Supplement Material

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)/medium199, 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-TARGET*plus* 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 sandwitch-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 [5-7] . 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 Na_3VO_4, 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), PKCε (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. PKCα, 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 Na_3VO_4 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/Lβ-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na_3VO_4 and 10 mmol/L $MgCl_2$). Immunocomplex 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) (CGPKGPGRRGRRRTSSFAEG) 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 Na_3VO_4, 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 $MgCl_2$, 10% glycerol, 0.1% NP40-alternative, 200 μmol/L Na_3VO_4, 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 respended 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 ^{32}P 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.

Langendroff heart perfusion

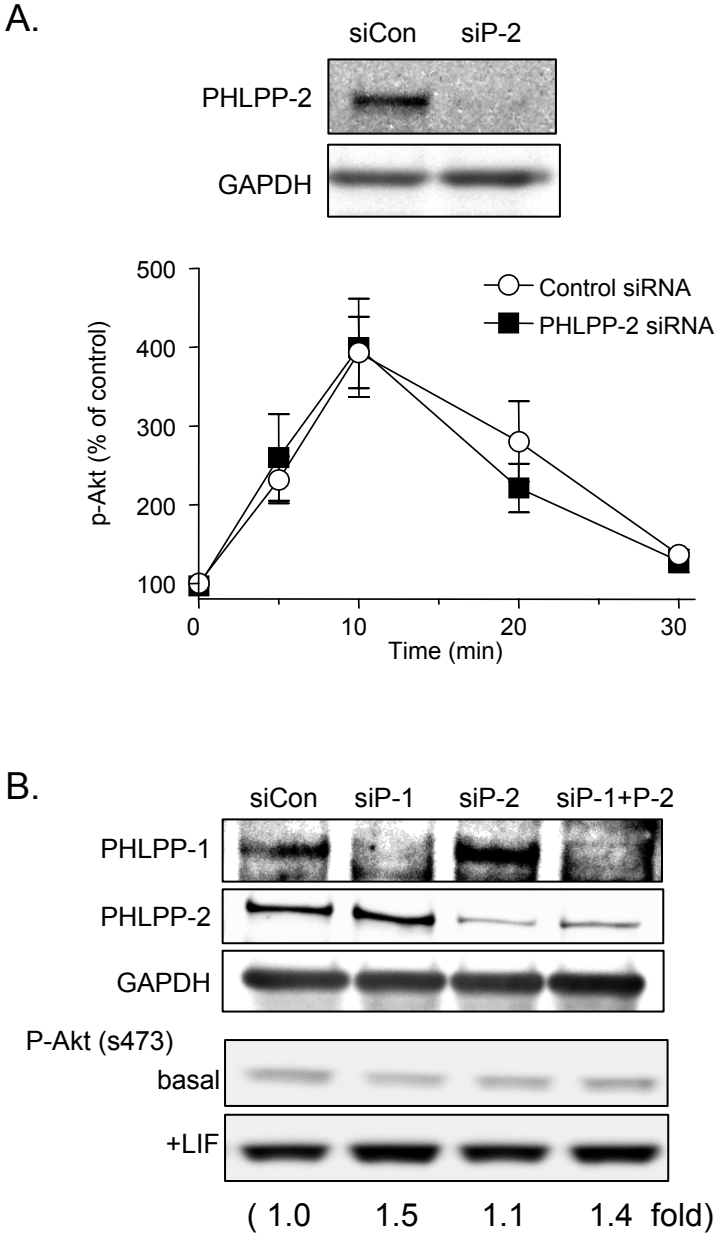
Adult male mice (C57BL/6, 8-10weeks age) were heparinized (500 units/kg, intraperitoneally) and anesthetized (2% isofluorane with 0.8% oxygen). Hearts were rapidly excised, washed in ice-cold modified Krebs-Henseleit solution (118 mmol/L NaCl, 24 mmol/L $NaHCO_3$,4 mmol/L KCl, 1 mmol/L NaH_2PO_4, 2 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 12 mmol/L glucose and 10 mmol/L Hepes,pH7.4), 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 ishcemia/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

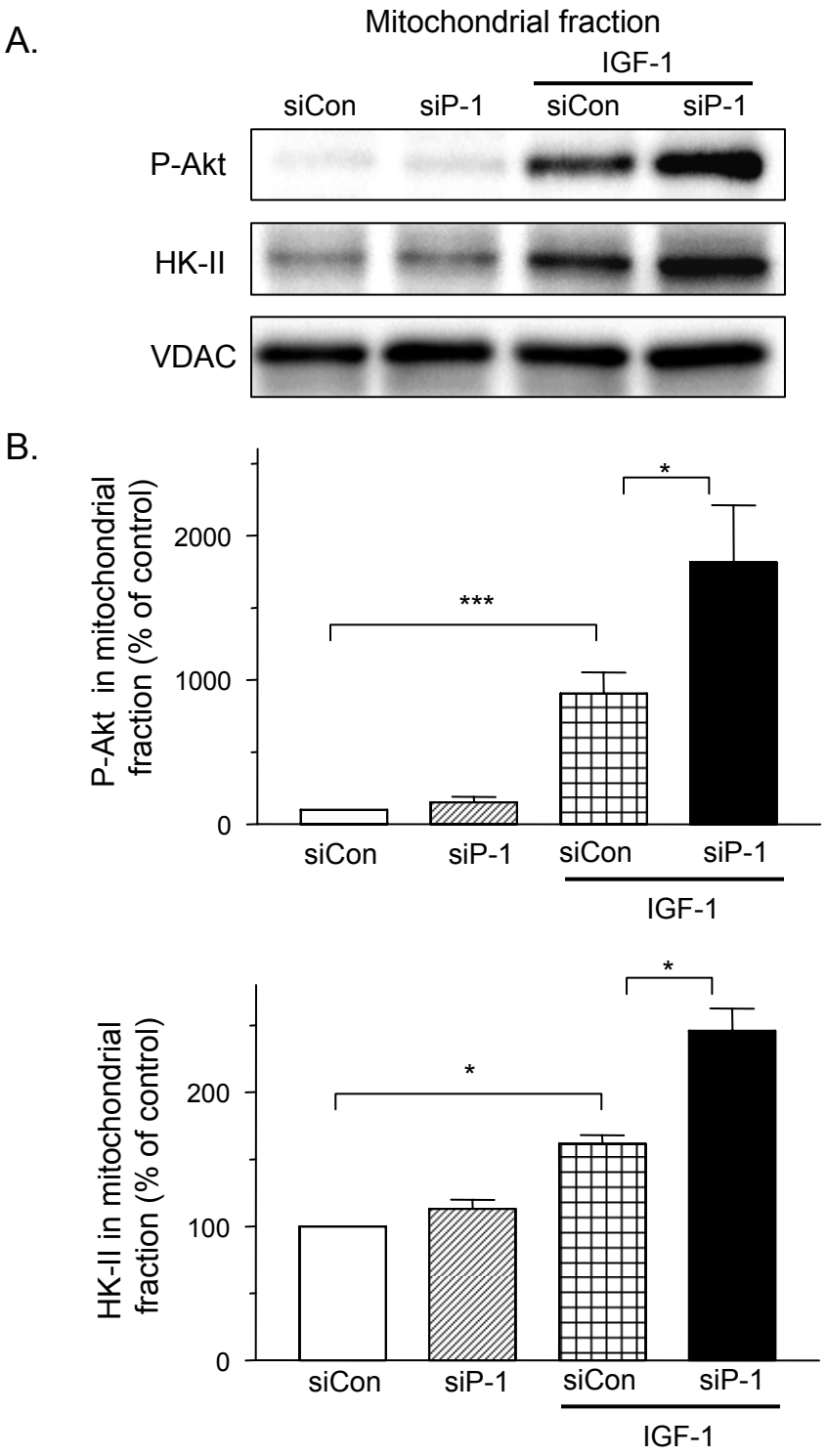
1. Means CK, Miyamoto S, Chun J, Brown JH. S1P1 receptor localization confers selectivity for Gi-mediated cAMP and contractile responses. *J Biol Chem*. 2008;283:11954-11963.
2. Means CK, Xiao CY, Li Z, Zhang T, Omens JH, Ishii I, Chun J, Brown JH. Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2007;292:H2944-2951.
3. Adams JW, Pagel AL, Means CK, Oksenberg D, Armstrong RC, Brown JH. Cardiomyocyte apoptosis induced by Galphaq signaling is mediated by permeability transition pore formation and activation of the mitochondrial death pathway. *Circ Res*. 2000;87:1180-1187.
4. Miyamoto S, Howes AL, Adams JW, Dorn GW, 2nd, Brown JH. Ca2+ dysregulation induces mitochondrial depolarization and apoptosis: role of Na+/Ca2+ exchanger and AKT. *J Biol Chem*. 2005;280:38505-38512.
5. Miyamoto S, Murphy AN, Brown JH. Akt mediates mitochondrial protection in cardiomyocytes through phosphorylation of mitochondrial hexokinase-II. *Cell Death Differ*. 2008;15:521-529.
6. Del Re DP, Miyamoto S, Brown JH. RhoA/Rho kinase up-regulate Bax to activate a mitochondrial death pathway and induce cardiomyocyte apoptosis. *J Biol Chem*. 2007;282:8069-8078.
7. Del Re DP, Miyamoto S, Brown JH. Focal adhesion kinase as a RhoA-activable signaling scaffold mediating Akt activation and cardiomyocyte protection. *J Biol Chem*. 2008;283:35622-35629.
8. Whittaker R, Glassy MS, Gude N, Sussman MA, Gottlieb RA, Glembotski CC. Kinetics of the translocation and phosphorylation of alphaB-crystallin in mouse heart mitochondria during ex vivo ischemia. *Am J Physiol Heart Circ Physiol*. 2009;296:H1633-1642.

Online Figure I



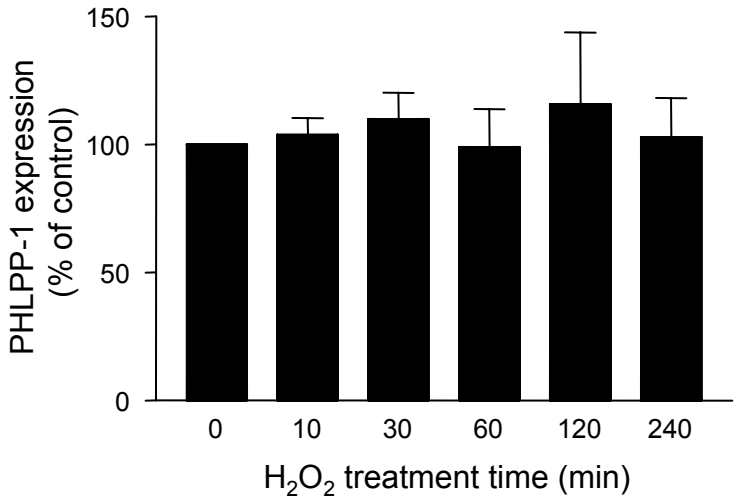
Online figure I. PHLPP-2 is expressed in the heart but knockdown of PHLPP-2 in NRVMs does not affect LIF-induced phosphorylation of Akt at S473. A. NRVMs were transfected with control (siCon) or PHLPP-2 siRNA (siP-2) for 48 hrs, treated with LIF, harvested and subjected to Western blotting for P-Akt (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



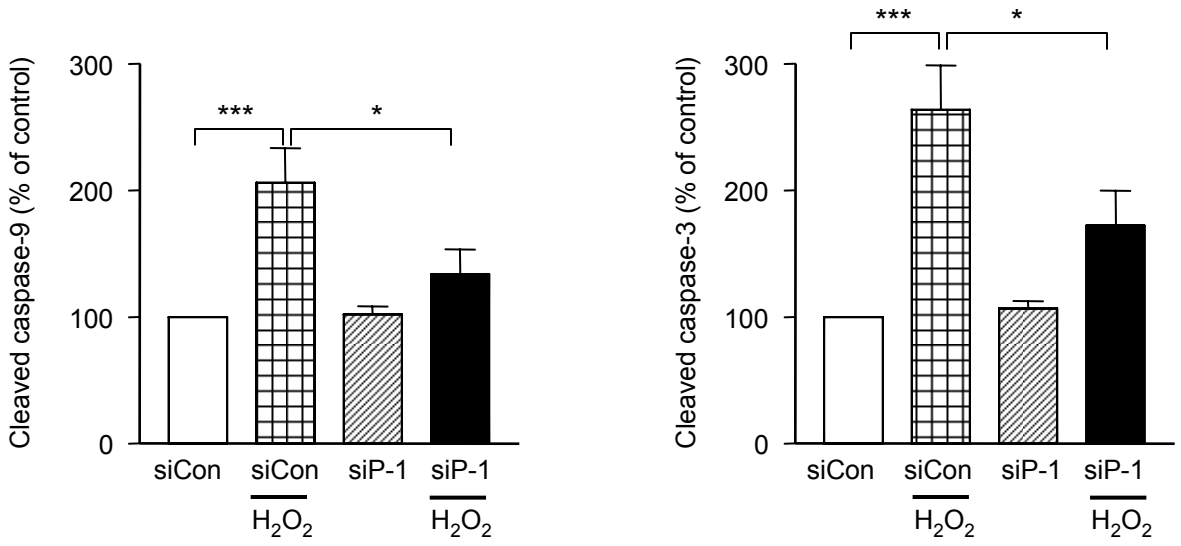
Online figure II. IGF-1 increases mitochondrial phosphorylated Akt and HK-II in mitochondria and this is potentiated by PHLPP-1 knockdown in NRVMs. NRVMs transfected with control siRNA (siCon) or PHLPP-1 siRNA (siP-1) were stimulated with IGF-1 (1 nmol/L, 10 min) and mitochondria were isolated. A, Representative 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



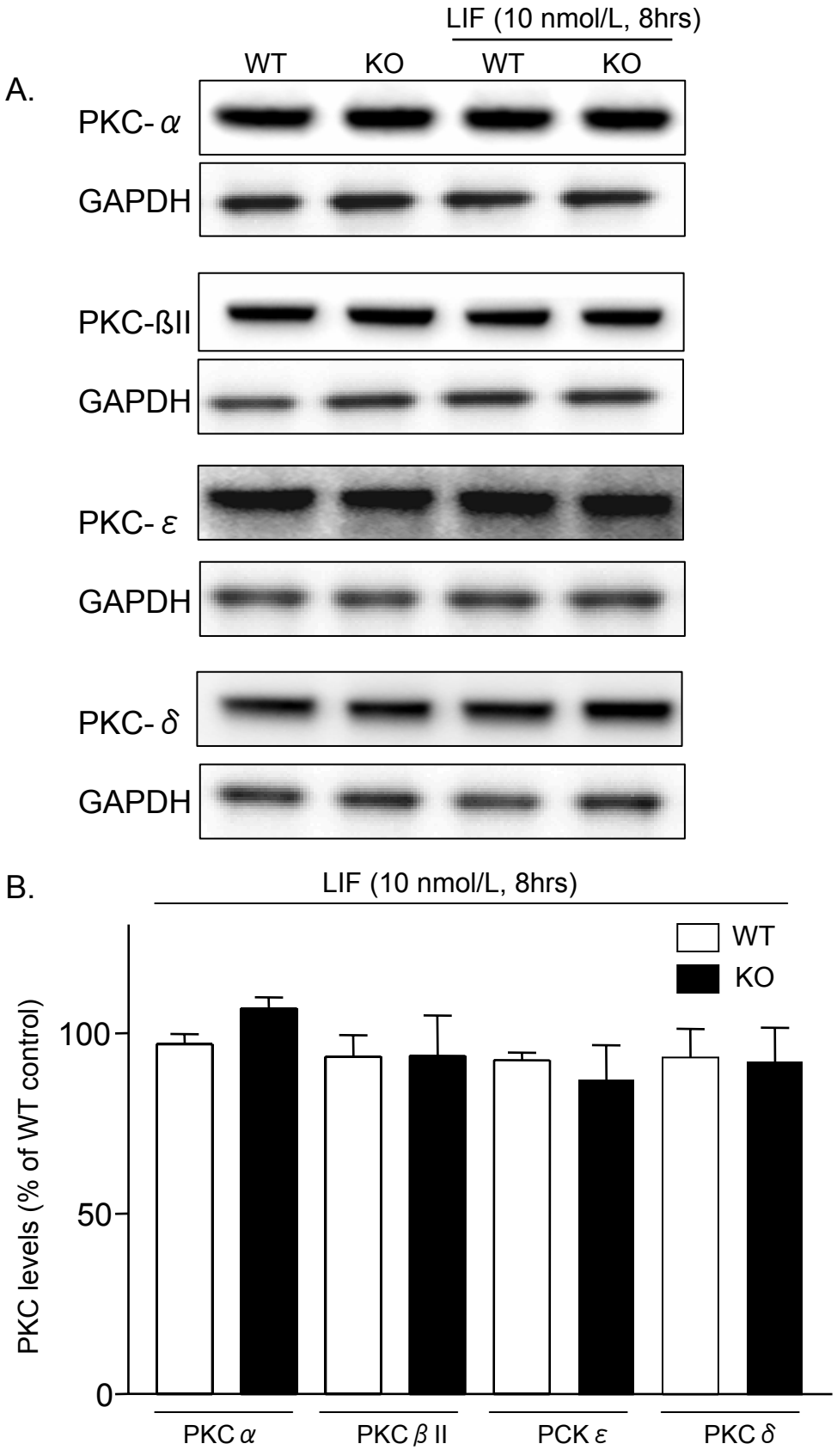
Online figure III. PHLPP-1 levels were not affected by 150 μmol/L H_2O_2 treatment in control siRNA treated NRVMs. Values represent averages ± S.E. (n=5).

Online
Figure IV



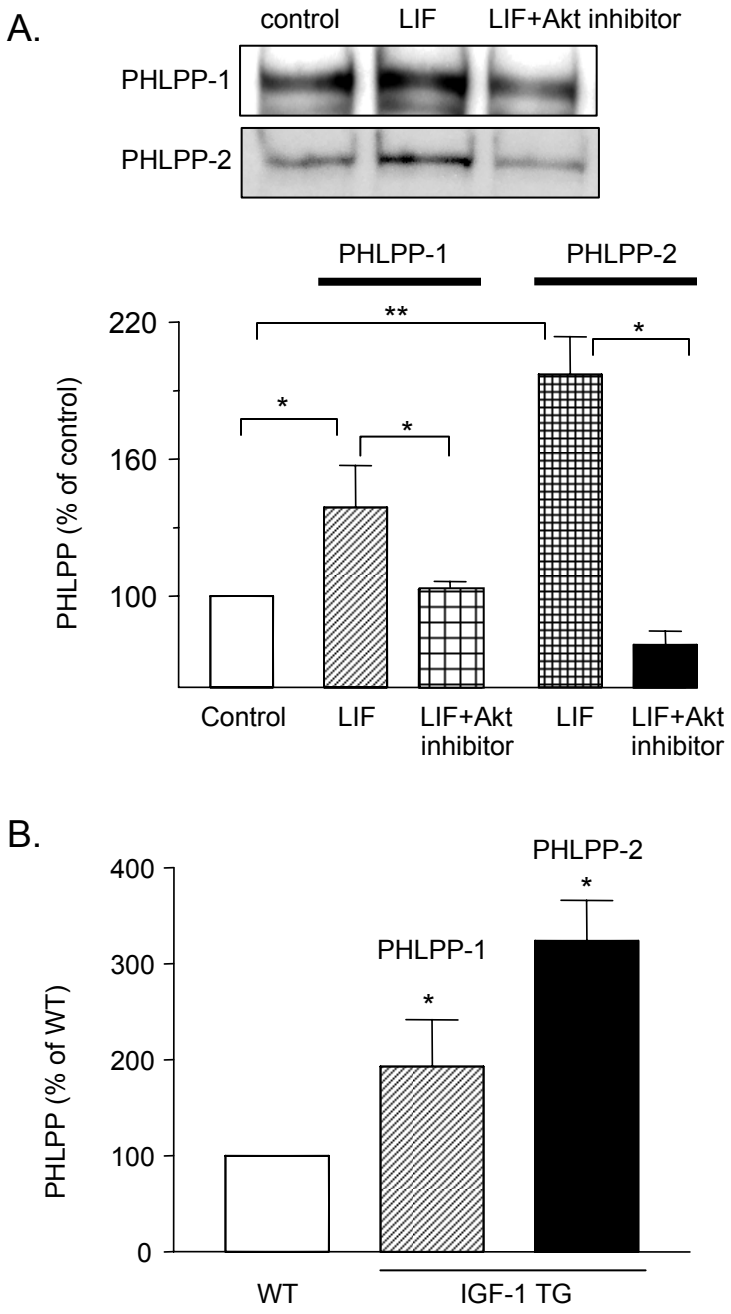
Online figure IV. PHLPP-1 knockdown attenuates caspase-9 and -3 activation induced by H_2O_2. NRVMs transfected with control (siCon) or PHLPP-1 siRNA (siP-1) and cultured for 18 hrs in the presence or absence of 150 μmol/L H_2O_2. Cells were harvested and subjected to Western blotting for cleaved caspase-9 and -3. Values represent averages ± S.E. (n=6-7). *, ***, $p<0.05$, $p<0.001$.

Online Figure V



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 PCK- 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



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). *, **, $p<0.05$, $p<0.01$.