PHLPP-1 Negatively Regulates Akt Activity and Survival in the Heart

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Rationale: The recently discovered PHLPP-1 (PH domain leucine-rich repeat protein phosphatase-1) selectively dephosphorylates Akt at Ser473 and terminates Akt signaling in cancer cells. The regulatory role of PHLPP-1 in the heart has not been considered.

Objective: To test the hypothesis that blockade/inhibition of PHLPP-1 could constitute a novel way to enhance Akt signals and provide cardioprotection.

Methods and Results: PHLPP-1 is expressed in neonatal rat ventricular myocytes (NRVMs) and in adult mouse ventricular myocytes (AMVMs). PHLPP-1 knockdown by small interfering RNA significantly enhances phosphorylation of Akt (p-Akt) at Ser473, but not at Thr308, in NRVMs stimulated with leukemia inhibitory factor (LIF). The increased phosphorylation is accompanied by greater Akt catalytic activity. PHLPP-1 knockdown enhances LIF-mediated cardioprotection against doxorubicin and also protects cardiomyocytes against H₂O₂. Direct Akt effects at mitochondria have been implicated in cardioprotection and mitochondria/cytosol fractionation revealed a significant enrichment of PHLPP-1 at mitochondria. The ability of PHLPP-1 knockdown to potentiate LIF-mediated increases in p-Akt at mitochondria and an accompanying increase in mitochondrial hexokinase-II was demonstrated. We generated PHLPP-1 knockout (KO) mice and demonstrate that AMVMs isolated from KO mice show potentiated p-Akt at Ser473 in response to agonists. When isolated perfused hearts are subjected to ischemia/reperfusion, p-Akt in whole-heart homogenates and in the mitochondrial fraction is significantly increased. Additionally in PHLPP-1 KO hearts, the increase in p-Akt elicited by ischemia/reperfusion is potentiated and, concomitantly, infarct size is significantly reduced.

Conclusions: These results implicate PHLPP-1 as an endogenous negative regulator of Akt activity and cell survival in the heart. (Circ Res. 2010;107:476-484.)

Key Words: Akt ■ PHLPP ■ phosphatase ■ heart ■ protection

Numerous studies have demonstrated that activation of Akt contributes to the cardioprotective effects of receptors tyrosine kinases,1,2 glycoprotein 130–linked receptors,3–5 and G protein–coupled receptors.6,7 These receptors activate phosphatidylinositol 3-kinase (PI3K) and the resultant increase in phosphatidylinositol (3,4,5) triphosphate (PIP₃) levels drives Akt translocation to the plasma membrane. Akt is subsequently activated through phosphorylation at Thr308 by the upstream kinase phosphoinositide-dependent kinase 1 (PDK1) and phosphorylation at Ser473 by a mechanism that depends on both TORC2 and the intrinsic catalytic activity of Akt.8,9 A recent study identified a PH domain-only protein, PHLDA3, that competes with the PH domain of Akt for binding of PIP₃.11 These molecules regulate the activation of Akt via various mechanisms but far less is known about mechanisms involved in terminating Akt activity by its dephosphorylation.

Protein phosphatase (PP)2A has been shown to dephosphorylate Akt at Thr308 and/or Ser473 in noncardiac cells.12,13 A pharmacological study also suggests that in retina PP2B (calcineurin) can dephosphorylate Akt at both sites.14 A more specific Akt-directed novel PP2C family member protein phosphatase, PHLPP (PH domain leucine-rich repeat protein phosphatase),15–17 has been recently identified. Two isoforms of PHLPP, PHLPP-1 and PHLPP-2, have been shown to selectively dephosphorylate the hydrophobic motif of Akt (Ser473), terminating Akt signaling.15,16 PHLPP levels

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are markedly reduced in several cancer cell lines, resulting in elevated Akt activation. Conversely heterologous expression of PHLPP in cancer cells can prevent Akt activation and promote apoptotic death. In cardiac myocytes, overexpression of PTEN has been shown to be proapoptotic, whereas genetic deletion of PTEN rescues hearts from ischemia/reperfusion (I/R) injury. These data support observations made in noncardiac cells which demonstrate that modulation of Akt activity regulates cell survival. A recent article showed that either PP2A or PP2B (calcineurin) can dephosphorylate Akt and thereby, regulate insulin signaling in cardiomyocytes. It has been generally believed that phosphatases such as PP2A and PP2B have poor substrate selectivity, eliciting dephosphorylation of diverse target molecules. In contrast, PHLPP has been reported to be a selective Akt phosphatase. In this study, we demonstrate a role for endogenous PHLPP-1 in regulation of cardiomyocyte Akt activity and survival in vitro and in vivo.

**Methods**

PHLPP-1 knockout (KO) mice were generated in the C57BL/6 strain as described previously. All mice used in the present study were male at 8 to 10 weeks of age. All procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. To knockdown PHLPP-1, presigned PHLPP-1 ON-TARGETplus small interfering (si)RNA for rat and control siRNA were purchased from Thermo Scientific. NRVMs were transfected with siRNA using DharmaFECT-I transfection reagent (Thermo Scientific) based on the instructions of the manufacturer, with additional details in the expanded Methods section (Online Data Supplement, available at http://circres.ahajournals.org). Results are reported as averages ± SEM. Statistical significance was determined using ANOVA followed by the Tukey post hoc test. P < 0.05 was considered statistically significant. For additional details regarding the methods used, see the Online Data Supplement.

**Results**

Northern blotting of various human tissues indicates that PHLPP-1 mRNA is most highly expressed in the brain, as previously reported, but is also present at significant levels in the heart (Figure 1A). To determine that PHLPP-1 protein is expressed in mouse, we evaluated PHLPP-1 expression by Western blotting in adult mouse tissues from wild-type (WT) and PHLPP-1 KO mice. PHLPP-1 appeared at the expected molecular weight of ~180 kDa and was highly expressed in the brain. PHLPP-1 was also clearly detected in the heart (Figure 1B), in isolated adult mouse ventricular myocytes (AMVMs) and in neonatal rat ventricular myocytes (NRVMs) (Figure 1C). PHLPP-2 mRNA was also present in isolated AMVMs. PHLPP-2 knockdown in NRVMs did not significantly affect phosphorylation of Akt at Ser473 (Online Figure I); thus, we focused our attention on examining the regulatory role of PHLPP-1 in cardiomyocytes.

To determine whether PHLPP-1 regulates Akt phosphorylation in cardiomyocytes, PHLPP-1 expression was inhibited using siRNA. Significant knockdown of PHLPP-1 was achieved by siRNA treatment of NRVMs (Figure 2A). To evaluate the functional effect of PHLPP-1 knockdown, NRVMs were stimulated with leukemia inhibitory factor (LIF) and levels of Akt phosphorylation at both Ser473 and Thr308 were examined (Figure 2B). LIF treatment elicited a greater than 3- to 4-fold peak increase in phosphorylation of Akt at Ser473 and Thr308 at 10 minutes and this declined to basal levels by 40 minutes. The magnitude of the response monitored by Akt Ser473 phosphorylation was significantly greater in cardiomyocytes treated with PHLPP-1 siRNA. Notably, LIF-induced phosphorylation of Akt at Thr308 was not significantly changed by the knockdown of PHLPP-1. PHLPP-1 knockdown-mediated potentiation of Akt phosphor-
ylation at Ser473 was also observed in NRVMs stimulated with insulin-like growth factor (IGF)-1 or sphingosine-1-phosphate (S1P) (Figure 2C), although T308 phosphorylation was not potentiated (data not shown). These data suggest that endogenous PHLPP-1 selectively regulates the extent of Akt phosphorylation at Ser473 in response to agonist, consistent with the initial study demonstrating that PHLPP-1 phosphatase activity is selective for Akt dephosphorylation at Ser473.15

The effect of phosphorylation of the Ser473 regulatory sites on Akt function is controversial. Accordingly, we assessed the effect of PHLPP-1 knockdown on Akt catalytic activity in our system using an in vitro kinase assay with a glycogen synthase kinase (GSK)3/β-peptide as substrate. As shown in Figure 2D, LIF increased Akt activity to a significantly greater extent in PHLPP-1 siRNA–treated cells compared to control cells. Treatment with PHLPP-1 siRNA did not affect expression of total Akt or of gp130, the receptor for LIF (data not shown). Thus, the relative increase in LIF-stimulated Akt activity in PHLPP-1 knockdown compared to control cells appears to result from the increase in Ser473 phosphorylation rather than upregulation of total Akt or the receptor. It has been demonstrated that PHLPP-1 deletion increased levels of conventional and novel protein kinase (PKC)α, βII, and ε in non cardiac cells.23 However, there were no significant changes in PKCα, βII, -δ, and -ε in NRVMs transfected with PHLPP-1 siRNA (Figure 2E).

Previous work from the Newton laboratory demonstrated that in cancer cell lines PHLPP-1 has selectivity for Akt-2 versus Akt-1.16 To determine whether PHLPP-1 differentially dephosphorylates Akt-1 and Akt-2 in cardiomyocytes, we immunoprecipitated either the Akt-1 or Akt-2 isoform from cells stimulated with LIF before Western blotting with P-Ser473 antibody (Figure 3A). Unexpectedly, PHLPP-1 knockdown induced comparable enhancement in Ser473 phosphorylation of Akt-1 and Akt-2, indicating that in cardiomyocytes PHLPP-1 dephosphorylates both Akt isoforms. The kinase activity assay likewise demonstrated that LIF-induced increases in both Akt-1 and Akt-2 catalytic activities are enhanced by PHLPP-1 knockdown (Figure 3B). It has also been reported that Akt-1 and Akt-2 have different substrate specificity, with GSK-3α preferentially phosphorylated by Akt-2 in noncardiac cells.16 We observed, however, that LIF-induced phosphorylation of GSK-3α and -3β were both significantly enhanced by PHLPP-1 knockdown (Figure 3C). These data indicate that PHLPP-1 can affect activation of and substrate phosphorylation by both of the major cardiac Akt isoforms.

To determine whether the increased Akt activity provided by PHLPP-1 knockdown translates into enhanced protection of cardiomyocytes, cells were transfected with control or PHLPP-1 siRNA and treated with doxorubicin, a chemotherapeutic agent known to exert significant cardiotoxic effects.
Several published reports have demonstrated that doxorubicin-induced cell death is prevented by agonists that stimulate Akt, including LIF.24 Thus, we determined whether PHLPP-1 knockdown enhances LIF-mediated protection against doxorubicin. A robust apoptotic response was induced by 18-hour treatment with 500 nmol/L doxorubicin, and LIF treatment was protective (Figure 4). The LIF-mediated protection was enhanced significantly in cardiomyocytes in which PHLPP-1 was knocked down. In the presence of Akt inhibition, the protective effects of LIF treatment and PHLPP-1 siRNA treatment were reversed, indicating that the observed effects of LIF and PHLPP-1 siRNA treatment are mediated through changes in Akt activity. These data provide evidence that the enhanced Akt phosphorylation and activity achieved by PHLPP-1 knockdown is functionally important in increasing the protective effects of an Akt activating ligand.

We recently reported that activated Akt translocates to mitochondria where it protects mitochondria against loss of function and attenuates cardiomyocyte cell death.5 We hypothesized that PHLPP-1 might also localize at mitochondria where it could locally regulate Akt activity and mitochondrial integrity. To explore this possibility, mitochondrial and cytosolic fractions were prepared as described in our previous work (Figure 5A). A significant amount of PHLPP-1 protein was found in the mitochondrial fraction. Treatment with PHLPP-1 siRNA was used to confirm the identity of the immunoreactive protein and also demonstrated that the protein detected in both fractions could be significantly downregulated.

To determine whether mitochondrial Akt activation is enhanced by PHLPP-1 knockdown, cardiomyocytes were treated with LIF, fractionated and Ser473 phosphorylated Akt levels examined. LIF increased phosphorylation of Ser473 in mitochondria of control cardiomyocytes and this response was 3-fold greater in PHLPP-1 siRNA–treated cells (Figure 5B). Increase in total Akt in mitochondrial fraction induced by LIF was not enhanced by PHLPP-1 knockdown (Figure 5C). Our previous study demonstrated that Akt increased the phosphorylation and association of hexokinase (HK)-II with mitochondria.5 Here, we demonstrate that PHLPP-1 knockdown also enhances the LIF-induced increase in HK-II association with mitochondria (Figure 5D). IGF-1–mediated increases in phosphorylated Akt and HK-II in the mitochondrial fraction were also significantly potentiated by PHLPP-1 knockdown (Online Figure II). These data support the notion that mitochondrial Akt activity is regulated by endogenous PHLPP-1. It has been demonstrated that nuclear Akt activity is increased in response to agonist stimulation.25 We determined whether nuclear Akt activity is controlled by PHLPP-1 by nuclear/cytosolic fractionation. PHLPP-1 was not detectable in the nuclear fraction (Figure 5E) and increase in phosphorylated Akt in the nuclear fraction induced by LIF was not potentiated by PHLPP-1 knockdown (Figure 5F).

Oxidative stress and I/R activate Akt and H₂O₂ treatment mimics this response in cardiomyocytes. Activation of Akt by H₂O₂ in cardiomyocytes is increased by PHLPP-1 knockdown, as demonstrated by enhanced and prolonged Akt Ser473 phosphorylation (Figure 6A). PHLPP-1 expression was not affected by H₂O₂ (Online Figure III). Phosphorylation...
tion of Akt at Thr308 was increased by H$_2$O$_2$, which was unaffected by PHLPP-1 knockdown (data not shown). H$_2$O$_2$-induced apoptosis was assessed after 20 hours of H$_2$O$_2$ treatment by measuring DNA fragmentation and found to be significantly attenuated by PHLPP-1 knockdown (Figure 6C). Addition of Akt inhibitors fully prevented the protective effect of PHLPP-1 knockdown, demonstrating that Akt activation is responsible for the effect of PHLPP-1 knockdown. H$_2$O$_2$-induced activation of caspase-9 and caspase-3 was also significantly attenuated by PHLPP-1 knockdown (Online Figure IV).

To extend our findings on the role of PHLPP-1 in response to oxidative stress, we examined the participation of PHLPP-1 in the isolated perfused heart subject to I/R. Robust time-dependent increases in phosphorylation of Akt at Ser473 were evident in Langendorff-perfused mouse hearts subject to ex vivo I/R (Figure 7). The peak increase was seen at 30 minutes of reperfusion and phosphorylation declined to basal levels by 120 minutes. Phosphorylation of Akt at Thr308 and GSK3 was also increased by ex vivo I/R in mouse hearts, suggesting increases in Akt kinase activity (data not shown). Phosphorylation of Akt was also observed in the mitochondrial fraction and showed kinetics similar to that observed in whole-heart homogenates. Mitochondrial association of HK-II, used as a readout for Akt activation at mitochondria, was also significantly attenuated by PHLPP-1 knockdown (Online Figure IV).

We then examined the effect of PHLPP-1 deletion on Akt phosphorylation in response to ex vivo I/R (Figure 8E). Akt phosphorylation was increased in response to I/R (30 minutes/30 minutes) and there was a significant enhancement of Akt Ser473 phosphorylation in the whole-heart homogenates from PHLPP-1 KO (Figure 8E, left). To determine whether phosphorylated Akt and HK-II at mitochondria are enhanced by PHLPP-1 deletion, mitochondria were isolated and subjected to Western blotting. Increases in phosphorylated Akt at Ser473 and HK-II in the mitochondrial fraction induced by I/R were markedly enhanced in PHLPP-1 KO (Figure 8E, middle and right). Mitochondrial total Akt was also increased.

**PHLPP-1 knockout mice were recently generated in our laboratories.** These mice lack PHLPP-1 expression in the heart (Figures 1B and 8A) but have no overt basal cardiac phenotype. AMVMs isolated from WT and PHLPP-1 KO mice were stimulated with LIF for various times to assess phosphorylation of Akt. There was a marked increase in LIF-induced Akt phosphorylation at Ser473 in AMVMs isolated from PHLPP-1 KO mice (Figure 8B), whereas increase in phosphorylation of Akt at Thr308 was not changed (data not shown). PHLPP-1 deletion also enhanced phosphorylated Akt at S473 induced by IGF-1 or S1P (Figure 8C), without affecting phosphorylation of Akt at T308. As observed in NRVMs transfected with PHLPP-1 siRNA, levels of PKCs were not changed in AMVMs isolated from KO and WT, in the presence or absence of LIF (Figure 8D; also Online Figure V).

**Figure 5. Mitochondrial distribution of PHLPP-1 and regulation of Akt at mitochondria.** A, NRVMs transfected with control siRNA (siCon) or PHLPP-1 siRNA (siP-1) were fractionated into mitochondrial and cytosolic fractions; equal portion of the total from each fractions were subjected to Western blotting with PHLPP-1. Voltage-dependent anion channel (VDAC) and Rho-GDI were used as mitochondrial and cytosolic markers, respectively. B, LIF-induced increase in phosphorylated Akt (S473) in the mitochondrial fraction is potentiated by PHLPP-1 knockdown (n=5). C, LIF induces total Akt (T-Akt) increases in mitochondrial fraction (n=5). D, LIF-induced increase in mitochondrial HK-II is further enhanced by PHLPP-1 knockdown (n=4). E, Cytosol/nuclear fractionation in NRVMs. PHLPP-1 was not detectable in the nuclear fraction. Lamin A/C and Rho-GDI were used as nuclear and cytosolic markers, respectively. F, LIF-induced phosphorylated Akt (S473) increase in the nuclear fractions is not significantly enhanced by PHLPP-1 knockdown (n=6). *P<0.05, **P<0.01, ***P<0.001.
in response to I/R but this was not increased by PHLPP-1 deletion. These results suggest that Akt activity is regulated by PHLPP-1 at mitochondria. To determine the functional importance of PHLPP-1 deletion, infarct size after 120 minutes reperfusion was measured by TTC staining. Remarkably, infarct size was smaller (by 45%), evidence that potentiation of Akt activation by PHLPP-1 deletion protects the heart against I/R injury (Figure 8F).

Discussion
Akt is an established survival signal in the heart. One approach to manipulating this pathway would be to increase Akt activation, but an equally feasible and potentially more selective approach would be to slow its inactivation. Mechanisms regulating the termination of Akt signals have, until recently, been poorly documented. PHLPP-1 was recently discovered to be an Akt phosphatase that selectively dephosphorylates Ser473 on Akt and can regulate tumor cell survival.15–17 The functional significance of PHLPP-1 expression in regulating physiological and pathophysiologic responses of other cell and tissue types has not, however, been examined. We demonstrate here that PHLPP-1 is expressed in cardiomyocytes, that it negatively regulates Akt activity through dephosphorylation of Akt at Ser473 and that it has functional effects on cardiomyocyte survival in vitro and in the ex vivo heart. Interestingly, our results suggest that PHLPP-1 distributes not only to cytosol but also to mitochondria where the extent of Akt activation can be locally regulated.

Analyzing constructs of Akt with Ala at one or the other of the phosphorylation positions (T308A or S473A), Alessi et al reported that mutation of either Ser473 or Thr308 reduced the rate of Akt activity by 85% and 95%, respectively, compared to WT Akt phosphorylated at both sites.26 Consistent with this, Akt selectively dephosphorylated at Ser473 by PHLPP has markedly reduced activity in in vitro assays using GSK3 as substrate.15 Thus, the intrinsic catalytic activity of Akt is reduced >80% in the absence of phosphorylation of Ser473. The data presented here confirm that increases in p-Akt at Ser473 are induced by PHLPP-1 knockdown or genetic deletion, in the
absence of concomitant increases in p-Akt at Thr308, and that this results in significant increases in Akt catalytic activity, supporting the significance of phosphorylation of Ser473 in regulating in vivo Akt kinase activity.

PHLPP-1 knockdown/knockout potentiates Akt phosphorylation at S473 induced by agonists (LIF, IGF-1 and S1P), suggesting the general importance of PHLPP-1 in regulation of Akt activity in cardiomyocytes. Knockdown of PHLPP-1 in NRVMs increases the LIF-induced Ser473 phosphorylation and activity of Akt-1 to the same extent as it affects Akt-2. PHLPP-1 knockdown also potentiates LIF-induced phosphorylation of both Akt-1 and Akt-2 substrates (GSK3-α and -β) in cardiomyocytes. In previous work, PHLPP-1 showed specificity for Akt-2. This may reflect differences in the properties of terminally differentiated cardiomyocytes versus tumor cells. It might also be related to our observations that PHLPP-2, although present in cardiomyocytes, is unable to regulate LIF-induced phosphorylation of Akt at Ser473, as indicated by the results of PHLPP-2 knockdown. With regard to the functions of Akt-1 and Akt-2, a protective role of Akt-1 has been well established in the heart, but genetic deletion of Akt-2 likewise revealed an involvement in protection against myocardial infarction. Thus, although the enhanced protective effects of PHLPP-1 knockdown observed in this study likely result from increased activity of Akt-1, it could reflect increases in activity of both Akt isoforms. It has been reported in colon cancer and normal breast epithelial cell lines that PHLPP-1 dephosphorylates PKCs, such as PKCα, -βII, -δ, and -ε, promoting degradation of the enzymes and this could contribute to the protection because protective role of PKC, especially PKCε, has been demonstrated. However, we did not observe changes in the levels of PKCs (α, βII, δ, and ε) in NRVMs transfected with PHLPP-1 siRNA or in AMVMs from PHLPP-1 KO, suggesting that potentiation of Akt signaling plays an important role in protective effects of PHLPP-1 knockdown/deletion.

Cytotoxic interventions such as doxorubicin have been shown to be counteracted by agonists such as LIF or IGF-1 which activate Akt. We demonstrate here that PHLPP-1 knockdown potentiates the protective effects of these ligands against doxorubicin toxicity through its effects on Akt. Oxidative stress, as induced by H2O2, is accompanied by Akt dephosphorylation at Ser473 induced by agonists (LIF, IGF-1 and S1P), which activate Akt. Akt has been considered as a reperfusion injury salvage kinases (RISK), counteracting cell damage through a compensatory protective pathway. This protective signaling response is also shown here to be potentiated by PHLPP-1 knockdown leading to enhanced cardiomyocyte survival. A more physiological form of oxidative stress is induced by exposing perfused hearts to global I/R. Akt has been shown to be activated in the adult heart by reperfusion following no flow ischemia and we demonstrate here that there is enhanced Akt activation by I/R when PHLPP-1 is genetically deleted. These findings suggest a physiological role for this phosphatase, whereas the concomitantly diminished infarct size demonstrates its functional importance. Thus, our results not only confirm that Akt is activated in response to oxidative stress and ex vivo I/R, but indicate that PHLPP-1 plays a role in this pathway and that the magnitude of this self-protecting signal can be enhanced by PHLPP-1 downregulation. These data suggest that PHLPP-1...
inhibitors could have therapeutic potential for protecting cardiomyocytes against I/R injury.

The mitochondrial death pathway plays a crucial role in heart diseases induced by I/R and doxorubicin toxicity. There is growing evidence that mitochondrial integrity can be regulated by reversible phosphorylation controlled by resident mitochondrial kinases/phosphatases or by kinases that translocate to mitochondria. We and others have shown that Akt activated at the plasma membrane redistributes to several cellular compartments including mitochondria and nucleus. More specifically, our previous work demonstrated that increases in mitochondrial Akt are responsible for preservation of mitochondrial integrity following stress induced by elevated Ca$^{2+}$ and ROS. This is attributable, at least in part, to increases in mitochondrial association of HK-II, a putative component of the permeability transition pore. We determined that HK-II has an Akt phosphorylation consensus sequence and is directly phosphorylated by Akt, providing a mechanism for increased association of HK-II with mitochondria. In the present study, we demonstrate for the first time that ex vivo I/R increases the accumulation of phosphorylated Akt at mitochondria. As evidence that the mitochondrial Akt is active, we find that the increase in phosphorylated Akt is accompanied by increased mitochondrial AKT-II association. Our subcellular fractionation studies also reveal that PHLPP-1 can localize at mitochondrial and that the accumulation of phosphorylated Akt at mitochondria is significantly increased by knockdown/knockout of PHLPP-1. Indeed, potentiation of Akt Ser473 phosphorylation by PHLPP-1 knockdown or deletion is considerably more prominent in the mitochondrial fraction than in whole cell/heart lysate. The observation that increase in mitochondrial AKT-II following Akt activation is significantly enhanced in PHLPP-1 knockdown/knockout further suggest that PHLPP-1 locally regulates Akt activity and thereby mitochondrial integrity. These results provide a previously undescribed mechanism by which mitochondrial function and integrity can be regulated through a dynamic balance between kinases and phosphatases.

Little is known about the mechanisms that regulate PHLPP, but changes in its expression level rather than its activation state may be most important in controlling Akt activity. Interestingly, we observed that 48 hours of LIF treatment upregulated both PHLPP-1 and PHLPP-2 expression in NRVMs (Online Figure VI). These increases were inhibited by treatment with an Akt inhibitor, implicating an Akt mediated feedback mechanism in regulation of PHLPP-1 and PHLPP-2 expression. Indeed, such a feedback mechanism was recently reported by Gao and coworkers, who demonstrated that that Akt activation prevents PHLPP-1 degradation by inhibiting its ubiquitination. Consistent with this, we also observed increased PHLPP-1 and PHLPP-2 expression in hearts from IGF-1 transgenic mice, which have elevated Akt activity (Online Figure VI). Further studies will be needed to determine whether these increases in PHLPP protein are attributable to transcriptional upregulation or to protein stabilization and whether they affect Akt activation and cardioprotection.

In conclusion, we demonstrate for the first time that PHLPP-1 is expressed in the heart and provide data indicating that it dephosphorylates Ser473 of Akt-1 and Akt-2, decreasing Akt kinase activity and facilitating cardiomyocyte death. Inhibition of Akt activity by PHLPP-1 can be observed at the level of mitochondria, contributing to control of mitochondrial integrity. Taken together, these data suggest that inhibition of PHLPP-1 would be beneficial in limiting ischemic heart diseases. Although therapeutic strategies leading to sustained Akt activation may have cardioprotective effects, they may also increase in cancer risk. In contrast, controlling Akt activity through pharmacological inhibition of PHLPP-1 for a relatively short time period after cardiac events should have beneficial effects by preventing the onset of cardiomyocyte loss and subsequent pathophysiological remodeling.

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Disclosures
None.

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Online expanded Methods

Cell Culture

Adult mouse ventricular myocytes were isolated from adult male mice (C57BL/6, 8-10 weeks age) \(^1,2\). Hearts were quickly removed, cannulated, and subjected to retrograde aortic perfusion at 37 °C, at a rate of 3 ml/min. Hearts were perfused for 4 min in Ca\(^{2+}\)-free buffer, followed by 8–10 min of perfusion with 0.25 mg/ml collagenase (Blendzyme 1, Roche). Hearts were removed from the cannula and the ventricle was dissociated at room temperature by pipetting with increasingly smaller transfer pipettes. Collagenase was inactivated once the tissue was thoroughly digested, by resuspending the tissue in medium containing 10% bovine calf serum. Calcium was gradually added back to a final concentration of 1 mmol/L and cells were plated on laminin-coated dishes in minimal essential medium/Hanks' balanced salt solution containing 5% serum. After 1 h, cells were washed and serum-free medium was added back. Cells were serum starved for 16-20 hrs prior to stimulation with agonists.

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-2-day-old Sprague-Dawley rat pups, digested with collagenase, and myocytes purified by passage through a Percoll gradient\(^3,4\). Myocytes were plated at density of 3.5x10\(^4\)/cm\(^2\) and maintained overnight in 4:1 Dulbecco's modified Eagle's medium (DMEM)/medium 199, supplemented with 10% horse serum, 5% fetal calf serum, and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). After an overnight culture, cells were transfected with siRNA (see below).

Transfection of cardiomyocytes with siRNA

Pre-designed PHLPP-1 ON-TARGETplus siRNA for rat (catalog number; J-094929-09) and control siRNA (catalog number; D-001810-02) were purchased from Thermo Scientific. NRVMs were transfected with siRNA using DharmaFECT-I transfection reagent (Thermo Scientific) based on manufacture's instruction. Two µmol/L siRNA were transfected into 1x10\(^6\) cells. siRNA and DharmaFECT-1 (4 µl and 12 µl for 1x10\(^6\) cells in 6 cm dish, respectively) were individually incubated in conical tubes containing 0.5 ml OPTI-MEM media (GIBCO) at room temperature for 5 min, mixed and incubated at room temperature for 20 min. Media in culture dishes were replaced with fresh media (3 ml for 6 cm dish) and siRNA/DharmaFECT-I mixtures (1 ml/dish) were added to culture dishes. After overnight incubation, cells were washed and cultured for another 48 hrs in serum free media (DMEM including 100 units/ml penicillin and 100 µg/ml streptomycin).

Cell Death ELISA Assay—DNA fragmentation indicative of apoptosis was assayed using the cell death detection ELISA PLUS (Roche Applied Science; catalog number/11 774 425 001). The assay is based on a quantitative sandwich-enzyme-immunoassay using mouse monoclonal
antibodies directed against DNA and histones to detect cytoplasmic histone-associated-DNA fragments. Cardiomyocytes were washed with ice-cold PBS twice and harvested in cytosolic extraction buffer (20 mmol/L Tris pH 7.6, 3 mmol/L EDTA, 3 mmol/L EGTA, 125 mmol/L NaCl, 20 mmol/L β-glycerophosphate and 0.4% Nonidet P-40 alternative plus protease and phosphatase inhibitors). Samples were nutated at 4°C for 10 min, spun down at 20,000 x g for 3 min and supernatants were saved. Supernatants (5 µl) were incubated with anti-histone-biotin antibody and anti-DNA-peroxidase antibody in a streptavidin-coated 96 well plate on an orbital shaker (60 rpm) at room temperature for 2 h. Subsequently wells were washed by incubation buffer (200 µl per well) 3 times, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid substrate (100 µl per well) was added and absorbance was measured at 405 nm by using plate reader. Akt inhibitors (inhibitor III/SH-6 and inhibitor V/triciribine) were purchased from EMD.

**Western blotting and Immunoprecipitation**

Cardiomyocytes were washed three times with ice-cold PBS, harvested in RIPA buffer; composed of 150 mM/L NaCl, 50 mM/L Tris (pH7.4), 1% NP-40, 1% of sodium deoxycholate, 0.1% of SDS, 0.2 mM/L EDTA, supplemented with 200 µmol/L Na3VO4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mmol/L PNPP and 1 mmol/L PMSF. Heart homogenate were prepared by using TissueMiser (Fisher Scientific). Samples were nutated at 4°C for 20 min, spun down at 20,000 x g for 10 min and supernatants were saved as whole cell/heart lysates. Protein concentration was measured by Micro BCA Protein Assay Kit (Thermo Scientific). Cell lysates were mixed with LDS sample buffer and reducing agent (Invitrogen), heated at 80°C for 10 min and equal amounts of protein (20-30 µg in NRVMs and AMVMs and 100-150 µg in whole heart homogenates) were loaded onto SDS-PAGE (Invitrogen NuPage system). Gels were run on ice and proteins were transferred to PVDF membranes (Millipore). Membranes were blocked by 5% milk TBS-Tween for 1 hour and probed at 4°C overnight, using the following antibodies. The PHLPP-1 antibody was from Bethyl Laboratories (#A300-660A, rabbit polyclonal). The total and phosphorylated Akt (rabbit polyclonal), phosphorylated GSK-3α/β (rabbit polyclonal), PKCe (rabbit monoclonal), LaminA/C (rabbit polyclonal), hexokinase-II (rabbit polyclonal) and gp130 (rabbit polyclonal) antibodies were from Cell Signaling technology. The VDAC antibody (rabbit polyclonal) was from EMD. The rho-GDI (mouse monoclonal) was from BD transduction laboratories. PKCa, β, and δ antibodies (rabbit polyclonal) were from Santa Cruz biotechnology. PHLPP-1 antibody was used at 1:2000 dilution in 5% BSA/TBS-tween and other antibodies were used at 1:1000 dilution in 5% BSA/TBS-tween. Blots were washed with TBS-tween (5 min x 5 times) and incubated with secondary antibodies (1:2000~1:5000 dilution) in 5% milk/TBS-Tween for 1 hour. For PHLPP-1 western blotting, anti-rabbit secondary antibody (Sigma, catalog number A6154) was used at 1:8000 dilution.
Total Akt, Akt-1 and Akt-2 were immunoprecipitated by using antibody to total Akt (Cell Signaling Technology, mouse monoclonal), Akt-1 (Cell Signaling Technology, mouse monoclonal) and Akt-2 (Santa Cruz Biotechnology, goat polyclonal), respectively. Cell lysates were precleaned with Protein A/G PLUS-agarose beads for 30 min at 4°C and 600-800 µg of total protein was then incubated with antibodies (4 µg) and protein A/G PLUS agarose (Santa Cruz Biotechnology) (30 µl of 50% slurry) at 4°C overnight. Immunocomplexes were washed with ice-cold RIPA buffer for 4 times, and beads were boiled in 2x LDS buffer to elute captured protein and subjected to Western blotting with phospho-Akt (S473) (Cell Signaling Technology, rabbit polyclonal).

**Akt kinase activity assay**
Akt kinase activity was assessed using nonradioactive Akt kinase assay kit (Cell Signaling Technology). Cardiomyocytes were harvested in Cell Lysis buffer (20 mmol/L Tris pH7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% triton-X100, 2.5mmol/L PNPP, 1mmol/L β-glycerophosphate, 1mmol/L Na3VO4 and 10 mg/mL leupeptin), and Akt, Akt-1 and Akt-2 were immunoprecipitated from cell lysates by using antibodies mentioned above. Immunocomplexes were spun down, washed twice with Cell Lysis buffer and washed twice with Kinase buffer (25 mmol/L Tris pH7.5, 5 mmol/L β-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na3VO4 and 10 mmol/L MgCl2). Immunocomplexes were resuspended in 50 µl Kinase buffer with 200 µmol/L ATP and 1 µg of the GSK-3 α/β crosstide, corresponding to residues surrounding GSK-3 α/β (Ser21/9) (CGPKGPGRGRRRTSSFAEG) as substrate. After incubation for 30 min at 30°C, reactions were terminated by adding 25 µl LDS buffer and reducing agent (Invitrogen), and boiling for 10 min. Samples were subjected to Western blotting with phosphorylated GSK-3α/β antibody (Cell Signaling Technology).

**Cytosol/mitochondria fractionation**
Cytosolic and mitochondrial fractions were prepared from neonatal rat ventricular myocytes (EMD, Cytosol /Mitochondria Fractionation Kit). 5,6 Cells were stimulated by agonists, collected in ice cold PBS, spun down at 600 x g for 5 min. PBS was carefully aspirated off and cells were resuspended in Cytosol Extraction Buffer Mix, briefly vortexed and incubated on ice for 10 minutes. Samples were centrifuged at 700 x g for 10 minutes to spin down nuclei and cell debris. Supernatants were transferred to new tubes and spun at 10,000 x g for 30 minutes to precipitate mitochondria. Supernatant was saved as the cytosolic fraction and the pellet was resuspended in RIPA buffer as the mitochondrial fraction.

**Isolation of mitochondria from adult mouse hearts**
Mitochondria were isolated from adult mouse hearts as previously described.5 Hearts were removed from Langendorff apparatus and the ventricle was homogenized by hand in isolation
buffer containing 70 mmol/L sucrose, 190 mmol/L mannitol, 20 mmol/L Hepes and 0.2 mmol/L EDTA, 1 µmol/L Na₃VO₄, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.5 mmol/L PNPP and 0.5 mmol/L PMSF. The homogenate was centrifuged at 600 x g for 10 min to remove nuclei and debris. The resulting supernatant was then centrifuged at 5000 x g for 15 min. The resulting mitochondrial pellet was washed by isolation buffer and re-centrifuged three times. After final spin, mitochondrial pellet was resuspended in RIPA buffer and subjected to Western blotting.

**Cytosol/Nuclear fractionation**

Cytosolic and nuclear fractions were prepared from NRVMs. Cells were washed by ice-cold PBS twice, collected by scraping in ice-cold PBS and spun down at 600 x g for 5min. Cells were resuspended in cytosolic extraction hypotonic buffer (10 mmol/L HEPES pH 7.6, 10 mmol/L NaCl, 1.5 mmol/L MgCl₂, 10% glycerol, 0.1% NP40-alternative, 200 µmol/L Na₃VO₄, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mmol/L PNPP and 1 mmol/L PMSF) and incubated on ice on an orbital shaker (70 rpm) for 15 min. Samples were spun down at 16000 x g for 5 min and supernatants were collected as cytosolic fractions. The pellets were resuspended in the buffer containing 20 mmol/L Tris pH 7.6, 3 mmol/L EDTA, 3 mmol/L EGTA, 250 mmol/L NaCl, 20 mmol/L β-glycerophosphate and 1% Nonidet P-40 alternative and protease-and phosphatase inhibitors, incubated on ice for 10 min. After spinning down at 2700 x g for 5min, supernatants were discarded (intracellular organelles including mitochondria). Pellets were resuspended in RIPA buffer, incubated on ice for 10 min, and spun down at 20000 x g for 5min. Resultant supernatants were collected as nuclear fractions.

**Northern blot**

The Multiple Tissue Northern (MTN®) Blot containing total RNAs prepared from different human tissues was obtained from Clontech. The hybridization experiment was performed according to the manufacturer suggested procedures. Briefly, a ~200 bp DNA fragment in the coding region of human PHLPP1 gene was amplified using PCR. The ³²P labeled probe was prepared using the PCR product as the template with the Random Primers DNA Labeling Kit (Invitrogen). The hybridized the blot was exposed to a film to detect the radioactive signals.

**Langendorff heart perfusion**

Adult male mice (C57BL/6, 8-10weeks age) were heparinized (500 units/kg, intraperitoneally) and anesthetized (2% isofluorane with 0.8% oxygen). Hearts were rapidly excised, washed in ice-cold modified Krebs-Henseleit solution (118 mmol/L NaCl, 24 mmol/L NaHCO₃, 4 mmol/L KCl, 1 mmol/L NaH₂PO₄, 2 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, 12 mmol/L glucose and 10 mmol/L Hepes, pH 7.4), and cannulated via the aorta on a 20-gauge stainless steel blunt needle. The hearts were mounted on a Langendorff apparatus and perfused with oxygenated
Krebs-Henseleit buffer at 37 °C at a constant pressure of 80 mmHg. Hearts were perfused for 30 min to allow for equilibration and subjected to no-flow ischemia for 30 min followed by reperfusion.7,8 To determine infarct size after 30 min ischemia/120 min reperfusion, the heart was removed from the apparatus and rinsed in ice-cold Krebs-Henseleit buffer, frozen at -20 °C for 2 hours and cut transversely into five slices of equal thickness. These samples were incubated in 1% 2,3,5-triphenyltetrazolium chloride(TTC)-containing Tris-HCl buffer (pH 7.8) at 37°C for 15 min to stain the viable myocardium and then were fixed in 10% formalin-phosphate buffered saline for 24 h. Infarct size (white area) was measured by NIH Image.

References
Online figure I. PHLPP-2 is expressed in the heart but knockdown of PHLPP-2 in NRVMs does not affect LIF-induced phosphorylation of Akt at S473. A. NRVMs were transfected with control (siCon) or PHLPP-2 siRNA (siP-2) for 48 hrs, treated with LIF, harvested and subjected to Western blotting for P-Akt (S473). Values represent averages ± S.E. (n=5). B. NRVMs were transfected with control (siCon), PHLPP-1 (siP-1) and/or PHLPP-2 siRNA (siP-2). Cells were stimulated with LIF and subjected to Western blotting for PHLPP-1, PHLPP-2, GAPDH and p-Akt (S473).
Online figure II. IGF-1 increases mitochondrial phosphorylated Akt and HK-II in mitochondria and this is potentiated by PHLPP-1 knockdown in NRVMs. NRVMs transfected with control siRNA (siCon) or PHLPP-1 siRNA (siP-1) were stimulated with IGF-1 (1 nmol/L, 10 min) and mitochondria were isolated. A, Representative blots. B, Quantified data of mitochondrial phosphorylated Akt (upper panel) and hexokinase II (lower panel)(n=5). *, ***, p<0.05, p<0.001.
Online figure III. PHLPP-1 levels were not affected by 150 µmol/L $H_2O_2$ treatment in control siRNA treated NRVMs. Values represent averages ± S.E. (n=5).
Online figure IV. PHLPP-1 knockdown attenuates caspase-9 and -3 activation induced by H$_2$O$_2$. NRVMs transfected with control (siCon) or PHLPP-1 siRNA (siP-1) and cultured for 18 hrs in the presence or absence of 150 µmol/L H$_2$O$_2$. Cells were harvested and subjected to Western blotting for cleaved caspase-9 and -3. Values represent averages ± S.E. (n=6-7). *, ***, p<0.05, p<0.001.
Levels of PKCs were not affected in AMVMs. Some AMVMs were treated with 10 nmol/L LIF for 8 hrs. A, Representative blots of PKC-α, βII, δ and ε in AMVMs in the presence or absence of LIF. B, Quantified data of PKC levels after 8 hrs LIF treatment.

**Online figure V.** Levels of PKCs were not affected in AMVMs. Some AMVMs were treated with 10 nmol/L LIF for 8 hrs. A, Representative blots of PKC-α, βII, δ and ε in AMVMs in the presence or absence of LIF. B, Quantified data of PKC levels after 8 hrs LIF treatment.
Online figure VI. Akt activation induces upregulation of PHLPP-1 and -2. A, NRVMs were treated with LIF for 48 hrs and Western blotting was carried out for PHLPP-1 and PHLPP-2. An Akt inhibitor, triciribine (1 µmol/L), was added to some samples before LIF treatment. Values represent averages ± S.E. (n=4). B, Western blotting of PHLPP-1 and PHLPP-2 in WT and IGF-1 transgenic mice (IGF-1 TG). Values represent averages ± S.E. (n=4). *, **, p<0.05, p<0.01.