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Inducible Expression of Active Protein Phosphatase-1 Inhibitor-1 Enhances Basal Cardiac Function and Protects Against Ischemia/Reperfusion Injury

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Abstract

Ischemic heart disease, which remains the leading cause of morbidity and mortality in the Western world, is invariably characterized by impaired cardiac function and disturbed Ca^{2+} homeostasis. Because enhanced inhibitor-1 (I-1) activity has been suggested to preserve Ca^{2+} cycling, we sought to define whether increases in I-1 activity in the adult heart may ameliorate contractile dysfunction and cellular injury in the face of an ischemic insult. To this end, we generated an inducible transgenic mouse model that enabled temporally controlled expression of active I-1 (T35D). Active I-1 expression in the adult heart elicited significant enhancement of contractile function, associated with preferential phospholamban phosphorylation and enhanced sarcoplasmic reticulum Ca^{2+} -transport. Further phosphoproteomic analysis revealed alterations in proteins associated with energy production and protein synthesis, possibly to support the increased metabolic demands of the hyperdynamic hearts. Importantly, on ischemia/reperfusion-induced injury, active I-1 expression augmented contractile function and recovery. Further examination revealed that the infarct region and apoptotic as well as necrotic injuries were significantly attenuated by enhanced I-1 activity. These cardioprotective effects were associated with suppression of the endoplasmic reticulum stress response. The present findings indicate that increased I-1 activity in the adult heart enhances Ca^{2+} cycling and improves mechanical recovery, as well as cell survival after an ischemic insult, suggesting that active I-1 may represent a potential therapeutic strategy in myocardial infarction.

Keywords

ischemia; reperfusion; protein phosphatase-1 inhibitor-1; phospholamban; ER stress

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Disclosures

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Ischemic heart disease remains the leading cause of cardiovascular disease and mortality in the Western world. In the United States alone, the incidence of myocardial infarction has reached an alarming 8.1 million.[1] Even though restoration of coronary flow alleviates the detriment of the ischemic insult, it is invariably accompanied by reperfusion-induced contractile dysfunction and cellular damage. The causes for these deleterious effects are multifactorial, but disturbed Ca^{2+} homeostasis has been proposed as a central contributor to postischemic injury.[2,3]

The sarcoplasmic reticulum (SR) is a crucial regulator of Ca^{2+} handling in the cardiomyocyte, and elucidation of its role in ischemia/reperfusion (I/R)-induced injury has been the focus of several investigations. Experimental evidence indicates that SR Ca^{2+} cycling is depressed in the postischemic myocardium[4,5]; yet the functional manifestations of this alteration remain controversial. On the one hand, it has been reported that intracellular Ca^{2+} overload, during reperfusion, and the subsequent futile cycles of Ca^{2+} release and reuptake by the SR are critical events that lead to contractile dysfunction, necrosis, and mitochondrial-dependent apoptosis.[6–8] As such, depressed SR function may be an adaptive mechanism aimed at alleviating these adverse effects. Conversely, the SR may act as an intracellular sink for excess cytosolic Ca^{2+}, and its compromised function may reduce the capacity of the cardiomyocyte to cope with intracellular Ca^{2+} overload.[3,9] Further efforts put forth to unravel the role of SR Ca^{2+}-handling proteins in I/R become very important not only to advance our fundamental understanding of SR function in the postischemic heart but also to identify novel targets, with potential clinical application.

An attractive protein in this respect is protein phosphatase (PP)1 inhibitor-1, which was the first recognized endogenous regulator of this phosphatase. Early studies revealed that, similarly to other PP1-interacting proteins, inhibitor-1 contains an RVXF motif sequence, which facilitates its interaction with PP1. Further studies indicated that inhibitor-1 is widely expressed in mammalian tissues. In fact, its significance has been described extensively in the brain and skeletal muscle, where it has been implicated in synaptic plasticity and hormonal regulation of glycogen metabolism, respectively.[10] In the heart, inhibitor-1 has been postulated as an integrator of multiple neurohormonal pathways associated with Ca^{2+} homeostasis and proper contractile function. In particular, on stimulation of the β-adrenergic axis, protein kinase (PK) A phosphorylates Thr35 in inhibitor-1, resulting in PP1 inhibition and amplification of the contractile response.[11,12] Inactivation of inhibitor-1 occurs by dephosphorylation of Thr35 by PP2A and PP2B, leading to relief of PP1 inhibition and restoration of basal function.[13] Importantly, inhibitor-1 can also be phosphorylated at Ser67 and Thr75 by PKC, but these phosphorylations enhance PP1 activity and diminish contractility.[14,15] Altogether, these data emphasize a key regulatory role for inhibitor-1 in cardiac physiology.

Notably, previous studies have shown that chronic increases in an active (T35D) and truncated (amino acids 1 to 65) form of inhibitor-1 that lacks the PKC sites, which mitigate the beneficial effects of inhibitor-1,[14,15] enhance Ca^{2+}cycling, and preserve cardiac performance in the failing heart.[12] In light of the contentious role of SR function in postischemic injury, we sought to address the effects of enhanced Ca^{2+} cycling, mediated by increased inhibitor-1 activity in the adult heart, on an ischemic insult. Our findings herein further support a beneficial role for enhanced Ca^{2+} cycling in I/R and implicate active inhibitor-1 as a potential therapeutic strategy for ischemic heart disease.

Materials and Methods

Generation of an Inducible Mouse Model With Expression of Active Inhibitor-1 (I-1c)

I-1c (amino acids 1 to 65; T35D) single transgenic mice (TG2) were crossed with mice carrying the tetracycline-controlled transactivator (tTA) gene (TG1) to generate double transgenic

(DTG) mice, with inducible I-1c expression. Animals were handled as approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

An expanded Materials and Methods section can be found in the online data supplement at http://circres.ahajournals.org.

Results

Generation of an Inducible Mouse Model With Expression of Active Inhibitor-1

To elucidate the effects of enhanced I-1 activity in the adult heart, we generated a mouse model with temporally regulated and cardiac-specific expression of a constitutively active and truncated (T35D; amino acids 1 to 65) form of I-1 (I-1c), using the Tet-off system. Double transgenic (DTG) mice are expected to express I-1c only on withdrawal of doxycycline (Dox) (Figure 1A). Control mice were also kept on the same diet regimen to eliminate any potential differential effects resulting from Dox administration. Immunoblotting confirmed the fidelity of this system. Specifically, in the absence of Dox, I-1c expression was effectively induced, at 1.65-fold of endogenous I-1 (Figure 1B, left blot), whereas in the presence of Dox, expression was successfully suppressed, in the DTG mice (Figure 1B, right blot). Importantly, the single transgenic I-1c mice (TG2) did not express I-1c either in the presence or absence of Dox (Figure 1B), indicating that the I-1c attenuated promoter did not exhibit leakage neither in the DTG mice, when the transactivator was sequestered by Dox, nor in the single I-1c transgenic mice, where the transactivator was absent. In addition, another line, line 2, was generated with similar (1.5-fold) increases in I-1c, compared to endogenous I-1 (data not shown). Importantly, Dox administration was not associated with any alterations in the levels of the Ca^{2+}-ATPase pump SERCA2a and phospholamban (PLN) (Figure 1C), further supporting the fidelity of the Tet-off system.

Active I-1 Expression in the Adult Heart Enhances Basal Contractility

Because I-1 has been proposed as a modulator of cardiac contractility, left ventricular contractile indices were assessed by use of invasive catheterization in vivo, to delineate any functional effects of I-1c expression in the adult heart. The results indicated that contractility was enhanced in a time- dependent manner, on Dox withdrawal. Specifically, a trend for enhanced relaxation was observed at 6 weeks (dP/dt_{min} was 3927±256 and 4583±291 mm Hg/sec, for wild-type [WT] and DTG respectively) and significant increases in basal contraction (25% increase in dP/dt_{max}; Figure 2A) and relaxation (37% increase in dP/dt_{min} [Figure 2B]; 24% decrease in Tau [Figure 2C]) parameters were obtained at 8 weeks in DTGs, as compared to WTs. This timeline of expression is similar to a previous report, using the Tet-off system.[16] Based on these results, subsequent experiments were conducted after 8 weeks of Dox withdrawal. In addition, contractility was enhanced to a similar extent in line 2 (Figure I in the online data supplement). Overall, these findings indicate that I-1c expression in the adult heart enhances cardiac performance.

Effects of Active I-1 Expression on Key Regulatory Phosphoproteins

Because phosphorylation of PLN, the ryanodine receptor, troponin I, and myosin-binding protein-C constitutes an important regulatory mechanism that governs cardiac contractility, we investigated these phosphoproteins as potential I-1c substrates. Quantitative immunoblotting indicated that phosphorylation of PLN at Ser16 and Thr17 was increased by 2-fold and 2.3-fold, respectively, in DTG, as compared to WT hearts. Interestingly, no changes were observed in the phosphorylation status of the other phosphoproteins examined (Figure 3A and 3B). Furthermore, no compensation was observed at the total protein level of PLN, the ryanodine receptor, troponin I, or myosin-binding protein-C (Figure 3A and 3C). These results were

confirmed independently in line 2 (data not shown), suggesting that I-1c shows remarkable specificity for PLN in vivo.

Active I-1 Enhances Ca^{2+} Uptake Into the Sarcoplasmic Reticulum

Because the degree of PLN phosphorylation profoundly affects the activation state of SERCA2a and contractility, oxalate-supported SR Ca^{2+}-transport was assessed in cardiac homogenates over a wide range of Ca^{2+} concentrations, similar to those present in vivo during relaxation and contraction. The results showed that I-1c expression significantly enhanced the affinity of SERCA2a for Ca^{2+} (the EC_{50} value decreased by 20.5%; Figure 4A and 4B). This alteration was similar to that obtained in PLN-heterozygous hearts but much smaller than the shift in the EC_{50} value in PLN-deficient hearts (58% decrease, compared to WT).[17] Furthermore, no differences in the maximal velocity of Ca^{2+} uptake were noted between the 2 groups (Figure 4A and 4C). These findings indicate that I-1c expression mediates disinhibition of SERCA2a by enhancing phosphorylation of PLN.

Phosphoproteomic Analysis of I-1c–Expressing Hearts

To gain further insights into the apparent specificity of I-1c for PLN, a phosphoproteomic approach was used to identify other potential I-1c–regulated targets. In particular, 2D gels were stained with a phosphospecific dye, namely ProQ Diamond, for detection of the phosphoproteome profile. Subsequently, the same gels were silver-stained to visualize any alterations at the whole proteome level. Analysis of 512 phosphoprotein and 1423 protein spots revealed that only 4 and 8 were altered in I-1c hearts, respectively (Figure 5A and 5B). Interestingly, none of the 4 phosphoproteins appeared to correspond to any of the altered proteins. Mass spectroscopic analysis positively identified 10 of these 12 spots. The altered proteins were grouped into 2 major categories: energy production and protein synthesis. In the first category, the phosphorylation levels of enoyl coenzyme A hydratase 1 (spot 34) and the protein levels of electron-transferring flavoprotein (spot 25), both of which are involved in the high-yield metabolic pathway of β-oxidation of fatty acids, were increased. Furthermore, creatine kinase (spot 30), which catalyzes the conversion of the high-energy compound phosphocreatine, was also increased. Interestingly, the level of glucose phosphate isomerase (spot 35), an important enzyme in glycolysis, the less efficient metabolic pathway, was downregulated. In the second category, the phosphorylation of the Tu translation elongation factor (spot 2), which is involved in protein translation, and the levels of inner mitochondrial membrane protein, which is involved in protein import, were increased. Notably, 3 different spots (spots 8, 9, and 18) were identified as inner mitochondrial membrane protein, which may represent previously reported spliced variants or posttranslational modifications.[18] The protein synthesis machinery was possibly increased to support assembly of the aforementioned energy production proteins. Finally, the phosphorylation of contrapsin (spot 6) was increased in I-1c hearts. Contrapsin is a serine-proteinase inhibitor, whose function is not well understood.[19] Although the observed phosphoprotein changes may be attributable to a direct action of I-1, this seems unlikely. Changes in these pathways were also observed in hyperdynamic PLN-deficient hearts,[20] and they may represent compensatory alterations to accommodate the increased energetic demands of these hearts.

Active I-1 Attenuates Contractile Dysfunction and Myocardial Infarction After I/R In Vivo

To examine the hypothesis that I-1c may be protective against postischemic injury, WT and DTG mice were subjected to I/R in vivo. WT hearts presented with contractile dysfunction post-I/R, as evidenced by depressed contraction (31% decrease in dP/dt_{max}; Figure 6A) and relaxation (37% decrease in dP/dt_{min} [Figure 6B]; 244% increase in Tau [Figure 6C]) parameters. However, DTG hearts exhibited enhanced cardiac function post-I/R and their contractile parameters were similar to those in WT hearts at preischemic conditions.

In addition to contractile dysfunction, the postischemic heart presents with irreversible cellular injury. To delineate any effects by I-1c in postischemic damage, I/R-induced myocardial infarction was assessed. The infarct-to-risk region ratio was 22.7±2.3 in WT hearts post-I/R, similar to previous reports,[21,22] whereas it was significantly attenuated (9.8±1.5, $P<0.005$) in DTG hearts (Figure 6D and 6E). These results indicate that enhanced I-1 activity attenuates postischemic contractile dysfunction and myocardial infarct size in vivo.

Active I-1 Improves Functional Recovery After I/R Ex Vivo

To further examine the potential benefits of I-1c, in the absence of neurohormonal influences, hearts were Langendorff-perfused and subjected to 40 minutes of global ischemia followed by 60 minutes of reperfusion. During the stabilization period, DTG hearts exhibited higher contractile parameters (Figure 7A and 7B), consistent with our results from in vivo recordings (Figure 2). On coronary flow interruption, contractile parameters dropped essentially to 0 in both groups. However, on reperfusion, DTG hearts exhibited better functional recovery, as compared to WT. Remarkably, after the first 5 minutes of reperfusion, contractile and relaxation parameters were increased by 1.7-fold and 1.8-fold, respectively, as compared to WT hearts, and function remained elevated over the 60 minutes of the reperfusion period (Figure 7A and 7B). Notably, the rates of contraction and relaxation, normalized to their preischemic values, were 51±0.07% and 39±0.05% in DTG hearts and only 34±0.09% and 24 ±0.06% in WT hearts, after 5 minutes of reperfusion, whereas no differences were noted after 60 minutes of reperfusion (Online Figure II, A and B). To elucidate the mechanisms, which may contribute to improved functional recovery, the phosphorylation levels of PLN, the target substrate of I-1c (Figure 3), were assessed. Phosphorylation at Ser16-PLN was not detectable post-I/R in either group, in agreement with the lack of β-adrenergic stimulation in ex vivo perfused hearts. However, phosphorylation at Thr17-PLN was significantly elevated in DTG hearts at 15 and 30 minutes, compared to WTs (Figure 7C), whereas there were no differences at 60 minutes postreperfusion. Collectively, these results show that I-1c ameliorates I/R-induced contractile dysfunction, at least partly, by increased PLN phosphorylation at its Thr17 site during early reperfusion.

Active I-1 Alleviates Cell Death After I/R by Attenuating the Endoplasmic Reticulum Stress Response

To delineate any cardioprotective effects conferred by I-1c in postischemic cellular damage, which may also contribute to the improved functional recovery, the extent of necrotic and apoptotic cell death was examined after I/R ex vivo. Lactate dehydrogenase efflux, an index of necrotic injury, was decreased by 60% (Figure 7D), and the extent of apoptosis, as assessed using an ELISA-based DNA fragmentation assay, was reduced to preischemic levels in DTG hearts post-I/R (Figure 7E). Because it has been previously reported that the endoplasmic reticulum (ER) stress response, is induced in the ischemic heart, at least partly by altered Ca^{2+} homeostasis, and contributes to cardiomyocyte apoptosis,[23] we hypothesized that improved Ca^{2+} cycling, mediated by I-1c expression, may attenuate the ER stress response. Thus, the ER stress response was evaluated at 15, 30, and 60 minutes postreperfusion. Although there was no induction observed at 15 and 30 minutes, there were significant increases in the levels of protein disulphide isomerase and Grp78 (glucose-regulated protein 78) by 2.8-fold and 1.6-fold, respectively, after 60 minutes (Figure 8A and 8B). Importantly, expression of both proteins was reduced to preischemic values in DTG hearts after 60 minutes of reperfusion, suggesting that induction of the ER stress response was prevented in DTG hearts. Consistent with activation of the ER stress response, expression of a downstream target, inositol-requiring enzyme 1α, was increased by 1.7-fold in WT hearts at this time point, whereas it was unaltered in DTG hearts (Figure 8C). Inositol-requiring enzyme 1α has been shown to be involved in the proteolytic processing of pro–caspase 12 into its active form, caspase-12. Consistently,

caspase-12 activity was increased by 1.7-fold in WT hearts at 60 minutes postreperfusion, whereas it was attenuated to preischemic values in DTG hearts (Figure 8D).

Furthermore, because mitochondrial-mediated apoptosis is a major determinant of cell injury in I/R, the potential effects of enhanced I-1 activity on this pathway were examined. Because it has been previously reported that Bad, an important regulator of mitochondrial-mediated cell death, is a PP1 substrate,[24] this protein was investigated as a potential I-1c–regulated phosphoprotein. The phosphorylation levels of Bad at Ser116 and Ser136 were similar between I-1c and WT hearts basally and post-I/R (Online Figure III, A and B). To further address a possible effect of I-1c on mitochondrial-mediated apoptosis, the activity of caspase-9, the downstream target of this pathway, was assessed. No differences were observed between the 2 groups at basal levels and after I/R (Online Figure III, C), suggesting that the antiapoptotic effects of I-1c may be primarily mediated through prevention of ER stress- and not mitochondrial-mediated cell death.

To further delineate the effects of I-1c on ER stress at the cellular level, adult rat cardiomyocytes were infected with Ad.I-1c and Ad.GFP (green fluorescent protein), as a control, and subjected to simulated I/R (sI/R). The extent of apoptosis, as assessed by DNA fragmentation, was increased by 2-fold in Ad.GFP-infected cells after sI/R, whereas it was reduced by 64.4% in I-1c–expressing myocytes (Figure 8E). Importantly, these effects were associated with an attenuated ER stress response, as evidenced by decreased (59.8%) levels of protein disulphide isomerase (Figure 8F). Overall, these results suggest that increased I-1 activity effectively attenuates induction of the ER stress response, which may mediate the cardioprotective effects of I-1c.

Discussion

Ischemic injury inevitably manifests in contractile dysfunction, impaired Ca^{2+} homeostasis, and cell death, on restoration of coronary flow. We provide evidence herein to show, for the first time, that augmenting Ca^{2+} cycling in the SR/ER, by enhancing inhibitor-1 activity in the adult heart, ameliorates the postischemic detriment at least at 2 different levels by facilitating mechanical recovery and ameliorating cell injury, through suppression of the ER stress response. These beneficial effects may be associated with enhanced PLN phosphorylation in the SR. These findings are consistent with the notion that, even though protein phosphatase-1 is present in multiple cellular compartments, it is differentially regulated by auxiliary proteins, such as inhibitor-1, which define its localization, substrate specificity, and catalytic activity.[25]

The observation that augmented SR Ca^{2+} transport and cycling, mediated by active inhibitor-1, improved postischemic injury has important implications on the role of Ca^{2+} cycling in myocardial I/R. Indeed, even though it is generally accepted that Ca^{2+} homeostasis is impaired and Ca^{2+} uptake into the SR is depressed in myocardial I/R,[4,5] the effects of enhanced Ca^{2+} uptake into the SR and concomitantly increased SR Ca^{2+} load, in the face of an ischemic insult remain controversial. On the one hand, it has been reported that such a maneuver is beneficial as it ameliorates postischemic injury. Consistent with this notion, transgenic expression of SERCA1a,[26] which exhibits higher kinetics than SERCA2a, as well as gene transfer of SERCA2a in rat and porcine animal models,[27,28] alleviated postischemic cardiac dysfunction and injury, whereas SERCA2a deficiency impaired relaxation and increased infarction on I/R.[29] In addition, it has been reported that phosphorylation of PLN at Thr17 is essential in facilitating recovery of contractility during early reperfusion,[30] further supporting the beneficial effects of dis-inhibition of the SR Ca^{2+} pump. On the other hand, there exists evidence that increasing SR Ca^{2+} cycling in the ischemic heart may be detrimental. Pharmacological inhibition of SERCA2a improved postischemic recovery and overexpression of histidine-rich Ca^{2+} -binding protein, an endogenous SERCA2a inhibitor, improved

functional recovery and cellular damage in the midst of I/R-induced injury.[21,31] In addition, PLN ablation exacerbated I/R-induced dysfunction,[32,33] further supporting the notion that enhancing Ca^{2+} cycling may have deleterious effects. It is intriguing to postulate that the extent of Ca^{2+} uptake and level of Ca^{2+} load in the SR are important determinants of the final outcome and that a tight balance needs to be attained. Indeed, in the present study, active inhibitor-1 moderately enhanced SR Ca^{2+} cycling, with beneficial effects. Future studies, using a gene therapy approach in higher mammalian species, may delineate the benefits of active I-1 therapy in ischemic heart disease.

The antiapoptotic effects elicited by active inhibitor-1 were associated with attenuation of ER stress-activated caspase-12 activity, whereas mitochondrial-dependent caspase-9 activity was similar in both groups. These results suggested that active inhibitor-1-mediated cardioprotection may be primarily dependent on attenuation of the ER stress or unfolded protein response (UPR). The initial intent of the UPR is to adapt to changing cellular conditions and reestablish proper ER function. Thus, adaptive, cytoprotective mechanisms are induced, including activation of transcriptional programs to increase the folding capacity of the ER, inhibition of protein synthesis, and degradation of misfolded proteins. However, prolonged or persistent induction of the ER stress pathways becomes maladaptive and initiates host defense mechanisms, including activation of the apoptotic cascade, which may lead to pathological disease states.[34] In fact, recent studies have reported that the UPR is induced in the failing heart and may be causally related to heart failure induction.[35,36]

Various stimuli, including disturbed Ca^{2+} homeostasis, have been shown to induce the UPR.[34,37] In fact, depletion of ER Ca^{2+} stores using thapsigargin, an inhibitor of SERCA2a, is a classic pharmacological way of inducing ER stress. The apparent importance of constant Ca^{2+} levels lies in the Ca^{2+} -dependent nature of ER chaperones, such as Grp78, for activation. As such, the altered Ca^{2+} homeostasis in the ischemic heart,[2,3] may adversely affect their function and induce the UPR.[38] In fact, it has been previously reported that ischemic insults induce the UPR in the cardiomyocyte, which activates apoptotic pathways.[23] Consistently, we found that the ER stress response was induced both in isolated cardiomyocytes and in whole hearts following I/R. However, active inhibitor-1 effectively attenuated ER stress activation. This may be attributable to its ability to, at least partially, restore Ca^{2+} homeostasis in the SR/ER by augmenting Ca^{2+} cycling in this organelle.

Collectively, the present findings indicate that enhancing Ca^{2+} cycling in the SR/ER by increasing inhibitor-1 activity alleviates postischemic injury by improving contractile recovery and attenuating cellular injury, suggesting that active inhibitor-1 may represent a novel therapeutic strategy in myocardial infarction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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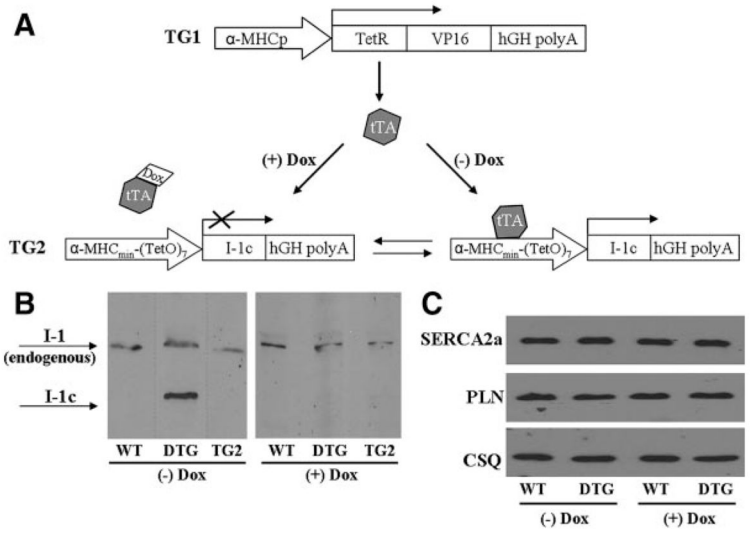

Figure 1.

Generation of a mouse model with inducible expression of I-1c. A, DTG mice were generated using the Tet-off system. TG1 drives expression of the tetracycline-regulated transactivator (tTA) by using the traditional *α*-myosin heavy chain promoter (*α*-MHCp). TG2 drives I-1c expression, using the attenuated *α*-MHCp, which is only active in the absence of Dox. B, Immunoblot analysis, after trichloroacetic acid precipitation of ≈400 mg of tissue, confirmed the validity of the system. DTG mice expressed I-1c at 1.65-fold of endogenous I-1 (WT, n=3; DTG, n=3) on Dox withdrawal for 8 weeks (left blot), whereas I-1c expression was suppressed in the presence of Dox (right blot). Single transgenic I-1c (TG2) mice did not express I-1c either in the presence (right blot) or absence of Dox (left blot). Dotted lines represent different blots. C, Immunoblot analysis for SERCA2a and PLN revealed that Dox administration did not have any off-target effects. Calsequestrin (CSQ) was used as a loading control (WT, n=4; DTG, n=4).

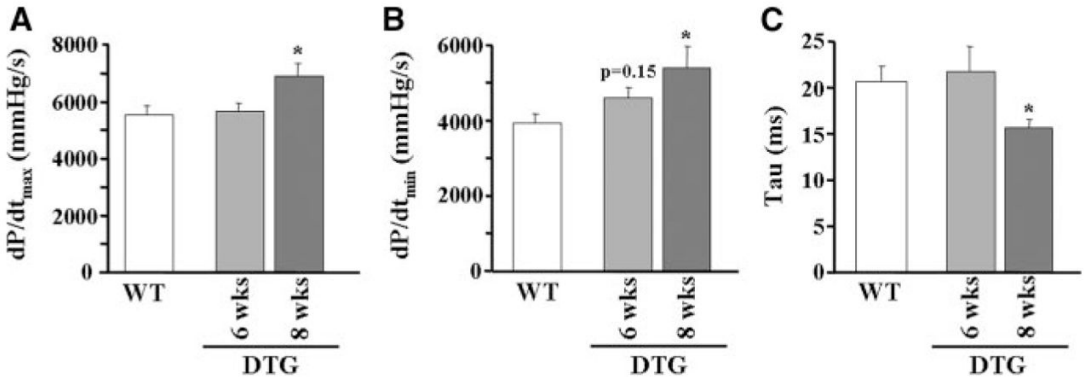

Figure 2.

I-1c expression in the adult heart enhances basal contractility. Dox was withdrawn from the diet of the mice for either 6 or 8 weeks, and cardiac function was examined using in vivo catheterization. Hearts expressing I-1c for 8 weeks exhibited enhanced rates of contraction (A) and relaxation (B), as well as a decreased time constant of relaxation τ (C) (WT, n=11; DTG [6 weeks], n=4; DTG [8 weeks], n=8). *P<0.05 vs WT.

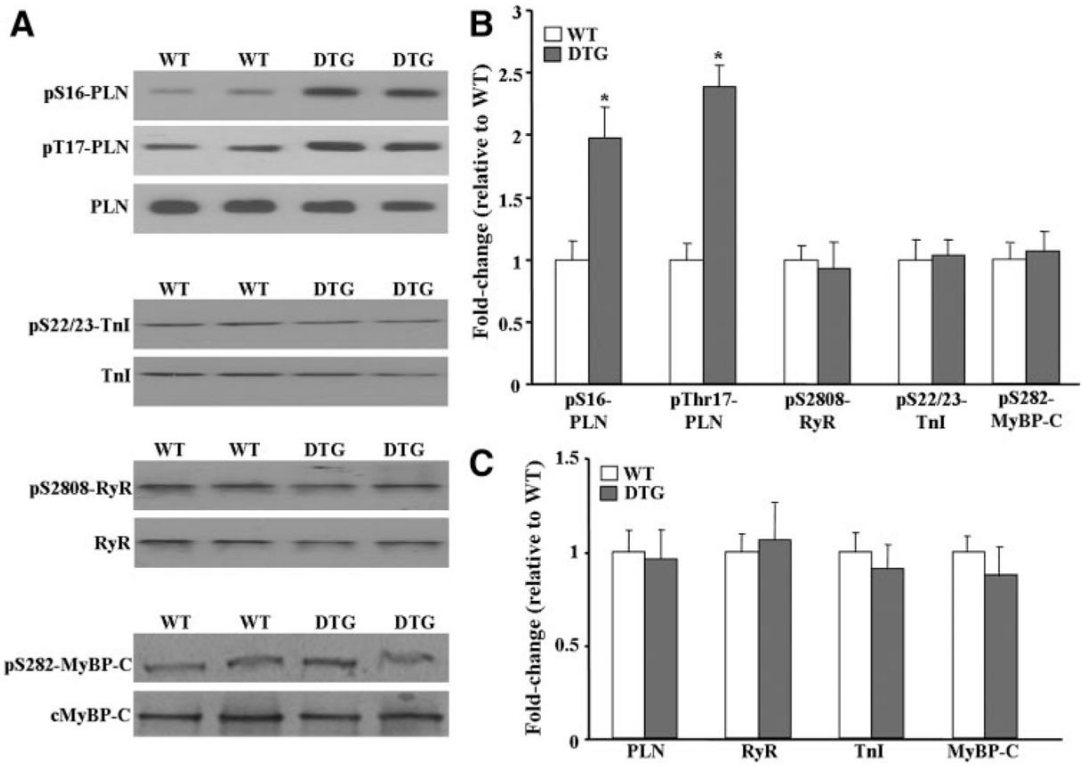

Figure 3.

$Ca2^{+}$ regulatory phosphoproteins in DTG hearts. A, Immunoblot analysis revealed that phosphorylation of PLN was increased in DTG hearts. No changes were observed in the levels of PLN, ryanodine receptor (RyR), troponin I (TnI), and myosin-binding protein-C (MyBP-C). B, Quantitative analysis of the phosphorylation levels of proteins shown in A, normalized to their respective total protein levels. C, Quantitative analysis of the total protein levels shown in A, normalized to calsequestrin (CSQ). Bars represent means±SEM. **P*<0.05 vs WT.

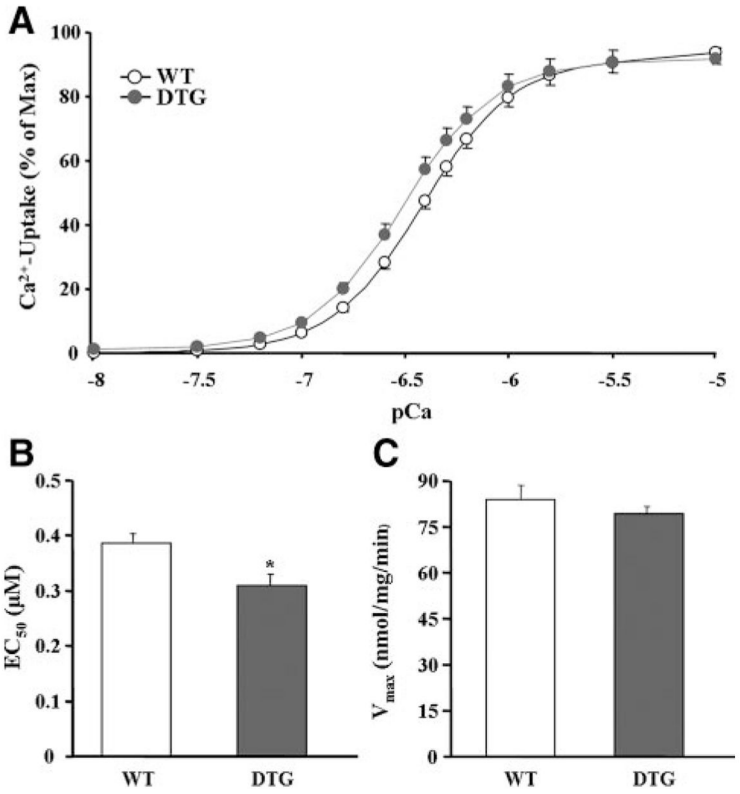

Figure 4.

I-1c expression enhances SERCA2a activity. A, The initial SERCA2a $Ca2^{+}$ uptake rates were assessed in cardiac homogenates over a wide range of $Ca2^{+}$ concentrations. The data were analyzed with Origin software, and the affinity of SERCA for $Ca2^{+}$ (B) and the maximal velocity (C) were calculated.

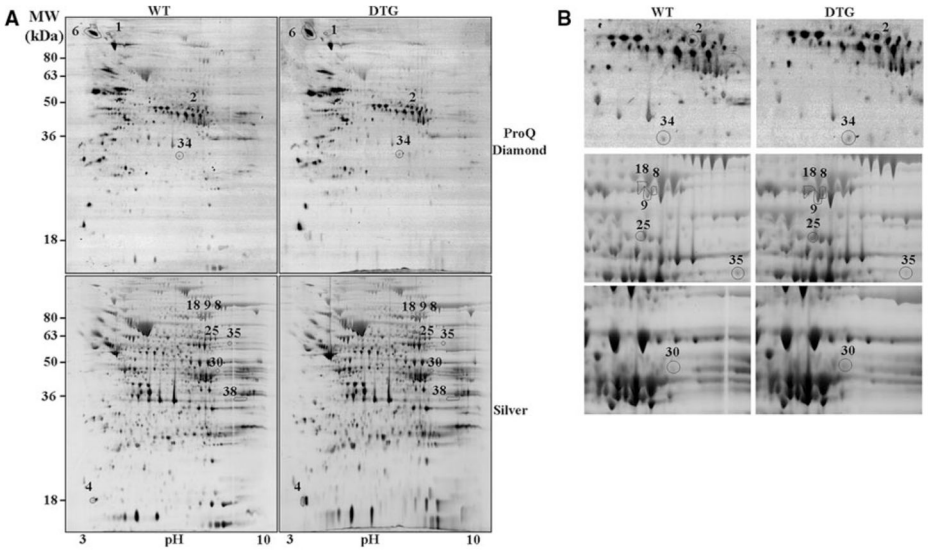

Figure 5.

Phosphoproteomic analysis of I-1c hearts. A, Representative images of ProQ-Diamond- and silver-stained 2D gels. B, Enlargement of some of the spots shown in A. The spot number, identity and fold change are indicated in Online Table I. WT, n=3; DTG, n=3.

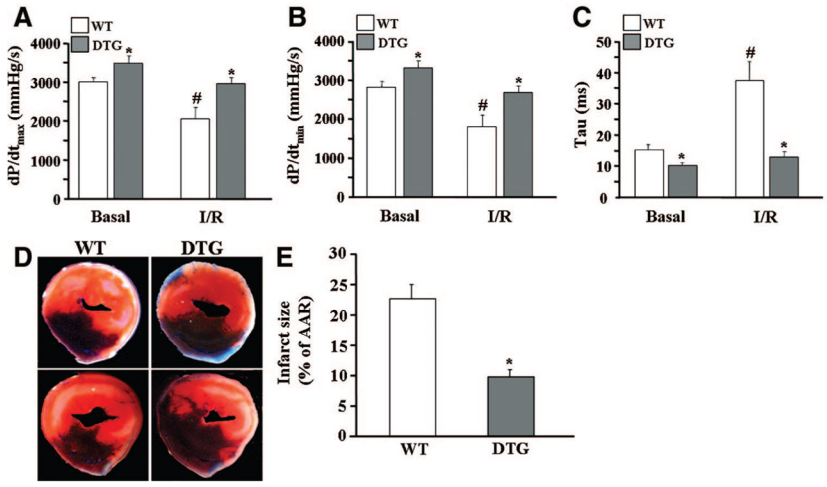

Figure 6.

I-1c attenuates contractile dysfunction and infarct size after I/R in vivo. Cardiac function was assessed after 30 minutes of ischemia, followed by 60 minutes of reperfusion, using in vivo catheterization. I-1c expression enhanced the rates of contraction (A) and relaxation (B) and decreased τ (C). **P*<0.05 vs WT under similar conditions, #*P*<0.05 vs WT basal (WT, n=4; DTG, n=4). D, Myocardial infarct size was assessed after 30 minutes of ischemia, followed by 24 hours of reperfusion. Hearts were infused with 2,3,5-triphenyltetrazolium chloride, followed by phthalo blue, and cut transversely into 2- to 3-mm-thick slices. Two representative myocardial sections from 1 heart in each group are shown. E, Quantification of infarct size. Bars represent means±SEM (WT, n=8; DTG, n=5). **P*<0.05 vs WT.

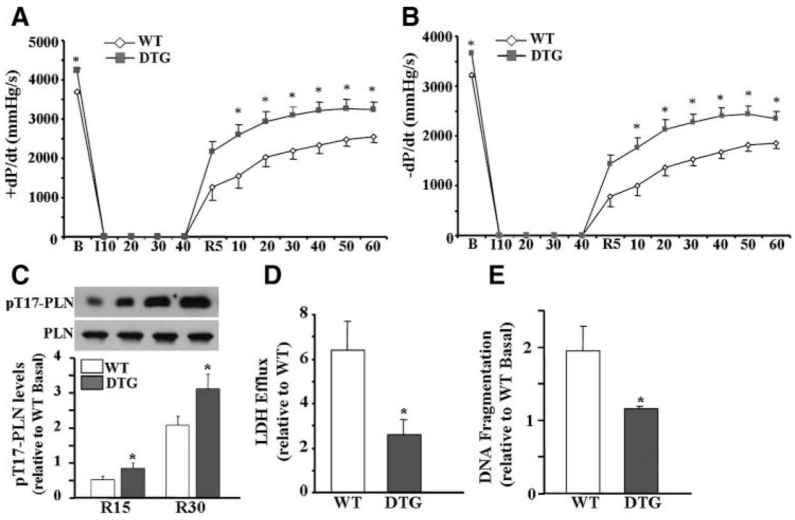

Figure 7.

I-1c improves postis-chemic cardiac performance in isolated perfused hearts. DTG hearts displayed enhanced maximal rates of contraction (A) and relaxation (B) post-I/R. Values represent means±SEM (WT, n=9; DTG, n=10). **P*<0.05 vs WT. C, Immunoblot analysis of pThr17-PLN on 15 and 30 minutes of reperfusion. Bars represent means±SEM (WT, n=3; DTG, n=3). **P*<0.05 vs WT. D, Lactate dehydrogenase (LDH) release, determined from the perfusate of hearts after 15 minutes of reperfusion. Bars represent means±SEM (WT, n=8; DTG, n=6). **P*<0.05 vs WT. E, DNA fragmentation, assessed at 120 minutes post-reperfusion. Bars represent means±SEM (WT, n=4; DTG, n=4). **P*<0.05 vs WT.

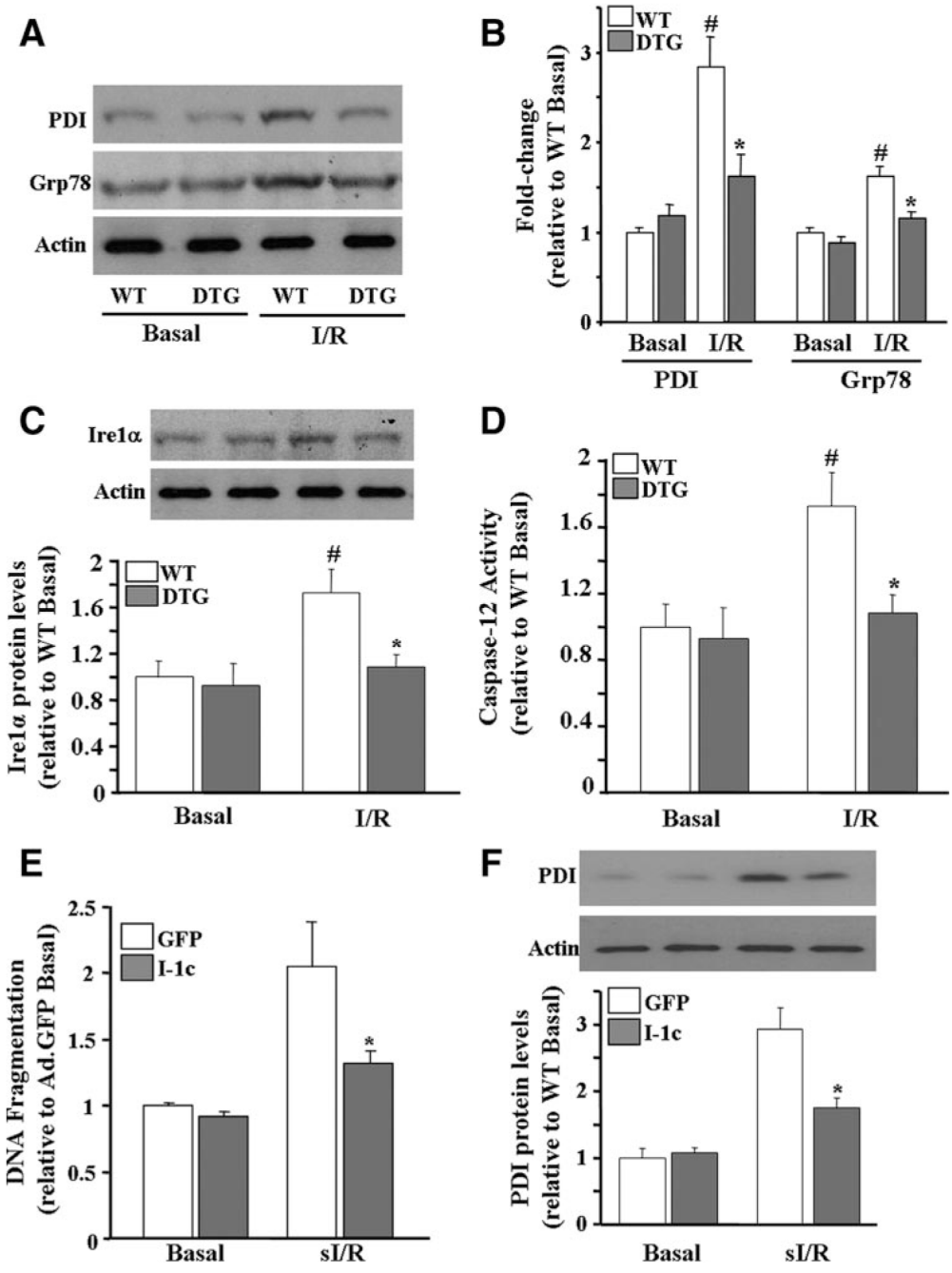

Figure 8.

I-1c attenuates the I/R-induced ER stress response. A, Immunoblot analysis of protein disulphide isomerase (PDI) and Grp78 revealed that the ER stress response was induced in WT hearts, whereas it was unaltered in DTG hearts on I/R ex vivo. B, Quantitative analysis of the blots in A, normalized to actin. C, Inositol-requiring enzyme 1α (Ire1α) increased in WT hearts post-I/R but remained unaltered in DTG hearts. D, Caspase-12 activity was increased post-I/R in WT hearts, but it was reduced to preischemic values in DTG hearts. Bars represent means ±SEM (WT, n=4; DTG, n=4). *P<0.05 vs WT I/R, #P<0.05 vs preischemic values in each group. E, DNA fragmentation was attenuated in Ad.I-1c–infected myocytes after sI/R (1 hour

of ischemia/3 hours of reperfusion). F, ER stress induction was attenuated in Ad.I-1c–infected myocytes. Bars represent means±SEM (WT, n=4; DTG, n=4). *P<0.05 vs Ad.GFP basal.