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

Persoulla Nicolaou, Patricia Rodriguez, Xiaoping Ren, Xiaoyang Zhou, Jiang Qian, Sakthivel Sadayappan, Bryan Mitton, Anand Pathak, Jeffrey Robbins, Roger J. Hajjar, Keith Jones, Evangelia G. Kranias

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. (Circ Res. 2009;104:00-00-00.)

Key Words: ischemia ▪ reperfusion ▪ protein phosphatase-1 inhibitor-1 ▪ phospholamban ▪ ER stress

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 interac-
tion 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 neurohumoral pathways associated with Ca2+ homeostasis and proper contractile function. In particular, on stimulation of the β-adrenergic axis, protein kinase (PKA) 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 (I-1c), which mitigate the beneficial effects of inhibitor-1,14,15 enhance Ca2+ 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 Ca2+ 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 Ca2+ 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

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 (right blot), 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).

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 Ca2+-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\text{min} was 3927\(\pm\)256 and 4583\(\pm\)291 mm Hg/sec, for wild-type [WT] and DTG respectively) and significant increases in basal contraction (25% increase in dP/dt\text{max}; Figure 2A) and relaxation (37% increase in dP/dt\text{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\text{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\text{50} value in PLN-deficient hearts (58% decrease, compared to WT).17 Furthermore, no differences in the maximal velocity of Ca\(^{2+}\) uptake were

![Image](https://example.com/image1.png)
was downregulated. In the second category, the phosphory-
ization 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 pro-
tein import, were increased. Notably, 3 different spots (spots
8, 9, and 18) were identified as inner mitochondrial mem-
brane protein, which may represent previously reported
spliced variants or posttranslational modifications.18 The
protein synthesis machinery was possibly increased to sup-
ter 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% de-
crease in dP/dtmax; Figure 6A) and relaxation (37% decrease
in dP/dtmin [Figure 6B]; 244% increase in τ [Figure 6C])
parameters. However, DTG hearts exhibited enhanced car-
diac 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 inter-
ruption, 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

![Figure 4](https://example.com/fig4.png)

**Figure 4.** I-1c expression enhances SERCA2a activity. A, The
initial SERCA2a Ca\(^{2+}\) uptake rates were assessed in cardiac
homogenates over a wide range of Ca\(^{2+}\) concentrations. The
data were analyzed with Origin software, and the affinity of
SERCA for Ca\(^{2+}\) (B) and the maximal velocity (C) were
calculated.

![Figure 5](https://example.com/fig5.png)

**Figure 5.** I-1c expression enhances SERCA2a activity. A, The
initial SERCA2a Ca\(^{2+}\) uptake rates were assessed in cardiac
homogenates over a wide range of Ca\(^{2+}\) concentrations. The
data were analyzed with Origin software, and the affinity of
SERCA for Ca\(^{2+}\) (B) and the maximal velocity (C) were
calculated.

![Figure 6](https://example.com/fig6.png)

**Figure 6.** I-1c expression enhances SERCA2a activity. A, The
initial SERCA2a Ca\(^{2+}\) uptake rates were assessed in cardiac
homogenates over a wide range of Ca\(^{2+}\) concentrations. The
data were analyzed with Origin software, and the affinity of
SERCA for Ca\(^{2+}\) (B) and the maximal velocity (C) were
calculated.

![Figure 7](https://example.com/fig7.png)

**Figure 7.** I-1c expression enhances SERCA2a activity. A, The
initial SERCA2a Ca\(^{2+}\) uptake rates were assessed in cardiac
homogenates over a wide range of Ca\(^{2+}\) concentrations. The
data were analyzed with Origin software, and the affinity of
SERCA for Ca\(^{2+}\) (B) and the maximal velocity (C) were
calculated.
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, 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 1, 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 1 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, 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 detrimental 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 Ca²⁺ transport and cycling, mediated by active inhibitor-1, improved postischemic injury has important implications on the role of Ca²⁺ cycling in myocardial I/R. Indeed, even though it is generally accepted that Ca²⁺ homeostasis is impaired and Ca²⁺ uptake into the SR is depressed in myocardial I/R, the effects of enhanced Ca²⁺ uptake into the SR and concomitantly increased SR Ca²⁺ 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,²¹ further supporting the beneficial effects of dis-inhibition of the SR Ca²⁺ pump. On the other hand, there exists evidence that increasing SR Ca²⁺ cycling in the ischemic heart may be detrimental. Pharmacological inhibition of SERCA2a improved postischemic recovery and overexpression of histidine-rich Ca²⁺-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,²² further supporting the notion that enhancing Ca²⁺ cycling may have deleterious effects. It is intriguing to postulate that the extent of Ca²⁺ uptake and level of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ homeostasis, have been shown to induce the UPR.³⁴,³⁷ In fact, depletion of ER Ca²⁺ stores using thapsigargin, an inhibitor of SERCA2a, is a classic pharmacological way of inducing ER stress. The apparent importance of constant Ca²⁺ levels lies in the Ca²⁺-dependent nature of ER chaperones, such as Grp78, for activation. As such, the altered Ca²⁺ 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.³⁹ 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 Ca²⁺ homeostasis in the SR/ER by augmenting Ca²⁺ cycling in this organelle.

Collectively, the present findings indicate that enhancing Ca²⁺ 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.

Acknowledgments

We thank K.D. Greis and M.A. Wyder from the University of Cincinnati Proteomics Laboratory for performing the phosphoproteomic studies.

Sources of Funding

This work was supported by NIH grants HL-26507, HL-64018, and HL-77101 (to E.G.K.); the Leducq Foundation (to E.G.K.); and AHA Predoctoral Fellowship 0715500B (to P.N.).

Disclosures

E.G.K. is a scientific founder of Nanocor. R.J.H. is a founder of Celladon and Nanocor.

References


Circulation Research. April 24, 2009
Inhibitor-1 Alleviates Ischemia/Reperfusion Injury

Nicolau et al


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

Persoulla Nicolaou, Patricia Rodriguez, Xiaoping Ren, Xiaoyang Zhou, Jiang Qian, Sakthivel Sadayappan, Bryan Mitton, Anand Pathak, Jeffrey Robbins, Roger J. Hajjar, Keith Jones and Evangelia G. Kranias

Circ Res. published online March 19, 2009;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2009/03/19/CIRCRESAHA.108.189811.citation

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/03/19/CIRCRESAHA.108.189811.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
SUPPLEMENT MATERIAL

MATERIALS AND METHODS

Generation of an inducible double transgenic mouse model with cardiac-specific expression of a truncated, active form of inhibitor-1 (I-1c)

The T35D-I-1 mutant was derived from the full-length mouse I-1, using site-directed mutagenesis. Subsequently, the truncated, constitutively active I-1 was generated by polymerase chain reaction (PCR) from the I-1-T35D cDNA, using the forward primer: 5’ – CAGA GGATCC ATG GAG CCC GAC AAC AGC CC – 3’; and the reverse primer: 5’ – CAGA GGATCC TCA TGA CAA GGT GGA CTT GAG AAG – 3’ (BamHI restriction enzyme sites underlined). The I-1c cDNA was cloned into a vector bearing an attenuated α-myosin heavy chain promoter (α-MHCp) in which the three GATA sites and the two thyroid responsive elements were replaced with seven repeats of the TetO sequence [α-MHCmin-(TetO)7]. The completed construct was submitted to the University of Cincinnati Transgenic Mouse Core for pronuclear microinjection. The I-1c single transgenic mice (TG2) were crossed with mice carrying the tetracycline-controlled transactivator (tTA) gene, which is under the regulation of the traditional α-MHCp (TG1). Mice carrying both transgenes are designated double transgenic (DTG). I-1c expression was effectively suppressed, using doxycycline (Dox) chow (625 mg/kg). Removal of Dox from their diet allowed for I-1c expression. The α-MHCmin-(TetO)7 promoter plasmid as well as the single transgenic TG1 mice were provided by Dr. Jeffrey Robbins of Cincinnati Children’s Hospital, OH. Animals were handled as approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Detection of inhibitor-1

Cardiac tissue from mice was enriched in I-1, using the trichloroacetic acid (TCA) method, as previously detailed (1). Briefly, 400 mg of frozen tissue was pulverized and
homogenized in ice-cold phosphate-buffered solution. Subsequently, TCA was added to a final concentration of 1.5% (w/v) and rotated for 1 hour at 4°C before centrifugation at 9,000 rpm for 30 min. The supernatant was adjusted to 15% TCA and rocked overnight at 4°C. This was centrifuged at 18,000 rpm and the pellet was resuspended in 0.5 mmol/L Tris-HCl, pH=8.0 and subjected to Western blotting, as described below.

**Immunoblotting**

Alterations in the phosphorylation and/or total level of proteins were analyzed from whole heart homogenates, with the exception of MyBP-C, which was analyzed from a myofibril protein preparation (2), using Western blotting. Briefly, 2-70 µg of protein were separated on polyacrylamide gels (6-15%) and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies, which were visualized by peroxidase-conjugated secondary antibodies (Amersham Biosciences) and enhanced chemiluminescence (Supersignal West Pico Chemiluminescent, Pierce). The bands were then quantified with densitometry, using ImageQuant 5.2. The antibodies were obtained from the sources listed: pSer16-PLN, pThr17-PLN and pSer2808-RyR (Badrilla), PLN (Upstate), RyR and actin (Sigma), pSer22/pSer23-TnI and TnI (Research Diagnostics Inc.), pSer282-MyBP-C (custom-made commercially, ProSci Inc.), MyBP-C (home-made, references 2, 3), I-1 and SERCA2a (custom-made commercially, Affinity Bioreagents), CSQ (Affinity Bioreagents), PDI (Alexis Biochemicals), Grp78 (Santa Cruz Biotechnology), Ire1α (Abcam), pSer116-Bad, pSer136-Bad and Bad (Cell Signaling).

**SR Ca\(^{2+}\)-uptake**
Initial SR Ca\textsuperscript{2+}-uptake rates were determined in cardiac homogenates, using the Millipore filtration technique and \textsuperscript{45}CaCl\textsubscript{2}, as previously described (4). Briefly, 100-250 \(\mu\)g of cardiac homogenate were incubated at 37\(^\circ\)C in a reaction buffer containing: 40 mmol/L imidazole, pH=7.0, 95 mmol/L KCl, 5 mmol/L NaN\textsubscript{3}, 5 mmol/L MgCl\textsubscript{2}, 0.5 mmol/L EGTA, and 5 mmol/L potassium oxalate. The initial uptake rates were determined over a wide range of Ca\textsuperscript{2+} concentrations (pCa 8 to 5). Ca\textsuperscript{2+}-uptake into SR vesicles was initiated by addition of 5 mmol/L ATP, and aliquots were filtered through a 0.45 \(\mu\)m Millipore filter after 0, 30, 60 and 90 seconds. The specific \textsuperscript{45}Ca\textsuperscript{2+}-uptake values were analyzed by non-linear regression, using the OriginLab 5.1 program to obtain the Ca\textsuperscript{2+} affinity (EC\textsubscript{50}) and the maximal Ca\textsuperscript{2+}-uptake rate (V\textsubscript{max}).

**In vivo catheterization**

To assess the effects of I-1c expression on left ventricular contractile parameters, hemodynamic and pressure volume loops were recorded during steady state, using a 1.4-French scale Millar catheter. Mice were anesthetized with sodium pentobarbital (80 mg/kg) and the right carotid artery was cannulated with a microtip pressure transducer catheter (SPR-839, Millar Instruments) connected to the MPVS-300 pressure-volume signal conditioning hardware, which provided analog outputs of ventricular pressure and volume signals for data acquisition over time. Analysis of the data was carried out, using Millar's PVAN software (Version 3.5).

**Two dimensional (2D) gel electrophoresis and image analysis**

Hearts were dounce-homogenized at 4\(^\circ\)C in a buffer consisting of 10 mmol/L Tris, supplemented with protease (Roche) and phosphatase inhibitor cocktails (Sigma). Cardiac homogenates were centrifuged at 800 x g for 10 min at 4\(^\circ\)C and the supernatant was subsequently
diluted 1:1 with extraction/lysis buffer (9 mol/L urea, 4% CHAPS, 0.1% SDS). 150 µg of protein per sample was then separated by 2D electrophoresis, using pre-cast immobilized pH gradient strips (Amersham), with pH ranges 3-10, as previously described (5). Protein concentration was determined, using the Non-Interfering Protein Assay (G-Biosciences, St Louis, USA). For isoelectric focusing (IEF), the samples were loaded on the immobilized pH gradient strip by in-gel sample rehydration, using GE healthcare destreak rehydration solution (GE healthcare) as a solubilizing agent overnight. Isoelectric focusing on an multiphor II isoelectrofocusing unit (Amersham/GE Healthcare) was conducted at 20°C, focused for 18:31 hours (0-300 V in 1 min (gradient), 300 V for 6 h; 300-3500 V in 1:30 h (gradient), 3500 V for 12 h) for a total of 47.5 kVhr. IPG strips were equilibrated 2 times for 15 min in 40 ml of equilibration buffer with DTT and 1 time for 15 min with equilibration buffer plus iodoacetic acid (IAA). The equilibration buffer consisted of: 6 mol/L urea, 112 mmol/L Tris/acetate, 30% v/v glycerol, 5% w/v SDS, 0.01% w/v bromophenol blue and freshly added DTT (2% w/v) or IAA (2.5% w/v); pH= 8.8). Strips were embedded on top of 10% T (total monomer), 2.2% C (cross-linking agent) duracryl double gels (220 x 220 x 1.5 mm; Nextgen Sciences Inc) after filling the chambers of the vertical SDS-PAGE unit (Genomic Solution) with appropriate buffers (anode buffer: 210 mmol/L Tris/acetate, pH=8.9; cathode buffer: 100 mmol/L Tris, 100 mmol/L tricine, 0.1% w/v SDS). The second dimensional electrophoresis was carried out at 8°C with constant current (50 mA per gel) for approximately 6 hours. The protein spots were visualized by either silver staining, using a Silver stain kit (Nextgen Sciences Inc) or ProQ-Diamond staining (Invitrogen/Molecular Probes). ProQ-stained gels were scanned with a FUJI FLA-5100 fluorescence imager (532nm excitation laser, FUJI LPG filter at 100 µm resolution). Silver-stained gels were scanned as 16 bit grayscale TIF images, using Image Scanner II, (Amersham/GE Healthcare) and digital
quantified with the differential display image analysis software Progenesis Same Spots (Nonlinear Dynamics Ltd). The value for each spot was calculated as a proportion of the total spot volume of all spots in the gel, following background subtraction and removal of other artifacts. Differential expression between the groups was determined as a fold-change and proteins with the most appropriate change in expression between the groups were selected for analysis by mass spectrometry.

**Protein digestion and peptide mass spectrometry**

Protein spots were excised, destained, dehydrated from the gels and subjected to a tryptic digestion as originally described by Shevchenko (6) with modifications as presented by Jarrold (7). The extracted peptides were concentrated in a Speed-Vac centrifuge (Savant) to a final volume of 10-15 µl. Peptides were desalted and purified utilizing the C18 ZipTips® (Millipore). Purified peptides were eluted with 2.5 µl of 0.3% trifluoroacetic acid in 60% v/v acetonitrile, followed by peptide concentration to a final volume of 0.5 µl by Speed-Vac centrifugation. Subsequently, 1 µl of matrix solution (0.1% trifluoroacetic acid in 50% v/v acetonitrile + 5 mmol/L ammonium phosphate monobasic + 5 µg/µl α-cyano-4-hydroxycinnamic acid) was added to each sample and transferred directly onto a matrix assisted laser desorption/ionization (MALDI) target plate (Applied Biosystems). MALDI-MS/MS analysis was performed on a 4800 MALDI-TOF-TOF instrument from Applied Biosystems operated in reflector positive mode followed by automate transition to a 2kV MS/MS mode to produce fragmentation spectra on the 15 most abundant peptides in each sample. Proteins were subsequently identified from the MS/MS fragmentation spectrum, using the GPSExplorer analysis software (Applied Biosystems) coupled to an in house MASCOT server (Matrix Science). Criteria for protein identification
included peptide composite ion scores of greater than 100 with a minimum of 2 MS/MS spectra per protein.

**Global ischemia ex vivo**

The role of I-1c in ischemic injury was assessed, using an isolated perfused heart model, as described previously (8, 9). Briefly, WT and DTG hearts were mounted on the Langendorff apparatus and perfused with Krebs-Henseleit physiological solution. A plastic, water-filled balloon was inserted in the left ventricle via the mitral valve and inflated to yield a left ventricular end-diastolic pressure (LVEDP) of 5-10 mmHg. The balloon was attached to a pressure transducer connected to a Heart Performing Analyzer (Micro-Med) and left ventricular developed pressure (LVDP) was monitored continuously. After a 30-min stabilization period, the hearts were subjected to 40 min of no-flow, global ischemia, followed by 15-120 min of reperfusion. Hearts were paced at 400 bpm except during ischemia.

**Regional ischemia in vivo**

I/R injury was induced *in vivo*, as described previously (8, 9). Briefly, mice were subjected to myocardial I/R by ligating the left anterior descending coronary artery (LAD) for 30 min, followed by release of the ligation. Contractile function was assessed after 60 min of reperfusion, using open-chested catheterization *in vivo*. Analysis of the data was carried out, using SonoSOFT software (Version 3.4.45). In separate experiments, the hearts were subjected to 30 min of ischemia and allowed to reperfuse for 24 hrs to assess infarct size. Specifically, the heart was removed from the animal, the aorta was cannulated and the heart was perfused with 1% 2, 3, 5-Triphenyltetrazolium chloride (TTC). The occluder, which had been left in place, was
re-tied and the heart was perfused with 5% phtalo blue. Hearts were frozen and cut into 5 or 6 transverse sections, with one section made at the site of the ligature. The slices were analyzed for infarct area, area at risk and total left ventricular area, using ImageJ software, available through the National Institute of Health (NIH). The infarct size was then expressed as a percentage of the area at risk.

**Lactate dehydrogenase (LDH) release**

Release of LDH was measured from the outflow of perfused hearts after I/R, using an assay kit, according to manufacturer’s instructions (Sigma). In particular, 100 µl of LDH assay mixture, consisting of equal volumes of LDH assay substrate, cofactor and dye solution, were added to 50 µl of perfusate in a 96-well plate. The plate was incubated in the dark for 30 min. The reaction was quenched by addition of 15 µl of 1N HCl and absorbance was measured in a spectrophotometer at 490 nm. Values were normalized to the volume of the effluent and the heart weight.

**DNA fragmentation**

DNA fragmentation was assessed, using a commercially available ELISA kit (Roche Applied Science), which measures cytosolic mono- and oligo-nucleosomes, as described previously (8, 9). Briefly, 50 µg of heart homogenate was combined with the immunoreagent in streptavidin-coated plates and incubated for 2 hours at room temperature. After extensive washing, color development was initiated with 100 µl of the colorimetric substrate, ABTS. The extent of apoptosis was quantified, using an ELISA plate reader at 405 nm.
Caspase activity

The activities of caspase-9 and 12 were assessed in a fluorometric assay, according to the manufacturer’s instructions (Biovision). In particular, 100 µg of protein were diluted in 50 µl of cell lysis buffer and incubated on ice for 10 min. 50 µl of 2X Reaction buffer (containing 10 mmol/L DDT) were added to the samples. The reaction was initiated with addition of 5 µl of the caspase-specific fluorescent substrate and the reaction was carried out at 37°C for 2 hours in the dark. Caspase activity was quantified in a fluorometer (excitation: 400 nm, emission: 505 nm).

Isolation of adult rat cardiomyocytes and simulated ischemia/reperfusion

Animals were handled according to the Institutional Animal Care and Use Committee at the University of Cincinnati. Myocytes from adult male Sprague-Dawley rats (~300 grams) were isolated by collagenase digestion, as previously described (1, 10). Myocytes were resuspended in modified culture medium (M199, Sigma), counted and plated on laminin-coated plates or dishes for 2 hours at 37°C in a humidified, 5% CO₂ incubator and subsequently infected with the adenoviruses at a multiplicity of infection (MOI) of 500 for 2 hours. Construction of the viruses has been previously described (11). At 24 hours post-infection, the cells were subjected to 1 hour of ischemia followed by 3 hours of reperfusion, as previously described (10). Specifically, the media was replaced with ischemic buffer containing 1.13 mmol/L CaCl₂, 5 mmol/L KCl, 0.3 mmol/L KH₂PO₄, 0.5 mmol/L MgCl₂, 0.4 mmol/L MgSO₄, 128 mmol/L NaCl, 4 mmol/L NaHCO₃, 10 mmol/L HEPES, pH=6.8, and placed in a chamber mimicking the hypoxic (1% O₂) and hypercapnic conditions (20% CO₂), observed during ischemia. The ischemic buffer was replaced with normal media and the cells were placed back in the humidified chamber at
atmospheric conditions, to allow reperfusion for 3 hours. The cells were harvested and stored at -80°C until further analysis.

**Statistical Analysis**

All the values are expressed as mean ± SEM for n experiments. Comparisons between two groups were evaluated by Student’s t-test for unpaired data. Statistical analysis of multiple groups was carried out by one-way ANOVA, with a Tukey test for post-hoc analysis. Results were considered statistically significant at p< 0.05.
REFERENCES:


**Online Figure I: Expression of I-1c in the adult mouse heart enhances basal contractility.**

Analysis of contractile parameters indicated that the maximum rates of contraction (A) and relaxation (B) were increased, while the time constant of relaxation (Tau) was decreased (C) by I-1c expression, in a temporal manner. 4 weeks of expression elicited significant increases in contractile parameters, similar to DTG mice without Dox. TG2 mice, carrying only the I-1c transgene and DTG mice kept on a Dox regimen displayed contractile parameters similar to WT, suggesting no leakage of the TG2 attenuated promoter. WT, n=8; TG2, n=7 and DTG, n=6, *p<0.05 vs. WT; #: p<0.05 DTG vs. DTG with no Dox.

**Online Table I: Phospho-proteomic analysis of I-1c hearts**

Phospho-proteomic analysis revealed that four phospho-protein and eight protein spots were altered in I-1c hearts (Figure 5). Mass spectroscopy analysis positively identified ten out of these twelve spots. The spot number, identity (ID) and fold change are indicated in the table. WT, n=3, DTG, n=3.

**Online Figure II: I-1c expression improves post-ischemic functional recovery, during early reperfusion in isolated perfused hearts.**

After 30 min of stabilization, hearts were subjected to 40 min of ischemia followed by 60 min of reperfusion. The graphs represent the percent recovery of the rates of contraction (A) and relaxation (B), normalized to pre-ischemic values, in WT and DTG hearts. Values represent mean ± SEM; WT, n=9, DTG, n=10; *p<0.05 vs. WT.
Online Figure III: I-1c expression does not alter the protein or phosphorylation levels of Bad and caspase-9 activity after I/R ex vivo.

A. Immunoblot analysis revealed that the protein levels of Bad were reduced after I/R, to a similar extent in WT and DTG Langendorff-perfused hearts. Phosphorylation of Bad was unaltered by the I/R treatment and was similar between the two groups. B. Quantitative analysis of the immunoblots shown in (A), normalized to Bad protein levels and actin, for Bad phosphorylation and Bad protein levels, respectively. C. Caspase-9 activity was enhanced to a similar extent in both WT and DTG hearts, in the post-ischemic heart. Bars represent mean ± SEM; WT, n=4, DTG, n=4; *p<0.05 vs. WT Basal.
Online Figure I

A. +dP/dt (mmHg/s)

B. -dP/dt (mmHg/s)

C. Tau (msec)
## Online Table I

<table>
<thead>
<tr>
<th>Spot #</th>
<th>ID</th>
<th>Fold-change</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>contrapsin</td>
<td>+1.52</td>
</tr>
<tr>
<td>2</td>
<td>Tu translation elongation factor, mitochondrial</td>
<td>+1.67</td>
</tr>
<tr>
<td>34</td>
<td>enoyl coenzyme A hydratase 1, peroxisomal</td>
<td>+75.2</td>
</tr>
<tr>
<td>18</td>
<td>inner membrane protein, mitochondrial</td>
<td>+1.96</td>
</tr>
<tr>
<td>9</td>
<td>inner membrane protein, mitochondrial</td>
<td>+1.91</td>
</tr>
<tr>
<td>8</td>
<td>inner membrane protein, mitochondrial</td>
<td>+1.68</td>
</tr>
<tr>
<td>25</td>
<td>electron transferring flavoprotein, dehydrogenase</td>
<td>+1.62</td>
</tr>
<tr>
<td>35</td>
<td>glucose phosphate isomerase 1</td>
<td>-1.54</td>
</tr>
<tr>
<td>30</td>
<td>creatine kinase, mitochondrial 2</td>
<td>+1.60</td>
</tr>
<tr>
<td>4</td>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit</td>
<td>+2.14</td>
</tr>
</tbody>
</table>
Online Figure II

A.

B.
Online Figure III

A.

<table>
<thead>
<tr>
<th></th>
<th>WT Basal</th>
<th>WT I/R</th>
<th>DTG Basal</th>
<th>DTG I/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>pS116-Bad</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pS136-Bad</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bad</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>DTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pS116-Bad</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>pS136-Bad</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Bad</td>
<td>0.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

C.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>DTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-9 Activity (relative to WT Basal)</td>
<td>0.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* indicates significant difference