the role of IGF-1-mediated ERK5 activation in cardiomyocyte apoptosis. As shown in Figure 2E, ISO stimulation of 48 hours significantly increased cardiomyocyte apoptosis determined by TUNEL staining, which is consistent with observations reported previously.⁵ The role of ERK5 activation in ISO-induced apoptosis was determined by overexpressing CA-MEK5 α in cardiomyocytes. As shown in Figure 2E, CA-MEK5 α blocked ISO-induced apoptosis, suggesting the critical role of ERK5 activation in regulating cardiomyocyte apoptosis.

To determine the role of ERK5 in the IGF-1-mediated inhibitory effect on apoptosis, we transduced Ad-LacZ or Ad-DN-ERK5 into cardiomyocytes. Twenty four hours after transduction, we pretreated the cells with vehicle or IGF-1. Twenty-four hours later, the cells were stimulated by ISO for 48 hours and cardiomyocyte apoptosis was measured. As shown in Figure 2E, we found that pretreatment of cells by IGF-1 completely inhibited ISO-induced apoptosis. However, the antiapoptotic effect of IGF-1 was completely inhibited by Ad-DN-ERK5, indicating that ERK5 is critical in mediating the IGF-1 antiapoptotic effect in cardiomyocytes.

ERK5/MEF2 Activation Is Critical for the IGF-1-Mediated Inhibition of PDE3A/ICER Feedback Loop

It is well established that MEF2 is an important downstream substrate for ERK5 kinase.³³ Therefore, we investigated the role of MEF2 on the IGF-1-mediated inhibitory effect on PDE3A/ICER feedback loop. First, we transduced cardio-

myocytes with an adenovirus containing a dominant negative form of MEF2C (R3T mutant, a mutation in the MADS box of MEF2C that eliminates DNA binding without affecting dimerization) (Ad-DN-MEF2) or Ad-LacZ as a control. The dominant negative form of R3T MEF2C mutant can dimerize with endogenous MEF2 proteins including MEF2A and MEF2C and inhibit their activities.³⁴ After 24 hours of transduction, cardiomyocytes were stimulated with IGF-1 or vehicle followed by ISO or vehicle stimulation as shown in Figure 2A. As shown in Figure 3A, IGF-1 inhibited ISO-mediated PDE3A reduction and ICER induction, and we found that Ad-DN-MEF2 transduction abolished this inhibitory effect of IGF-1 on PDE3A reduction and ICER induction. To further confirm the importance of MEF2 activation, we cotransduced Ad-CA-MEK5 α and Ad-DN-MEF2, and, after 24 hours of transduction, we stimulated the cells with ISO. As shown in Figure 3B, activation of ERK5 inhibited ISO-mediated PDE3A reduction and ICER induction. Transduction of Ad-DN-MEF2 did not inhibit Ad-CA-MEK5 α -mediated ERK5 activation as expected (Figure 3B, third panel from the bottom). Transduction with Ad-DN-MEF2 abolished the inhibitory effect of IGF-1 on ISO-mediated PDE3A downregulation and ICER upregulation, suggesting a critical role for MEF2 activation in IGF-1/ERK5-mediated inhibition on PDE3A/ICER feedback loop.

Role of ERK5 in the Regulation of ICER Protein Stability, Which Inhibits ERK5-Mediated Antiapoptotic Effect and PDE3A Expression

In this study, we found that activation of ERK5 can induce PDE3A expression and inhibit ICER induction. Previously, we have reported that overexpression of ICER inhibits PDE3A expression and forms a PDE3A/ICER feedback loop.⁸ The expression level of ICER is regulated by CREB-dependent ICER gene transcription as well as proteasome-dependent ICER protein ubiquitination and degradation.³⁵

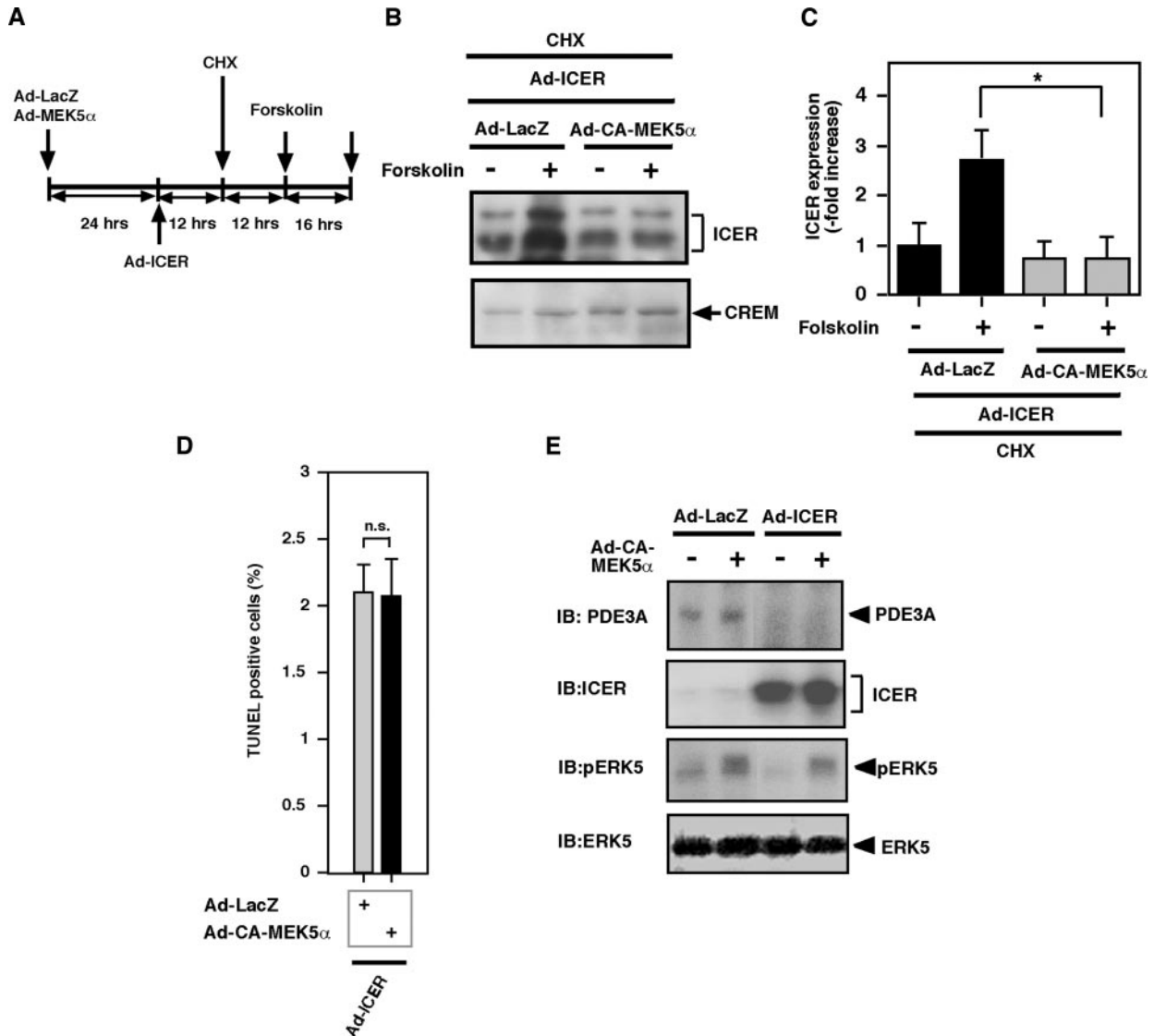


Figure 4. Role of ERK5 activation on ICER stability and PDE3A/ICER feedback loop regulation. **A**, Schematic diagram showing experimental protocol. Cardiomyocytes were transduced with Ad-LacZ or Ad-CA-MEK5 α for 24 hours and then transduced with Ad-ICER. Twelve hours after Ad-ICER transduction, cells were pretreated with cycloheximide, then stimulated with forskolin (10 μ mol/L) or vehicle for 16 hours. **B**, Western blotting showing ICER and cAMP-responsive element modulator (CREM) protein expression in cardiomyocytes treated with the above protocol. **C**, The intensity of the band representing ICER was normalized to the intensity of Ad-LacZ and vehicle-treated control samples, which was designated as 1. Data represent mean \pm SD of 3 samples. * P <0.05. **D**, Effect of ERK5 activation on ICER-mediated apoptosis. Cardiomyocytes were transduced with Ad-LacZ, Ad-CA-MEK5 α , or Ad-ICER at 50 multiplicities of infection for 24 hours. ERK5 activation could not reverse ICER-mediated apoptosis. Data represent mean of 3 repeats (mean \pm SD). Similar results were obtained from at least 2 independent experiments. **E**, Cardiomyocytes were transduced with Ad-LacZ, Ad-ICER, or Ad-CA-MEK5 α . After 24 hours of transduction, expression of PDE3A, ICER, and ERK5 activation were detected by Western blotting. IB indicates immunoblot.

We have found the critical role of PKA activation in stabilizing the ICER protein.⁸ However, the role of ERK5 activation on ICER protein stability remains unknown. To determine the role of ERK5 activation on forskolin-mediated ICER protein stability. As shown in Figure 4A, cardiomyocytes were transduced with Ad-LacZ or Ad-CA-MEK5 α for 24 hours and then transduced with Ad-ICER for 12 hours (this time allowing a sufficient expression of ICER; data not shown). After Ad-ICER transduction, cells were treated with cycloheximide for 12 hours and then stimulated with forskolin for 16 hours. As shown in Figure 4B and 4C,

in the presence of lacZ, forskolin increased ICER expression, although cells were pretreated with cycloheximide. ICER was induced exogenously by Ad-ICER transduction and forskolin-mediated ICER induction was investigated under the treatment with the protein synthesis inhibitor cycloheximide. Therefore, the increase of ICER by forskolin was solely attributable to ICER protein stabilization but not attributable to the regulation of transcriptional machinery or RNA stability, as we described previously.⁸ CA-MEK5 α significantly decreased forskolin-mediated ICER stabilization in both cardiomyocytes and CHO cells (Figure 4B and 4C), indicating that ERK5 plays an important role in destabilizing the ICER

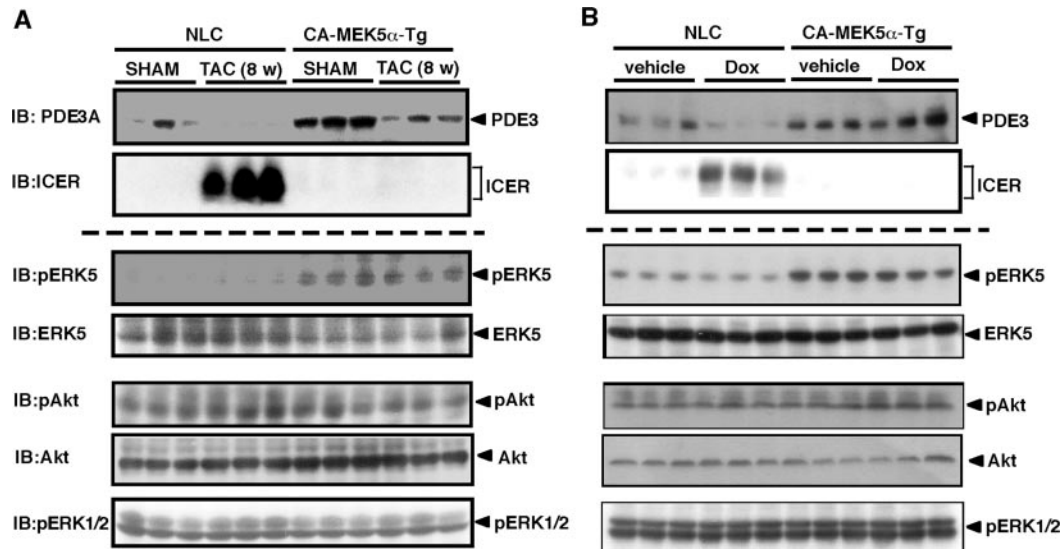


Figure 5. Pressure overload and Dox-mediated PDE3A reduction and ICER induction in NLC and CA-MEK5 α -Tg mice. A, Western blot showing PDE3A and ICER expression and ERK5, Akt, and ERK1/2 kinase activity in sham and 8-week TAC mouse hearts in NLC and CA-MEK5 α -Tg mice. B, Western blot showing PDE3A and ICER expression and ERK5, Akt, and ERK1/2 kinase activity in vehicle and Dox-treated (5 days) hearts in NLC and CA-MEK5 α -Tg mice. IB indicates immunoblot.

protein, which is opposite of the effect of PKA activation on ICER stability. The exact mechanism of ICER stabilization induced by PKA remains unclear. It is possible that ERK5 activation inhibits the process of PKA-mediated ICER stabilization. However, further investigation is required to address this question.

Although ERK5 can regulate ICER destabilization, the involvement of ICER as a downstream effector of ERK5 activation during the ERK5-mediated antiapoptotic effect and PDE3A expression remains unclear. As shown in Figure 2D, we found that activation of ERK5 could inhibit ISO-mediated ICER induction. To determine the contribution of ERK5-mediated ICER reduction to the antiapoptotic effect, we cotransduced cardiomyocytes with Ad-LacZ or Ad-CA-MEK5 α with Ad-ICER and assessed apoptosis. In agreement with our previous reports,⁵ we found that apoptosis was evoked by ICER overexpression (Figure 4D). However, Ad-CA-MEK5 α transduction could not inhibit apoptosis induced by ICER overexpression (Figure 4D). Furthermore, we found that activation of ERK5 by CA-MEK5 α could not restore PDE3A reduction by ICER overexpression (Figure 4E), suggesting that the reduction of ICER and induction of PDE3A is a critical downstream event mediated by ERK5 activation, which is the mechanism for the observed ERK5 antiapoptotic effect in cardiomyocytes.

Pressure Overload and Doxorubicin-Mediated Reduction of PDE3A and ICER Induction Were Inhibited in the CA-MEK5 α -Tg Heart

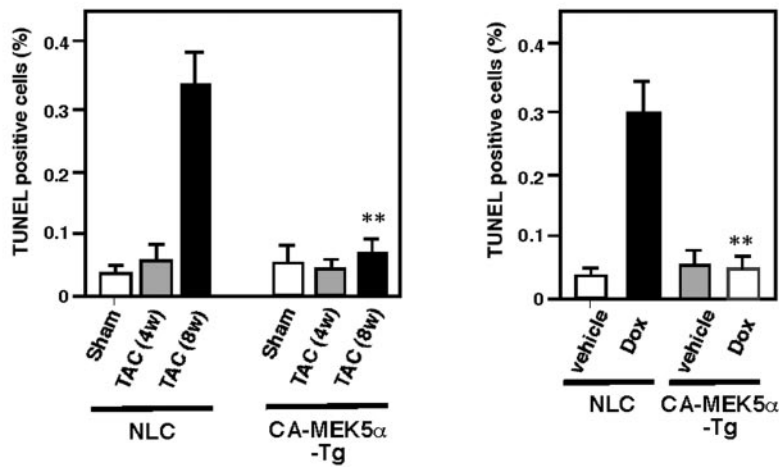
Previously, we found that PDE3A reduction and subsequent ICER induction is critical for ISO-mediated apoptosis and that pressure overload by TAC decreased PDE3A expression and increased ICER expression.⁵ Therefore, we next examined whether ERK5 activation by CA-MEK5 α prevents pressure overload-induced PDE3A reduction and subsequent ICER induction. As shown in Figure 5A

(functional data in Table I of the online data supplement; Figure 7), pressure overload inhibited PDE3A expression and significantly increased ICER induction in nontransgenic littermate control (NLC) mice. In contrast, PDE3A expression was preserved in CA-MEK5 α -Tg mice, and we did not find any significant increase of ICER expression induced by pressure overload in CA-MEK5 α -Tg mice. There were no significant differences in Akt and ERK1/2 activation among NLC and CA-MEK5 α -Tg mice. To determine the general role of ERK5 activation on PDE3A/ICER feedback loop in heart failure, we also examined PDE3A and ICER expression in a Dox-mediated mouse heart failure model. As shown in Figure 5B, we also found a significant reduction of PDE3A and ICER induction after 5 days of Dox treatment, which were completely abolished in CA-MEK5 α -Tg mice. No significant differences of Akt and ERK1/2 activation among NLC and CA-MEK5 α -Tg mice were observed in this model. These data support the critical role of ERK5 activation in regulating the PDE3A/ICER feedback loop in a heart failure model.

Cardiac Expression of CA-MEK5 α Reduces Apoptosis Induced by Pressure Overload or Dox

Apoptosis is believed to be important for the transition from hypertrophy to heart failure.⁴ It has been reported that although apoptotic cells are not detectable in normal mouse LV, they are widespread in the LV after 8-week TAC.³⁶ Similarly, we found very few TUNEL-positive cells in control and 4-week TAC mouse hearts. In contrast, in hearts after 8-weeks of TAC, the number of in situ TUNEL-positive cells was increased to $0.33 \pm 0.06\%$ (Figure 6A, left). We found significantly decreased TUNEL-positive cells in CA-MEK5 α -Tg mice after 8 weeks of TAC (Figure 6A, left). In a Dox-mediated heart failure model, we also found that Dox-induced apoptosis was significantly inhibited in CA-MEK5 α -Tg mice, supporting the general antiapoptotic role of

A TUNEL



B Cleaved Caspase-3

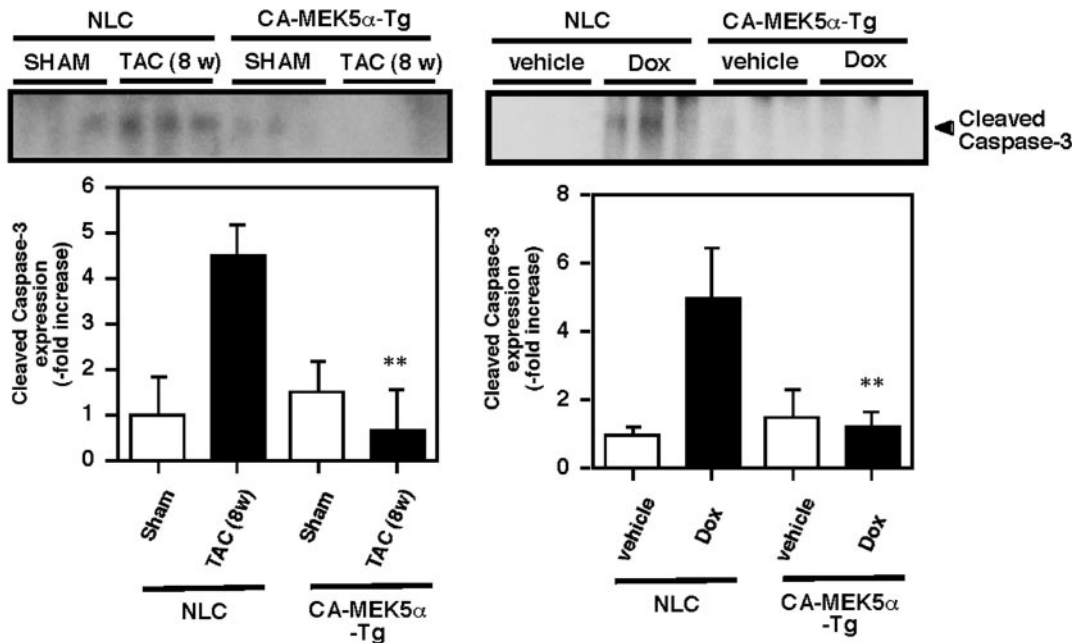


Figure 6. Pressure overload- and Dox-induced myocardial apoptosis in NLC and CA-MEK5-Tg mice. A, Pressure overload-mediated (left) or Dox-mediated (right) percentage of TUNEL-positive nuclei in NLC and CA-MEK5 α -Tg mice. B, Pressure overload-mediated (left) or Dox-mediated (right) cleaved caspase-3 expression in NLC and CA-MEK5 α -Tg mice. The intensity of the band representing cleaved caspase-3 was normalized to the intensity of 1 control sample that was designated as 1. Data represent mean \pm SD of 3 samples. ** $P < 0.01$ compared with NLC sham (left) or vehicle treatment (right).

ERK5 activation in heart failure (Figure 6A, right). To confirm these data, we further evaluated the process of apoptosis in vivo by examining cleaved caspase-3 expression. As shown in Figure 6B, cleaved caspase-3 expression was significantly increased in both TAC and Dox-mediated heart failure models, and activation of ERK5 by CA-MEK5 α abolished cleaved caspase-3 expression in these heart failure models. Of note, these same samples did not have any significant differences in ERK1/2 and Akt expression or activation (Figure 5). These data further support the antiapoptotic role of ERK5 activation during heart failure.

CA-MEK5 α -Mediated ERK5 Activation Prevents Dox or TAC-Mediated Cardiac Dysfunction

Because we found a critical inhibitory role for ERK5 activation on PDE3A and the ICER feedback loop and apoptosis, we next investigated whether ERK5 activation can inhibit the development of heart failure in vivo. Figure 7A shows a representative echocardiogram after vehicle or Dox administration in NLC and CA-MEK5 α -Tg mice. As shown in supplemental Table I, left ventricular (LV) end-systolic diameter was markedly increased in NLC mice after Dox injection, but significantly smaller in Dox-injected CA-

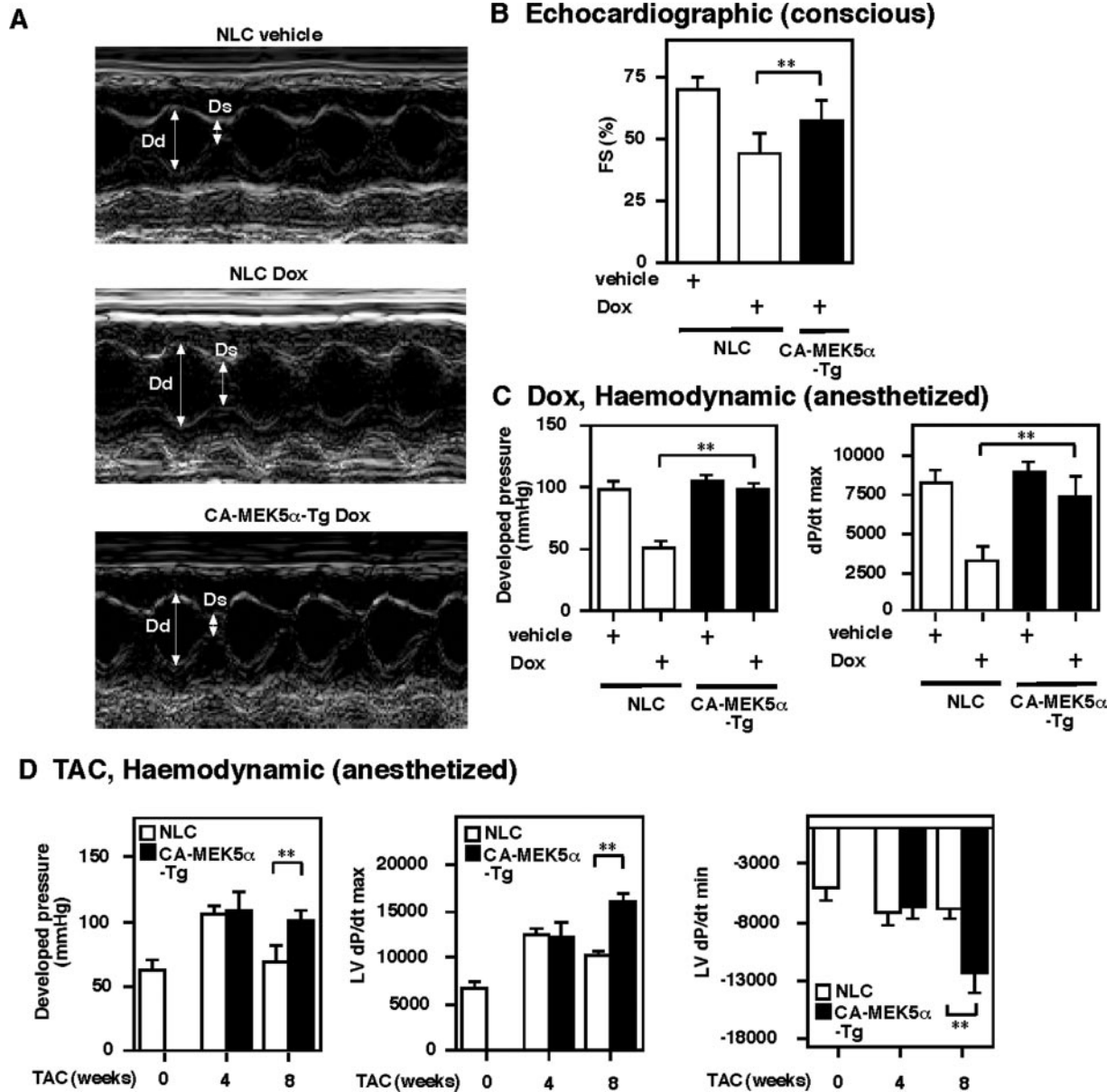


Figure 7. Cardioprotective role of ERK5 activation in Dox- and TAC-mediated heart failure model. A, Representative M-mode echocardiogram of NLC and CA-MEK5 α -Tg mice treated with vehicle or Dox. Dd indicates diameter of diastole; Ds, diameter of systolic. B, Percentage of fractional shortening (FS) in NLC and CA-MEK5 α -Tg mice after vehicle or Dox treatment. C and D, Hemodynamic measurements in NLC and CA-MEK5 α -Tg mice after Dox treatment (C) or TAC (D). All data are expressed as mean \pm SD. ***P*<0.01.

MEK5 α -Tg mice compared with Dox-injected NLC mice. Fractional shortening was also better preserved in Dox-injected CA-MEK5 α mice than Dox-NLC (supplemental Table I; Figure 7B). In agreement with our previous reports, there was no difference in basal cardiac function between NLC and CA-MEK5 α -Tg mice.²⁰ After Dox injection, we observed a significant decrease of developed pressure and dP/dt_{max} in NLC mice. In contrast, Dox-injected CA-MEK5 α -Tg mice had normal developed pressure and dP/dt_{max} (Figure 7C, supplemental Table II).

To further determine the role of ERK5 activation in heart failure, we performed TAC in both NLC and CA-MEK5 α -Tg mice. As shown in supplemental Table III, LV weight was increased by 37% in 4-week TAC mice and by 89% in 8-week TAC mice compared with age-matched controls. At

these stages of hypertrophy, there were no differences between CA-MEK5 α -Tg and NLC in body weight, LV weight, and LV/body weight ratio. In the 4-week TAC mice, absolute LV systolic pressure was elevated in both CA-MEK5 α -Tg and NLC mice. Interestingly, in the 8-week TAC NLC mice, absolute LV systolic pressure was lower than in the 4-week TAC mice despite a greater magnitude of hypertrophy. However, we found that in CA-MEK5 α -Tg mice, absolute LV systolic pressure did not decrease compared with 4-week TAC CA-MEK5 α -Tg mice. Furthermore, in the 8-week TAC mice, LV systolic developed pressure per gram (LV weight) was significantly depressed in NLC mice compared with the sham-operation group. In contrast, LV systolic developed pressure per gram was significantly higher in the 8-week TAC CA-MEK5 α -Tg mice. Furthermore, we found that the

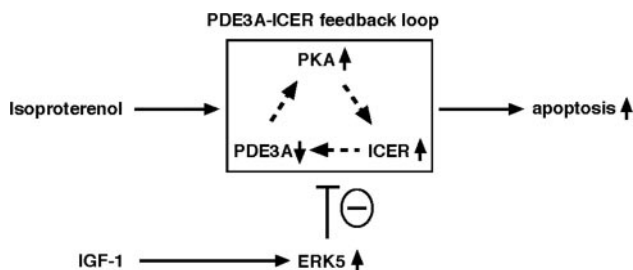


Figure 8. Scheme of ERK5/MEF2 activation and PDE3A/ICER feedback loop. ISO induced sustained downregulation of PDE3A expression and upregulation of ICER, which is regulated by PDE3A/ICER-positive feedback loop. IGF-1-induced ERK5 activation leads to PDE3A expression caused by MEF2-dependent PDE3A promoter activation and inhibits induction of PDE3A/ICER autoregulatory positive-feedback loop, which plays a key role in cardiomyocyte apoptosis.

absolute values for developed pressure, dP/dt_{max} , and dP/dt_{min} in the 8-week TAC CA-MEK5 α -Tg mice were greater than those in the 8-week TAC NLC mice (Figure 7D, and supplemental Table III). Heart rate was similar between the groups (supplemental Table III). These data suggest the presence of depressed systolic performance in the 8-week TAC NLC mice compared with the 4-week TAC NLC mice, and ERK5 activation induced by CA-MEK5 α -Tg inhibits the development of cardiac dysfunction by pressure overload.

Discussion

We have previously reported that the PDE3A expression and activity was significantly decreased during the transition from compensated hypertrophy to decompensated heart failure.⁵ Because PDE3A expression and activity as well as subsequent inhibition of ICER expression are crucial for inhibiting apoptosis, the reduction of PDE3A may have a significant impact on pressure overload- and Dox-mediated apoptosis and subsequent cardiac dysfunction. In fact, we found that activation of ERK5 significantly inhibited PDE3A reduction as well as ICER induction and subsequent apoptosis and partially normalized cardiac function. These data suggest an important role for the PDE3A/ICER (Figure 8) feedback loop in the transition from compensated hypertrophy to decompensated heart failure.

To our knowledge this is the first report to show that IGF-1 activates ERK5 in cardiomyocytes. IGF-1/ERK5 inhibits the ISO-mediated PDE3A/ICER feedback loop and plays a critical role in regulating ISO-induced apoptosis. Based on both DN-ERK5 and ERK5 siRNA results (Figure 2), IGF-1-mediated ERK5 activation is definitely “necessary” to inhibit ISO-mediated reduction of PDE3A. Furthermore, we found that the antiapoptotic effect of ERK5 activation was lost by overexpression of ICER by Ad-ICER transduction, suggesting that ERK5 activation acts upstream of the PDE3A/ICER feedback loop, consistent with the IGF-1 results reported in Figures 2 and 3. The ability of IGF-1 to inhibit cell death by both apoptosis and necrosis has previously been shown in experimental ischemic cardiomyopathy in vivo.³⁷ In addition, Welch et al have reported that cardiac specific IGF-1 expression prevented apoptosis and improved hemodynamics in tropomodulin-overexpressing transgenic mice.²⁶ Because

IGF-1 activates phosphatidylinositol 3-kinase, which in turn phosphorylates the downstream effector molecule Akt and inhibits apoptosis, Akt has been the main focus of the antiapoptotic effect of IGF-1.³¹ However, the role of Akt in heart failure becomes complicated, because overexpression of Akt in the heart may become maladaptive.^{29,30} Although future studies will be required for the dependency of phosphatidylinositol 3-kinase on ERK5 activation, our data suggest a critical role for ERK5 activation in relaying the IGF-1-mediated cardioprotective effect. Furthermore, because we found the possible involvement of other kinases in regulating PDE3A expression (C.Y., and J.-i.A., unpublished data, 2006), we assume that activation of ERK5 kinase alone may not be “sufficient” to increase PDE3 expression. Therefore, CA-MEK5 α did not change the basal expression of PDE3A (Figure 2D) but only prevented its loss and induction of ICER after ISO treatment (Figure 2B and 2C). In contrast, we observed increased PDE3 expression in CA-MEK5 α -Tg mice in vivo (Figure 5A), suggesting that ERK5 may be among the critical factors to regulate PDE3 expression in vivo. To better understand the role of ERK5 activation in IGF-1 signaling and PDE3A expression, further studies would be required using IGF-1 transgenic mice with knock out of ERK5.

An expanded Discussion section is available in the online data supplement

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Disclosures

None.

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Activation of Extracellular Signal-Regulated Kinase 5 Reduces Cardiac Apoptosis and Dysfunction via Inhibition of a Phosphodiesterase 3A/Inducible cAMP Early Repressor Feedback Loop

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Expanded methods***Reagents and Adenovirus vectors:***

Adenovirus harboring antisense ICER-II γ (Ad-ICER-AS) was a kind gift from Dr. Sadoshima¹. Although there are four isoforms of ICER (ICER-I, ICER-I γ , ICER-II, ICER-II γ), the function of each isoform is indistinguishable and Ad-ICER-AS inhibits expression of all ICER isoforms¹. 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. Molkenin (Cincinnati Children's Hospital Medical Center), respectively. Adenovirus expressing constitutively active form of MEK5 α (Ad-CA-MEK5 α), 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 previously². 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 °C. 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, 10 μ M 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 described³. 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 Na₃VO₄, 10 μ g/ml aprotinin, 5 μ g/ml pepstatin, 20 μ g/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 previously⁴. ICER protein corresponds to a group of four proteins of approximately 16-18 kDa and 13-14 kDa⁵.

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 previously⁶. 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 previously⁶. Briefly, 10-12 week-old FVB male mice (\approx 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 previously⁶. 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 catheter (Millar Instruments) was inserted into the left atrium, advanced through the mitral valve, and secured in the LV as described previously⁶. 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 Louis MO). Protein concentration was determined with the

Bradford protein assay (Bio-Rad). Protein (30 μ g) 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 MEK5 α (CA-MEK5 α , S311D/T315D) cDNA was subcloned into a pBluescript-based Tg vector between the 5.5-kb murine- α MHC promoter and 250-bp SV-40 polyadenylation sequences, and we generated three different lines of CA-MEK5 α -Tg and all three lines showed a similar phenotype and MEK5 α expression as we reported previously⁷. Both non-transgenic littermate control (NLC) and CA-MEK5 α 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 2×10^5 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 \pm 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-MEK5 α 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 destabilization (Fig.4) will require further investigation. Since it has been reported that ERK1/2 targets ICER to ubiquitin-mediated destruction⁴, 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 loop⁹, 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 apoptosis⁹. 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 β 1-adrenergic receptor expression¹⁰, 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 β 1-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 MEK5 α and MEK5 β

The importance of ERK5 activation in pressure overload- and Dox-induced cardiomyocyte apoptosis was also provided *in vivo* by using CA-MEK5 α -Tg mice. Nicol *et al.*¹¹ reported that cardiac-specific expression of activated MEK5 β 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 MEK5 α , but Nicol et al. used MEK5 β , and 2) Mouse strain: we used FVB, but Nicol et al. used C3HB6. MEK5 β is 89 amino acids shorter than MEK5 α at the N-terminus (which does not contain PB-1 domain), and we recently reported a novel functional difference in the way full-length MEK5 α and the shorter MEK5 β splice variant regulate ERK5 activity ¹². In fact only MEK5 α activated ERK5 while MEK5 β exhibited a dominant-negative phenotype inhibiting CA-MEK5 α or growth factor-induced activation of ERK5¹². Seyfried et al. reported that MEK5 β , 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 Seyfried 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 (n = 8)	α -Tg
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-matched nontransgenic littermate control mice, LVEDd indicates left ventricle end-diastolic dimension; LVESd, left ventricle end-systolic dimension, %FS, percent fractional 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-matched 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-matched 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.