Effects of MEK5/ERK5 Association on Small Ubiquitin-Related Modification of ERK5: Implications for Diabetic Ventricular Dysfunction After Myocardial Infarction

Tetsuro Shishido,* Chang-Hoon Woo,* Bo Ding,* Carolyn McClain, Carlos A. Molina, Chen Yan, Jay Yang, Jun-ichi' Abe

Abstract—Diabetes mellitus (DM) contributes to the exacerbation of left ventricle (LV) dysfunction after myocardial infarction (MI). Activation of ERK5, an atypical mitogen activated protein kinase with transcriptional activity, inhibits apoptosis and LV dysfunction after doxorubicin treatment. SUMOylation has been proposed as a negative regulator of various transcription factors. In the current study, we investigated the role of ERK5-SUMOylation in ERK5 transcriptional activity as well as on DM-mediated exacerbation of LV dysfunction and apoptosis after MI. ERK5 wild-type transcriptional activity was inhibited by Ubc9 (SUMO E2 conjugase) or PIAS1 (E3 ligase), but not in the ERK5-SUMOylation-site defective mutant (K6R/K22R). H₂O₂ and high glucose, 2 well-known mediators of diabetes, induced ERK5-SUMOylation, and the K6R/K22R mutant, dominant negative form of Ubc9, and siRNA-PIAS1 reversed H₂O₂-mediated reduction of ERK5 transcriptional activity in cardiomyocytes, indicating the presence of SUMOylation-dependent ERK5 transcriptional repression. Constitutively active form of MEK5α (CA-MEK5α) inhibited ERK5-SUMOylation independent of kinase activity, but dependent on MEK5-ERK5 association. To investigate the pathological role of ERK5-SUMOylation in DM mice after MI, we used cardiac specific CA-MEK5α transgenic mice (CA-MEK5α-Tg). MI was induced in streptozotocin (STZ)-injected (DM+MI group) or vehicle-injected mice (MI group) by ligating the left coronary artery. The ERK5-SUMOylation was increased in the DM+MI, but not in the MI group. ERK5-SUMOylation, the exacerbation of LV dysfunction, and the number of TUNEL-positive cells in DM+MI was significantly inhibited in CA-MEK5α-Tg mice. Of note, we could not detect any difference of cardiac function after MI in non-diabetic CA-MEK5α-Tg and non-transgenic littermate control mice. These results demonstrated that ERK5 transcriptional activity is subject to downregulation by diabetes-dependent SUMOylation, which resulted in a proapoptotic condition contributing to poor post-MI LV function. (Circ Res. 2008;102:1416-1425.)

Key Words: ERK5 | SUMOylation | diabetes | myocardial infarction | apoptosis

Diabetes is an independent risk factor for both mortality and morbidity after myocardial infarction (MI). A number of clinical studies have shown that the post-MI left ventricular (LV) function is significantly worse in diabetic compared with nondiabetic patients. However, what is lacking is a plausible relationship between diabetes and any of the known regulators of myocyte apoptosis known to play a significant role in the post-MI cardiac dysfunction. Previously, we have demonstrated that downregulation of phosphodiesterase 3A (PDE3A) is associated with apoptosis and induction of inducible cAMP early repressor (ICER), a proapoptotic transcriptional repressor, providing a mechanistic framework for how angiotensin II (Ang II) regulates myocyte apoptosis. Sustained elevation of ICER favors apoptosis through inhibition of cAMP response element binding protein (CREB)-mediated transcription and down-regulation of Bcl-2. Interactions between PDE3A and ICER constitute an autoregulatory positive feedback loop (PDE3A-ICER feedback loop) likely to determine the fate of injured myocytes. ERK5, an atypical mitogen activated protein kinase with transcriptional activity, negatively regulates the executor PDE3A-ICER feedback loop and subsequent apoptosis. Our recent data indicated that ERK5 transcriptional activity itself is subjected to downregulation by reactive oxygen species (ROS) and advanced glycosylation endproducts (AGE)-
dependent small ubiquitin-related modification (SUMO), and inhibits KLF2 and eNOS expression in endothelial cells.\textsuperscript{8} ERK5-SUMOylation at the NH2-terminal region (K6 and K22) significantly inhibits COOH-terminal ERK5 transcriptional activity.\textsuperscript{8} Posttranslational modification by SUMO commonly regulates the assembly and disassembly of protein complexes, protein localization, stability, and function.\textsuperscript{9} SUMOylation is known to be highly substoichiometric because it often generates intermediates that result in new protein interactions or conformational states that persist even after SUMO removal.\textsuperscript{9}

In the current study, we showed that ERK5 is 1 of the major targets of SUMOylation in diabetic hearts. We found that SUMOylation-dependent ERK5 transcriptional repression induced by ROS and high glucose. Constitutively active form of MEK5α (CA-MEK5α) inhibited ERK5-SUMOylation independent of its kinase activity but dependent on MEK5-ERK5 association. Diabetic hearts demonstrating exacerbation of LV dysfunction after myocardial infarction (MI) were accompanied by enhanced ERK5-SUMOylation. The inhibition of ERK5-SUMOylation by CA-MEK5α significantly improved the cardiac function after MI in diabetic mice but not in nondiabetic mice. These data suggested the importance of ERK5-SUMOylation on ROS-mediated ERK5 transcriptional repression, which contributes to poor cardiac function after MI in diabetes.

Materials and Methods
Details on reagents, antibodies, plasmid and adenovirus vectors construction, cell culture, mammalian 1-hybrid analysis and transfection of cells, immunoprecipitation (SUMOylation assay) and Western blot analysis, transfection of the PIAS1 siRNA, analysis of apoptosis, animal models, streptozotocin (STZ) injections, coronary ligation surgery, echocardiographic analysis and in vivo hemodynamic measurements, and statistical analysis are also provided in the online Data Supplement (available online at http://circres.ahajournals.org).

Results
Streptozotocin (STZ)-Induced Hyperglycemia Exacerabtes LV Dysfunction and Failure After an Experimental MI
Diabetes adversely affects LV dysfunction after MI and leads to a higher incidence of heart failure, but the exact mechanism of diabetes-mediated exacerbation of LV dysfunction remains largely unknown. First, we established a diabetic mouse model in our laboratory and demonstrated the development of heart failure after MI as previously described.\textsuperscript{10}
Four groups of mice were studied. Diabetes mellitus (DM) was induced in male FVB mice (5 to 8 weeks old and 25 to 35 g body weight, The Jackson Laboratory, Bar Harbor, ME) by intraperitoneal injection of STZ (200 mg/kg body weight). Tail vein blood glucose samples were measured 6 days after injection to ensure induction of hyperglycemia. As a control, vehicle (0.1 mol/L citrate buffer, pH 4.5) was injected in another group of mice. At 7 days after injection, MI was induced in STZ-injected (DM/MI group) or vehicle-injected mice (MI group) by ligating the left coronary artery. We have carefully characterized the fasting glucose level after different doses of intraperitoneal injection of STZ in FVB mice and found that one injection of 200 mg/kg body weight STZ resulted in fasting blood glucose (FBG) of around 200 mg/dL at 1 week after injection. Because mice with extremely high glucose level (> 400 mg/dL) did not tolerate the coronary ligation surgery well, we chose a STZ dose that resulted in this moderately high FBG as the diabetic mice model for our study. A sham operation without ligating the coronary artery was also performed in additional groups of STZ-injected mice (DM group) and saline-injected mice (control group). All 4 groups of mice (control, MI, DM, and DM/MI) were followed up for another 1 week (1-week post-MI study). STZ treatment significantly increased random blood sugar (BS) levels 2 weeks after injection (Figure 1A, left panel). Of note, we did not observe any significant body weight difference between the MI and DM/MI groups at 1 week after injection and at the time of surgery (Figure 1A, right panel), and at 1 week after surgery (supplemental Table I). Survival at 1 week after MI was significantly lower in the DM/MI versus MI group (Figure 1B).

Figure 2. ERK5-SUMOylation was increased in DM/MI mice and induced by H$_2$O$_2$ and high glucose in cardiomyocytes. Heart samples from 4 groups of mice (control, MI, DM, and DM/MI) were immunoprecipitated with anti-ERK5 antibody, and Western blot analysis was performed with anti-SUMO2/3 antibody (top). A significant decrease in ERK5 phosphorylation was observed in both MI and DM/MI groups (2nd from top) but with no difference in the amount of total ERK5 (3rd from top). Immunoblot with SUMO2/3 antibody (4th from top) and Ubc9 antibody (bottom) confirmed no significant differences in the general SUMOylation of proteins and the Ubc9 expression between samples. B. Heart samples from control and DM/MI mice without or with preoperative insulin treatment subjected to IP with ERK5 antibody and IB with anti-SUMO2/3 antibody. The preoperative insulin treatment decreased ERK5-SUMOylation (arrow) in the DM/MI group. Cardiomyocytes were treated with H$_2$O$_2$ (C, D) or high glucose and mannitol (E) and probed for SUMOylated ERK5. C, Cells treated for the indicated time were lysed and immunoprecipitated using anti-ERK5 antibody then blotted with anti-SUMO2/3 (top) and ERK5 (bottom) antibodies. D, H$_2$O$_2$-mediated ERK5-SUMOylation was measured at one hour at the indicated concentration of H$_2$O$_2$ (top). No significant difference of ERK5 expression among the samples was observed (bottom). E, After treatment of cardiomyocytes with high glucose or mannitol, cells were lysed and immunoprecipitated with anti-ERK5 antibody and Western blot analysis was performed with anti-SUMO2/3 antibody (top). No significant difference of free forms of SUMO2/3 (middle) and Ubc9 (bottom) expression among the samples was observed. Data are representative of results from experiments performed on at least 3 separate occasions.
We could not detect any difference in LV weight/tibial length (TL) between the MI and DM/H11001 MI groups. But lung weight/TL was increased in the MI group, which was significantly exacerbated in the DM/H11001 MI group (Figure 1C), suggesting a more severe congestive heart failure in the DM/H11001 MI group than MI group. Echocardiography 1 week after ligation showed LV dilatation and dysfunction in the MI group but with both measures exaggerated in the DM/H11001 MI group (Figure 1D and 1E; supplemental Table I).

ERK5-SUMOylation in DM+MI Model

Recently, we have reported the importance of ERK5-SUMOylation on regulating its transcriptional activity.8 H2O2 and advanced glycation end products (AGE), 2 well-known mediators of diabetes, negatively regulated ERK5 transcriptional activity via ERK5-SUMOylation in endothelial cells. Because ERK5 demonstrates a cardio-protective effect,7 we investigated the effect of STZ-mediated hyperglycemia and MI on the ERK5-SUMOylation. As shown in Figure 2, we found a slight increase of ERK5-SUMOylation in both the DM (with sham operation) and MI (without STZ treatment) groups. In contrast, the ERK5-SUMOylation was significantly exaggerated in the DM+MI group. These data suggested the possible role of ERK5-SUMOylation on the exaggerated LV dysfunction and development of heart failure after MI in DM mice. In contrast, a reduction of ERK5 phosphorylation was observed in both MI and DM/H11001 MI groups compared with non-MI control and DM groups. No significant difference in ERK5 phosphorylation was observed in MI versus DM/H11001 MI groups, suggesting that ERK5 phosphorylation and its subsequent kinase activation may not be involved in the exaggerated LV dysfunction after MI in DM mice.

We investigated the possible cardio-toxic effect of STZ, as opposed to hyperglycemia, by administering insulin (60 U/kg subcutaneous b.i.d. NPH human insulin [Humulin N; Eli Lilly]) 1 to 2 days before the coronary ligation surgery to normalize the FBG and determined infarct size, cardiac function, and the biochemical markers such as ERK5-SUMOylation after MI as previously described.13 We demonstrated that preoperative insulin treatment of STZ-injected mice decreased the FBG to 157.6±49.5 mg/dL. Such preoperative insulin-treatment did not result in significant differences in basal cardiac function from nondiabetic MI control.
In addition, we could not detect any increase in ERK5-SUMOylation after insulin-treatment, which was observed in the hyperglycemic DM/H11001 MI mice (Figure 2B). Therefore, we concluded that the exacerbation of cardiac damage and ERK5-SUMOylation in DM/H11001 MI model was attributable to hyperglycemia and not to STZ toxicity.

One of the major concerns of this study is whether the observed differences between the groups can be accounted for by metabolic perturbations (particularly malnutrition and weight loss in the diabetic animals), or by the direct effects of STZ on the myocardium. We carefully examined this issue and found that both MI alone and DM+MI animals had equally low body weights, but DM+MI mice showed greater cardiac damage (Figure 1A; supplemental Table I).

H2O2 Inhibited ERK5 Transcriptional Activity and Induced Apoptosis via ERK5-SUMOylation

Because we found ERK5-SUMOylation in the DM+MI group, and ERK5-SUMOylation could decrease ERK5 transcriptional activity and possibly its cardio-protective effect, we investigated whether H2O2 and high glucose can induce endogenous ERK5-SUMOylation. As shown in Figure 2C and 2D, H2O2 significantly increased ERK5-SUMOylation in a time- and dose-dependent manner. High glucose, but not mannitol (20 mmol/L) as a control, also increased ERK5-SUMOylation (Figure 2E). Previously, we reported that H2O2 and advanced glycation end products (AGE) inhibited ERK5 transcriptional activity via ERK5-SUMOylation in endothelial cells. Because we found that H2O2 and high glucose could induce ERK5-SUMOylation in cardiomyocytes (Figure 2C through 2E), we investigated whether ERK5-SUMOylation was important in the H2O2-mediated reduction of ERK5 transcriptional activity in cardiomyocytes. If SUMOylation of ERK5 underlies the H2O2-mediated reduction of its transcriptional activity, inhibition of SUMOylation by coexpression of the dominant negative form of Ubc9 (DN-Ubc9) should interfere with this reduction in transcriptional activity. As shown in Figure 3A, H2O2 (30 μmol/L) decreased ERK5 transcriptional activity by approximately 56%, but this reduction in the transcriptional activity was less in DN-Ubc9 transfected cardiomyocytes in a DN-Ubc9 dose-dependent manner.

Next, we compared the H2O2 (30 μmol/L)-induced reduction of transcriptional activity in the wild-type and SUMOylation sites-mutant (K6R/K22R) ERK5. As shown in Figure 3B, H2O2-induced reduction of ERK5 transcriptional activity was significantly less in the ERK5-K6R/K22R mutant. To further confirm the involvement of ERK5-SUMOylation in transcriptional regulation, we tested whether deletion of PIAS1 using PIAS1 siRNA could inhibit the H2O2-mediated reduction of ERK5 transcriptional activity. PIAS1 siRNA inhibited PIAS1 expression and H2O2-mediated ERK5-SUMOylation in cardiomyocytes (Figure 3C). Furthermore, PIAS1 siRNA significantly prevented H2O2-mediated reduction of ERK5 transcriptional activity (Figure 3D). Taken together, these data strongly suggested the critical role of ERK5-SUMOylation in H2O2-mediated reduction of ERK5 transcriptional activity.

We have reported previously the importance of ERK5 in the regulation of cardiomyocyte apoptosis. Therefore, we investigated whether ERK5-SUMOylation was involved in the H2O2-mediated apoptosis. As shown in Figure 4, in this model the deletion of PIAS1 significantly inhibited H2O2-mediated apoptosis. To further support the critical role of ERK5-SUMOylation in regulating cardiomyocyte apoptosis, we generated 2 adenovirus vectors expressing the ERK5 wild-type and ERK5 K6R/K22R mutant. Sixteen hours after transduction with adenovirus vector containing Xpress tagged-ERK5 wild-type (Ad-ERK5-WT) or ERK5 K6R/K22R mutant (Ad-ERK5 K6R/K22R), cardiomyocytes were treated with H2O2 (100 μmol/L) for 24 hours, and apoptosis

![Figure 4. Role of PIAS1 in H2O2-mediated cardiomyocyte apoptosis.](image-url)
was measured by TUNEL staining. We found that expression of ERK5-SUMOylation site-mutant significantly inhibited H₂O₂-induced apoptosis (Figure 4C). No difference in the amount of ERK5 wild-type and mutants was confirmed in samples by immunoblotting with anti-Xpress antibody (Figure 4C). Of note, we needed to increase the concentration of H₂O₂ from 30/₉₂₆₂₉ mol/L to 100/₉₂₆₂₉ mol/L in this set of experiments because we could not detect significant increase of TUNEL positive cells in Ad-ERK5 WT tranduced cells with 30/₉₂₆₂₉ mol/L H₂O₂ treatment, probably because of the protective effect of ERK5 wild-type overexpression.

Interaction of MEK5α With ERK5 Inhibits ERK5-SUMOylation but not MEK5α-Mediated ERK5 Kinase Activation

It has been reported that ERK5 activation inhibits MEF2-SUMOylation via MEF2-Ser179 phosphorylation and increases MEF2 transcriptional activity. Therefore, we investigated whether MEK5 activation induced by CA-MEK5α can similarly inhibit Ubc9 and SUMO3-mediated ERK5-SUMOylation. As shown in Figure 5A, CA-MEK5α significantly inhibited CA-MEK5α-mediated reduction of ERK5-SUMOylation. Cells were cotransfected with HA-SUMO3, Ubc9, DN-Ubc9, and Xp-tagged ERK5 (WT or ERK5-AEF kinase dead mutant) with CA-MEK5α or control cDNA as indicated and performed ERK5-SUMOylation assay as described above. No difference in the amount of ERK5 wild type and mutants (2nd from the top), CA-MEK5α (3rd from top), or Ubc9 (4th from top) was observed in samples by Western blot analysis. The expression of free forms of SUMO2/3 and SUMO conjugated 30 to 280 kDa proteins were detected by immunoblotting with anti-SUMO2/3 antibody (bottom).

Figure 5. Association of MEK5 with ERK5, but not ERK5 kinase activation and phosphorylation, inhibits ERK5-SUMOylation. A, CA-MEK5α-mediated reduction of ERK5-SUMOylation was observed in both ERK5 wild type and kinase dead TEY motif mutant (ERK5-AEF). CHO cells were cotransfected with HA-SUMO3, Ubc9, DN-Ubc9, and Xp-tagged ERK5 (WT or ERK5-AEF kinase dead mutant) with CA-MEK5α or control cDNA as indicated and performed ERK5-SUMOylation assay as described above. No difference in the amount of ERK5 wild type and mutants (2nd from the top), CA-MEK5α (3rd from top), or Ubc9 (4th from top) was observed in samples by Western blot analysis. B, Both MEK5 binding site truncated mutants of ERK5 (ERK5-Δaa72–139, and ERK5-Δaa90–130) did not associate with MEK5α. Cells were cotransfected with Xp-tagged ERK5 (WT or ERK5-Δaa72–139, and ERK5-Δaa90–130) with HA tagged CA-MEK5α or control cDNA as indicated. After 24 hours of transfection, whole cell extract was immunoprecipitated with anti-HA antibody and Western blot analysis was performed with anti-HA (upper) or ERK5 (lower) antibody. No difference in the amount of ERK5 wild type and mutants or HA-CA-MEK5α was observed in samples by Western blot analysis. C, Both MEK5 binding site truncated mutants of ERK5 (ERK5-Δaa72–139, and ERK5-Δaa90–130) inhibited CA-MEK5α-mediated reduction of ERK5-SUMOylation. Cells were cotransfected with HA-SUMO3, Ubc9, DN-Ubc9, and Xp-tagged ERK5 (WT or ERK5-Δaa72–139, and ERK5-Δaa90–130) with CA-MEK5α or control cDNA as indicated and performed ERK5-SUMOylation assay as described above. No difference in the amount of ERK5 wild type and mutants (2nd and 3rd from the top), or CA-MEK5α (4th from the top) was observed in samples by Western blot analysis. Activated (phosphorylated) form of ERK5 showed band shift as previously described (3rd from the top and bottom). Data are representative of results from experiments performed on at least 3 separate occasions.
provides a novel mechanism of MEK5α activation of ERK5 via inhibition of ERK5-SUMOylation.

To determine the effect of MEK5α-mediated ERK5 phosphorylation on ERK5-SUMOylation, we mutated the TEY phosphorylation motif of ERK5, which is critical for ERK5 kinase activation, and investigated whether these mutations can affect the CA-MEK5α-mediated inhibition of ERK5-SUMOylation. As shown in Figure 5A (lanes 4 through 6), although it is well known that the ERK kinase cannot be activated without the TEY motif phosphorylation, mutation of this motif had no effect on CA-MEK5α-mediated reduction of ERK5-SUMOylation. This suggested that both ERK kinase activation and TEY motif phosphorylation were not involved in the CA-MEK5α-induced inhibition of ERK5-SUMOylation. Of note, despite the presence of CA-MEK5α-induced inhibition of ERK5-SUMOylation, no significant difference in the SUMO-conjugated 75- and 110-kDa proteins and other SUMO-conjugated proteins between 30 to 280 kDa with or without CA-MEK5α transfection was noted. These data suggested that CA-MEK5α may have a unique regulatory role in ERK5-SUMOylation distinct from the general SUMOylation process induced by Ubc9/SUMO3.

Residues 78 to 139 of ERK5 constitute the MEK5α-binding domain. To investigate the contribution of MEK5α-ERK5 association on CA-MEK5α-induced inhibition of ERK5-SUMOylation, we generated 2 deletion mutants of the kinase activation site, Δ72 to 139 and Δ90 to 130, with respective deletions of the numbered residues. Coimmunoprecipitation studies confirmed the lack of association between these Δ-mutants and MEK5α (Figure 5B). Cotransfection of HA-SUMO3 and Ubc9 induced ERK5-SUMOylation, which was inhibited by CA-MEK5α induction (Figure 5C, lanes 1 through 3). As shown in Figure 5C (lanes 4 through 7), both MEK5 binding site Δ-mutants were SUMOylated, but CA-MEK5α no longer substantially inhibited ERK5-SUMOylation compared with WT. These data indicate the critical role of ERK5-MEK5 association, but not ERK5 kinase activation and TEY motif phosphorylation, on CA-MEK5α-mediated inhibition of ERK5-SUMOylation.

**ERK5-SUMOylation, PDE3A-ICER Feedback Loop, and Apoptosis in DM+MI Mice Was Significantly Inhibited in CA-MEK5α-Tg Mice**

As shown in Figure 2, we found that ERK5-SUMOylation was significantly increased in DM+MI mice heart. However, the involvement of ERK5-SUMOylation on diabetes-mediated exacerbation of LV dysfunction after MI remains unclear. Because CA-MEK5α-mediated MEK5-ERK5 association inhibited ERK5-SUMOylation in vitro we investigated whether DM-mediated ERK5-SUMOylation might be inhibited in cardiac specific CA-MEK5α-Tg mice. As shown in Figure 6A (top panel), ERK5-SUMOylation was significantly increased at 1 week after MI in diabetic nontransgenic littermate control (NLC) mice. In contrast, we did not find any significant MI-induced increase in ERK5-SUMOylation in diabetic CA-MEK5α-Tg mice. Previously, we have reported on the critical role of ERK5 activation in regulating the PDE3A/ICER feedback loop in a heart failure model.

Because we found that ERK5-SUMOylation inhibited ERK5 transcriptional activity, we determined whether the induction of PDE3A/ICER feedback loop in DM+MI mice can also be inhibited in the CA-MEK5α-Tg mice. As shown in Figure 6C, we found a reduction of PDE3A and ICER induction at 1 week after MI in diabetic NLC mice but significantly inhibited in CA-MEK5α-Tg mice. ERK5 phosphorylation was decreased in diabetic NLC mice after MI as we have shown in Figure 2A, but was recovered in CA-MEK5α-Tg mice (Figure 6A, 2nd panel from the top). No significant differences in Akt and activation between NLC and CA-MEK5α-Tg mice were observed in this model (Figure 6B, top and 2nd from the top). In contrast to ERK5, we found that ERK1/2 phosphorylation was increased in both diabetic NLC and CA-MEK5α-Tg mice after MI, which suggested the different regulatory mechanism of ERK1/2 compared with ERK5 after MI in DM mice (Figure 6B, 3rd from the top and top right).
Cardiac Dysfunction One Week After MI in DM, but not in Non-DM Mice, Was Rescued in CA-MEK5α-Tg Mice

Because we found an inhibitory role for CA-MEK5α on ERK5-SUMOylation and subsequent PDE3A and the ICER feedback loop and apoptosis, we next investigated whether the inhibition of ERK5-SUMOylation by CA-MEK5α can prevent the exacerbation of LV dysfunction and heart failure after MI in DM mice in vivo. No survival difference was noted between NLC and CA-MEK5α-Tg mice at the 1-week time point after MI in nondiabetic mice (Figure 8A). In contrast, in STZ-treated DM mice, the survival rate for CA-MEK5α was significantly higher than for NLC mice (Figure 8B). Random BS levels were elevated in both NLC and CA-MEK5α-Tg mice 1 week after STZ injection and at the time of surgery, but we did not find any difference in the body weight among these groups (Figure 8C).

One week after coronary ligation, both LV weight/TL and lung weight/TL were increased in diabetic NLC mice. In CA-MEK5α-Tg mice lung weight/TL was significantly decreased, consistent with the idea that MEK5α activation reduces cardiac dysfunction and heart failure after MI in diabetic mice (Figure 8D; supplemental Table I). In NLC mice, echocardiographic analysis showed that both LVEDD and LVEDS after MI in DM mice were greater than in sham, and FS% also significantly decreased in DM+MI group (Figure 8E; supplemental Table I). Increased in LVEDD and LVEDS and the reduction of FS% after MI in diabetic NLC mice were significantly prevented in CA-MEK5α-Tg mice (Figure 8E). Of note, there was no significant difference in cardiac function (FS%) between NLC and CA-MEK5α-Tg mice at the same time point after MI in nondiabetic mice (Figure 8F), confirming the unique role of MEK5α activation on DM-mediated exacerbation of LV dysfunction after MI. In agreement with echocardiographic data, we observed a significant decrease in developed pressure (DP) and dP/dt max in diabetic NLC mice after MI. In contrast, diabetic CA-MEK5α-Tg mice showed significantly higher DP and dP/dt max (Figure 8G; supplemental Table II). Thus physiological data also confirmed the critical role of MEK5 activation in ameliorating the exacerbation of cardiac dysfunction after MI by DM.

Discussion

In this study we found that ERK5-SUMOylation was significantly increased after MI in diabetic mice. Diabetic CA-MEK5α-Tg mice demonstrated reduced ERK5-SUMOylation and improved LV function and lung weight/TL after MI in comparison to the NLC mice. These data strongly suggested that the activation of MEK5, which inhibited diabetes-mediated ERK5-SUMOylation after MI, was cardioprotective against STZ-induced exacerbation of LV dysfunction after MI. Because both H2O2 and high glucose increased ERK5-SUMOylation, it is most likely that ROS production
may be involved in this process, but the exact mechanism remains unclear. DN-Ubc9 and PIAS1 siRNA impaired ERK5-SUMOylation suggesting the involvement of Ubc9 and PIAS1 in ERK5-SUMOylation. Recently, the involvement of PIAS1 phosphorylation on regulating downstream events has been reported. PIAS1 can act by selectively inhibiting the recruitment of NF-κB/STAT1 to the endogenous gene products. IKKε is associated with PIAS1 and mediates the Ser 90 phosphorylation of PIAS1, which in turn is required for PIAS1 to block the promoter binding of p65.

In this report, the importance of both Ser 90 phosphorylation and SUMO ligase activity of PIAS1 to repress transcriptional...
activity was proposed. However, the mutant of S90A PIAS1 did not decrease its ability to induce protein SUMOylation compared with wild type, suggesting additional regulatory mechanism of PIAS1 E3 ligase activity in addition to SUMOylation. The possible link between phosphorylation and SUMOylation is intriguing provided that ROS or high glucose–mediated kinase activation could be subject to PIAS1 phosphorylation coupled to ERK5-SUMOylation. Future research should focus on identification of the molecular mechanism of ROS and high glucose–mediated kinase activation could be subject to PIAS1 phosphorylation coupled to ERK5-SUMOylation because this process appears to be involved in the exacerbation of diabetes-mediated LV dysfunction after MI.

An expanded Discussion section is available in the Online Data Supplement.

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Disclosures
None.

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Expanded materials and methods

Reagents.

D-glucose and H$_2$O$_2$ was purchased from J.T. Baker (Phillipsburg, NJ), and mannitol was from Sigma (St. Louis, MO).

Antibodies.

Antibodies against phospho-ERK5 and ERK5 were purchased from Cell Signaling Technology (Danvers, MA) and UBI (Lake Placid, NY). Flag and tubulin antibodies were purchased from Sigma, St. Louis, MO, HA, Ubc9, PIAS1, and PML antibodies from SantaCruz, Santa Cruz, CA, Xpress antibodies from Invitrogen, Carlsbad, CA, and SUMO2/3 antibodies from ABGENT, San Diego, CA.

Plasmid and adenovirus vector construction.

Mouse ERK5 and the constitutively active form of MEK5$\alpha$ (CA-MEK5$\alpha$) were cloned as described previously $^1$. The plasmids encoding human HA-SUMO3, Ubc9, and DN-Ubc9 (C93S) were kind gifts from Dr. Ellis Jaffray (University of Menchester, UK). pcDNA3-Myc-PIAS1 was a kind gift from Dr. Masahide Takahashi (Nagoya University, Japan). Gal4-ERK5 and VP16-ERK5 were created by inserting the mouse ERK5 isolated from pcDNA3.1-ERK5 into BamH1 and Not1 sites of the pBIND and pACT vectors, respectively. The double and deletion mutations of ERK5 were created with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The specific binding region was selected as aa71~aa139 or aa90~aa130 based on
previous paper. All constructs were verified by DNA sequencing. Adenovirus expressing Xpess-tagged ERK5 wild type (Ad-ERK5 WT) and ERK5 K6R/K22R mutant (Ad-ERK5 K6R/K22R) were generated using ViraPower Adenoviral Expression System (Invitrogen).

Cell Culture.

Primary cultures of neonatal rat cardiomyocytes were performed as described previously. 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 with 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% fetal bovine serum and 10μM cytosine 1-β-D-arabinofuranoside (Sigma), which was added to inhibit the growth of contaminating non-myocytes. More than 90% of cells were cardiomyocytes (positive for α-actinin). CHO cells maintained in F-12 medium (Gibco, CA) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Mammalian one-hybrid analysis and transfection of cells.

CHO or cardiomyocytes were plated in 12-well plates at 5 x 10^4 cells/well. For the mammalian one-hybrid assay, cells were transfected in Opti-MEM (Invitrogen, CA) with lipofectamine mixture containing the pG5-luc vector and various pBIND plasmids (Promega, WI). After 4 hrs, cells were washed and fresh F12 or DMEM supplemented with 10% fetal bovine serum was added. 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 was 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 hrs after transfection except as indicated, and the luciferase activity was assayed with the dual luciferase kit (Promega, WI) using a TD-20/20 Luminometer (Turner Designs, CA). In case of the mammalian one-hybrid analysis for the ERK5 transcriptional activity, cells were transfected with pG5-luc vector, pBind-ERK5 and pcDNA3 or pcDNA3-CA-MEK5α and then exposed to H₂O₂ or AGE for the indicated times. Transfections were performed in triplicate, and each experiment was repeated at least two times.

**Immunoprecipitation (SUMOylation assay) and Western blot analysis.**

Cells were collected in phosphate-buffered saline containing 10 mM N-ethylmaleimide (NEM), and cell extracts were prepared in modified radioimmunoprecipitation assay 1 (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 % Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol, 1:200-diluted protease inhibitor cocktail (Sigma, MO), 1 mM PMSF and 10 mM NEM, and 0.1 mM iodoacetamide). 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 same modified RIPA buffer as described above. Immunoprecipitation with a mouse monoclonal anti-FLAG, HA, Xpress antibody, or polyclonal ERK5 antibody was performed as described previously ⁴. Bound proteins were released in 2x sodium dodecyl sulfate (SDS) sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto a Hybond enhanced chemiluminescence nitrocellulose membrane, and visualized by using the enhanced chemiluminescence detection reagents.
(Amersham Pharmacia Biotech) according to the manufacturer's instructions. Immunoblotting with ICER antibody and PDE3A was performed as previously described.

Transfection of the PIAS1 siRNA.

The PIAS1 siRNA was purchased from Invitrogen (Carlsbad, CA). The mouse and rat specific PIAS1 target sequence was 5′–AAGCUCUAGAAUGAUCCGG-3′. A non-specific control siRNA from Invitrogen (Carlsbad, CA) was used as a negative control. The cardiomyocytes were transiently transfected with 40 nM of medium GC control RNA or PIAS1 siRNA using Lipofectamine 2000 and plus reagent (Invitrogen) following protocols provided by the manufacturer. The cells were harvested 36 to 48 h after siRNA transfection, and protein expression were measured by using immunoblotting with antibodies against PIAS1 (SantaCruz, CA).

Analysis of apoptosis.

Cardiomyocyte apoptosis was measured by the terminal deoxynucleotidyl 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. For TUNEL method, cells were also stained with anti-cardiomyocyte-specific sarcomeric α-actinin antibody to distinguish cardiomyocytes from contaminating fibroblasts and only anti-cardiomyocyte-specific sarcomeric α-actinin antibody positive cells were counted. An average of 1000 anti-cardiomyocyte-specific sarcomeric α-actinin antibody positive cells from random fields were analyzed. All measurements were performed blinded and at least three independent experiments were performed.
Apoptotic nuclei were detected in LV myocardium by in situ terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-conjugated dUTP nick end-labeling as described \(^1,2\). In brief, cryostat sections (5 µm) from the left ventricle were mounted onto glass slides, fixed in 10% buffered formalin, then embedded in paraffin. After deparaffinization, sections were treated with 8 µg/mL proteinase K for 5 min at room temperature. End-labeling was carried out according to the manufacturer’s instructions, and tissue sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) staining (Sigma) and anti α-actinin mouse monoclonal antibody (Sigma) with subsequent anti-mouse TRITC-conjugated secondary antibody (Sigma) to identify cardiomyocyte and non-myocyte nuclei. Sections were examined at 40x using a fluorescence microscope capable of imaging 3 distinct channels without adjustment of the microscope stage position. 10 random fields per section were examined.

**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 in FVB strains and all three lines showed a similar phenotype and MEK5α expression as we reported previously \(^1\). The number of the animals was described in each figure and figure legend.

**Streptozotocin (STZ) injections:**

At age 8-10 weeks, mice (FVB, CA-MEK5α-Tg, or non-transgenic littermate control (NLC)) made diabetic by IP injection of a single dose of freshly prepared STZ solution (200 mg/kg body
wt in citrate saline, pH 4.5) using 26.5 Gauge needle after overnight fasting. Diabetic status was confirmed by blood glucose. To rule out toxic effects of STZ on cardiomyocytes, 2 STZ-injected mice per time-point were treated with insulin; Starting 2 days after STZ injection, mice was treated with 60 units/kg subcutaneously b.i.d. NPH human insulin (HumulinN; Eli Lilly, Indianapolis, IN). As a control, vehicle (0.1 mol/l citrate buffer, pH 4.5) was injected in another group of mice.

**Coronary ligation surgery:**

Mice received a single dose of the analgesic Buprenorphine (Buprene) at a dose of 0.05 mg/Kg SQ prior to surgery. If necessary, another dose was administered 6-12 hours later. Also, beginning one day prior to surgery, mice received acetaminophen at a dose of 1.5 mg/mL of drinking water as analgesia. This allowed the mice to get accustomed to the different taste in the water before surgery. Mice was initially anesthetized with 2% halothane in 100% O₂ in an animal induction box for the initial procedure. An approved scavenging device was used to prevent gas buildup and the possibility of explosion or potential toxic effects. Maintenance anesthesia was 0.5 to 1.5% halothane.

Myocardial infarction was induced in the mouse by ligating the left coronary artery. After intubation, a midline incision is made between the sternum and the left internal mammalian artery. The heart was exteriorized, and the left coronary artery was ligated intramurally 2 mm from its origin with a 9-0 proline suture. The suture was clamped (using a micro-serrifines clamp) to occlude the coronary artery. After confirming that there was no bleeding, the chest was closed in two layers; the ribs (inner layer) were closed with 5-0 coated vicryl sutures in an interrupted pattern. The skin was closed using 6-0 nylon or silk sutures in a cuticular manner.
The anesthesia was stopped and the mouse was allowed to recover for several minutes before the endotracheal tube is removed. A sham operation involved an identical procedure, except that the suture is passed through the myocardium without occlusion. As the end point procedure, we used ketamine (80 mg/kg, IP) and xylazine (5 mg/kg, IP).

**Echocardiographic analysis and in vivo hemodynamic measurements.**

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 heart beats per trace, and three traces from each animal. The pooled data were analyzed for statistical significance. In vivo hemodynamic measurements were performed as previously described 7-9.

**Statistical Analysis.**

Data are reported as mean ± S.D or mean ± S.E.M. as indicated. Statistical analysis was performed with the StatView 4.0 package (ABACUS Concepts, Berkeley, CA). Differences were analyzed with a one-way or a two way repeated–measure analysis of variance as appropriate, followed by Schéffe’s correction for multiple comparisons. P values less than 0.05 are indicated by * and less than 0.01 by **.
Expanded discussion

As we described in the introduction, the involvement of PIAS family in apoptosis has been proposed, but its contribution in heart remained unclear. In this study, we found that knock down of PIAS1 using PIAS1 siRNA significantly inhibited H$_2$O$_2$-mediated apoptosis in cardiomyocytes, suggesting the critical role of PIAS1 in apoptosis in cardiomyocytes. Since ERK5 transcriptional activity is critical for regulating PDE3A-ICER feedback loop as well as its subsequent apoptosis in heart failure, PIAS1-mediated ERK5-SUMOylation and inhibition of its transcriptional activity could contribute, at least in part, to cardiomyocytes apoptosis. In addition, the inhibition of ERK5-SUMOylation in CA-MEK5α-Tg mice prevented cardiac injury and apoptosis after MI in DM mice, providing in vivo evidence of the cardio-protective role of MEK5α activation and subsequent inhibition of ERK5-SUMOylation. Further studies will be required to determine the exact pro-apoptotic role of PIAS1 in heart failure.

Previously, we reported that CA-MEK5α-Tg can be cardio-protective after ischemia/reperfusion injury in an ex vivo Langendorff preparation within 3 hrs of reperfusion, possibly due to regulating mitochondrial connexin 43 function as we previously reported. However, in this study we could not detect improvement of cardiac function one week after MI in non-diabetic mice in vivo. We would like to emphasize here that our MI model in a current study is a “permanent” coronary ligation and therefore not a ischemia/reperfusion model. Our permanent ischemia model is a good model to determine the process of cardiac remodeling after ischemia but not a good model to investigate ischemia/reperfusion injury. In the permanent ligation model it is very difficult to decrease the infarct size by modifying gene expression against chronic ischemia. In fact a previous report demonstrated a protective effect of gene
modification only at 3-4 weeks after coronary ligation, and the difference was seen in the remodeling area but not in the infarcted area in the permanent coronary ligation model 14.

Therefore, we may observe the protective role of CA-MEK5α if we extend our investigation to 3-4 weeks after MI, but at one week after MI, the protective effect of CA-MEK5α on cardiac remodeling process was not observed. In contrast, we believe that DM accelerates the development of apoptosis in the non-infarcted area enabling the detection of the protective effect of CA-MEK5α-Tg mice even at the one week post-MI time point in the permanent coronary ligation model. Further investigation will be necessary to clarify the role of ERK5-SUMOylation in the chronic phase of cardiac remodeling after MI.

Although we found that the inhibition of ERK5-SUMOylation, apoptosis and DM + MI-mediated cardiac dysfunction in CA-MEK5α-Tg mice, it is still possible that CA-MEK5α-mediated inhibition of cardiac dysfunction may not be due to the inhibition ERK5-SUMOylation in CA-MEK5α-Tg mice. However, we found that 1) knock down of PIAS1 using PIAS1 siRNA significantly inhibited H₂O₂-mediated apoptosis and ERK5-SUMOylation, and 2) mutation of ERK5-SUMOylation sites of ERK5 (K6R/K22R) significantly inhibited H₂O₂-mediated apoptosis compared with wild type ERK5 in vitro. Taken together these data suggest that PIAS1-mediated ERK5-SUMOylation could contribute to cardiac apoptosis and may lead to subsequent cardiac dysfunction in vivo. To directly prove the importance of ERK5-SUMOylation in vivo, we will need to generate a double transgenic mice expressing PIAS1 and ERK5 K6R/K22R mutant and determine the cardiac dysfunction after myocardial infarction.
References


Table S1. Physiological parameters and Echocardiogram measurements after one week of surgery

<table>
<thead>
<tr>
<th></th>
<th>Control + Sham</th>
<th>Control + MI</th>
<th>DM + Sham</th>
<th>DM + MI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=11</td>
<td>n=12</td>
<td>n=11</td>
<td>n=13</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>24.4 ± 0.8</td>
<td>22.2 ± 1.0</td>
<td>23.5 ± 1.1</td>
<td>21.6 ± 0.7</td>
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<tr>
<td>Random Blood Sugar (mg/dl)</td>
<td>119 ± 8</td>
<td>116 ± 8</td>
<td>326 ± 17 **</td>
<td>284 ± 16 ** ††</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>106 ± 3</td>
<td>121 ± 6</td>
<td>103 ± 4</td>
<td>128 ± 6 *</td>
</tr>
<tr>
<td>Left Ventricle (LV) Weight (mg)</td>
<td>77 ± 2</td>
<td>91 ± 5</td>
<td>77 ± 4</td>
<td>98 ± 4 **</td>
</tr>
<tr>
<td>Lung Weight (mg)</td>
<td>128 ± 5</td>
<td>166 ± 7 **</td>
<td>132 ± 5</td>
<td>210 ± 10 ** ††</td>
</tr>
<tr>
<td>Tibial Length (TL) (mm)</td>
<td>18.6 ± 0.2</td>
<td>18.3 ± 0.2</td>
<td>18.3 ± 0.1</td>
<td>18.3 ± 0.1</td>
</tr>
<tr>
<td>LV/TL (mg/mm)</td>
<td>4.19 ± 0.10</td>
<td>4.98 ± 0.27</td>
<td>4.23 ± 0.21</td>
<td>5.35 ± 0.24 ** ††</td>
</tr>
<tr>
<td>Lung Weight/LT (mg/mm)</td>
<td>6.91 ± 0.25</td>
<td>9.12 ± 0.44 **</td>
<td>7.20 ± 0.27</td>
<td>11.54 ± 0.54 ** ††</td>
</tr>
</tbody>
</table>

Heart Rate (bpm)          | 609 ± 23       | 516 ± 25     | 560 ± 26  | 553 ± 23 |
Tibial Length (TL) (mm)    | 18.8 ± 0.3     | 18.1 ± 0.1   | 18.4 ± 0.1| 18.3 ± 0.1|
AWd (mm)                  | 0.68 ± 0.02    | 0.51 ± 0.34 **| 0.66 ± 0.02| 0.49 ± 0.03 **|
PWd (mm)                  | 0.73 ± 0.23    | 0.75 ± 0.03  | 0.75 ± 0.02| 0.66 ± 0.02 |
LVEDd (mm)                | 3.20 ± 0.07    | 3.88 ± 0.11 **| 3.23 ± 0.10| 4.41 ± 0.09 ** ††|
LVEDd/TL (mm/mm)          | 0.170 ± 0.004  | 0.214 ± 0.007 **| 0.176 ± 0.005| 0.242 ± 0.006 ** ††|
LVESd (mm)                | 1.35 ± 0.06    | 2.84 ± 0.17 **| 1.38 ± 0.10| 3.69 ± 0.09 ** ††|
LVESd/TL (mm/mm)          | 0.071 ± 0.003  | 0.157 ± 0.010 **| 0.075 ± 0.006| 0.202 ± 0.005 ** ††|
%FS (%)                   | 58.0 ± 1.3     | 27.1 ± 3.1 **| 57.6 ± 1.9| 16.3 ± 1.1 ** ††|

MI: myocardial infarction; DM: diabetes melittus; LVEDd; left ventricle end-diastolic dimension, LVESd; left ventricle end-systolic dimension, AWd; left ventricle anterior wall end-diastolic dimension, PWd; left ventricle posterior wall end-diastolic dimension. mean ± SEM, * p<0.05 and ** p<0.01 vs.Control + Sham, † p<0.05 and †† p<0.01 vs.Control + MI

Table S2. Physiological parameters, echocardiogram, and hemodynamic measurements after one week of surgery in NLC and CA-MEK5α-Tg mice

<table>
<thead>
<tr>
<th></th>
<th>Control + Sham in NLC</th>
<th>DM + MI in NLC</th>
<th>DM + MI in CA-MEK5α-Tg</th>
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<tbody>
<tr>
<td></td>
<td>n=8</td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>27.3 ± 1.0</td>
<td>24.6 ± 1.1</td>
<td>24.3 ± 0.5</td>
</tr>
<tr>
<td>Blood Sugar (mg/dl)</td>
<td>121 ± 9</td>
<td>297 ± 19 **</td>
<td>313 ± 23 **</td>
</tr>
<tr>
<td>Tibial Length (TL) (mm)</td>
<td>17.9 ± 2</td>
<td>18.1 ± 1</td>
<td>18.2 ± 1</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>93 ± 2</td>
<td>142 ± 4 **</td>
<td>135 ± 6 **</td>
</tr>
<tr>
<td>Left Ventricle (LV) Weight (mg)</td>
<td>71 ± 2</td>
<td>108 ± 4 **</td>
<td>97 ± 4 ** ††</td>
</tr>
<tr>
<td>LV/TTL (mm)</td>
<td>3.99 ± 0.12</td>
<td>5.93 ± 0.21 **</td>
<td>5.34 ± 0.22 **</td>
</tr>
<tr>
<td>Lung Weight (mg/mm)</td>
<td>114 ± 3</td>
<td>224 ± 20 **</td>
<td>155 ± 6 * ††</td>
</tr>
<tr>
<td>Lung Weight/TL (mg/mm)</td>
<td>6.38 ± 0.19</td>
<td>12.3 ± 1.08 **</td>
<td>8.52 ± 0.34 ** ††</td>
</tr>
</tbody>
</table>

Heart Rate (bpm)          | 593 ± 1               | 565 ± 23       | 586 ± 11                 |
ATd (mm)                  | 0.65 ± 002            | 0.51 ± 003 **  | 0.54 ± 0.02 **           |
PWd (mm)                  | 0.76 ± 0.09           | 0.65 ± 0.03    | 0.73 ± 0.03              |
LVEDd (mm)                | 3.12 ± 0.65           | 4.34 ± 015 **  | 3.88 ± 0.13 †            |
LVEDd/TL (mm/mm)          | 0.174 ± 0004          | 0.240 ± 0009 **| 0.213 ± 0007 ††          |
LVESd (mm)                | 1.30 ± 0.07           | 3.68 ± 0.23 ** | 2.93 ± 0.20 ** ††        |
LVESd/TL (mm/mm)          | 0.073 ± 0004          | 0.202 ± 0013 * | 0.160 ± 0011 ††          |
%FS (%)                   | 58.5 ± 1.7            | 15.0 ± 1.9 **  | 25.4 ± 3.4 ** ††         |

Heat Rate (bpm)          | 361 ± 16              | 379 ± 13       | 377 ± 27                 |
LV systolic pressure (mmHg) | 111.2 ± 5.0          | 51.4 ± 2.7 **  | 70.9 ± 4.3 * ††          |
dp/dt (mmHg/s)            | 3824 ± 58             | 1553 ± 203 **  | 2831 ± 228 ** ††         |
-dp/dt (mmHg/s)           | 2921 ± 192            | 992 ± 109 **   | 1968 ± 249 * ††          |
LV developed pressure (mmHg) | 105.5 ± 4.5          | 46.1 ± 4.2 **  | 69.9 ± 4.7 * ††          |

NLC: age-matched nontransgenic littermate control mice, CA-MEK5α-Tg; CA-MEK5α- transgenic mice, MI: myocardial infarction, DM: diabetes melittus, LVEDd; left ventricle end-diastolic dimension, LVESd; left ventricle end-systolic dimension, AWd; left ventricle anterior wall end-diastolic dimension, PWd; left ventricle posterior wall end-diastolic dimension, mean ± SEM, * p<0.05 and ** p<0.01 vs.Control + Sham, † p<0.05 and †† p<0.01 vs. DM + MI in NLC