Supplemental data

Materials and Methods

Primary cultures of neonatal rat ventricular cardiac myocytes

Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old Crl: (WI) BR-Wistar rats (Charles River Laboratories) as described previously[1]. A cardiac myocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient. Cells were cultured in complete medium (CM) containing Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 5% horse serum, 4 μg/ml transferrin, 0.7 ng/ml sodium selenite (Life Technologies, Inc.), 2 g/liter bovine serum albumin (fraction V), 3 mM pyruvic acid, 15 mM HEPES, 100 μM ascorbic acid, 100 μg/ml ampicillin, 5 μg/ml linoleic acid and 100 μM 5-bromo-2'-deoxyuridine (Sigma).

Construction of adenoviruses

Recombinant adenovirus vectors were constructed as described [2]. pBHGlox∆E1,3Cre (Microbix), including the ∆E1 adenoviral genome, was co-transfected with the pDC shuttle vector containing the gene of interest into 293 cells. We made replication-defective human adenovirus type 5 (devoid of E1) harboring full length PKN (Ad-PKN), and a kinase-negative form of PKN (K644D; Ad-DNPKN). Adenovirus harboring beta-galactosidase (Ad-LacZ) was used as a control. In the *in vitro* study, transduction of full length PKN was used instead of CAPKN, because we were unable to generate adenovirus harboring CAPKN.

Echocardiography

Echocardiography was performed after mice were anesthetized with 12 μl/g body weight of 2.5% avertin as described previously [3].

Ischemia/reperfusion

Pathogen-free mice were housed in a temperature-controlled environment with 12 hr light/dark cycles, where they received food and water ad libitum. Mice (3 months old) were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). A rodent ventilator (model 683; Harvard Apparatus Inc) was used with 65% oxygen during the surgical procedure. The animals were kept warm using heat lamps and heating pads. Rectal temperature was monitored and maintained between 36.5 and 37.5°C. The chest was opened by a horizontal incision through the muscle between the ribs (third intercostal space). Ischemia was achieved by ligating the anterior descending branch of the left coronary artery (LAD) using an 8-0 nylon suture, with a

silicon tubing (1 mm OD) placed on top of the LAD, 2 mm below the border between left atrium and left ventricle (LV). Regional ischemia was confirmed by ECG change (ST elevation). After occlusion for 45 min, the silicon tubing was removed to achieve reperfusion. Treatment of mice with epoxomicin was conducted as described previously [4]. Treatment with propranolol, a beta adrenergic receptor antagonist, was conducted by intraperitoneal injection at a dose of 3 mg/kg five minutes before ischemia. For treatment with N-(2-mercaptopropionyl)-glycine (MPG), an antioxidant, MPG (100 mg/kg/hr) was infused intravenously through a cannula inserted into the jugular vein from15 min before ischemia throughout the experiment, as described previously[5, 6].

Assessment of area at risk and infarct size

After I/R, the animals were reanesthetized and intubated, and the chest was opened. After arresting the heart at the diastolic phase by KCl injection, the ascending aorta was canulated and perfused with saline to wash out blood. The LAD was occluded with the same suture, which had been left at the site of the ligation. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into 1-mm thick cross sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 10 min. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured using Adobe Photoshop (Adobe Systems Inc.), and the values obtained were averaged. The percentage of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from all sections. AAR/LV and infarct area/AAR were expressed as a percentage.

Evaluation of apoptosis in tissue sections

DNA fragmentation was detected *in situ* using the TUNEL assay as described previously [2]. Briefly, deparaffinized sections were incubated with proteinase K, and DNA fragments were labeled with fluorescein-conjugated dUTP using TdT (Roche Molecular Biochemicals). Nuclear density was determined by manual counting of DAPI-stained nuclei in six fields for each animal using the 40x objective, and the number of TUNEL-positive nuclei was counted by examining the entire section using the same power objective.

Langendorff perfused hearts

Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). Hearts were excised and perfused with modified Krebs-Henseleit bicarbonate buffer (NaCl 118 mmol/L, KCl 4.7 mmol/L, $CaCl_2$ 2.0 mmol/L, $MgSO_4$ 1.2 mmol/L, KH_2PO_4 1.2 mmol/L, $NaHCO_3$ 25.0

mmol/L, glucose 11.0 mmol/L, 37°C) using Langendorff-perfused method. After stabilization, hearts were subjected to 45 min of global ischemia followed by 60 min of reperfusion. LV developed pressure (LVDP), LV end-diastolic pressure (LVEDP), positive and negative dP/dt were measured.

Viability of the cells

Viability of the cells was measured by Cell Titer Blue (CTB) assays (Promega). In brief, cardiac myocytes (1 $X10^5$ per 100μl) were seeded onto 96-well dishes. After 24 hr, the medium was changed to serum free medium. Cardiac myocytes were transduced with adenovirus harboring PKN, DNPKN, or LacZ for 24 hours, or shRNA against PKN or control shRNA for 72 hours, and then treated with hydrogen peroxide for 8 hours. Viable cell numbers were measured by the CTB assay. The CTB assays were performed according to the supplier's protocol. The experiments were conducted in triplicate at least three times.

Analysis of DNA fragmentation by ELISA

Histone-associated DNA fragments were quantified using Cell Death Detection ELISA (Roche) according to the manufacturer’s protocol.

Immunoblot analysis

For immunoblot analysis, heart homogenates and cardiac myocyte lysates were prepared in RIPA lysis buffer containing 50 mmol/L Tris·HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 1 mmol/L EDTA, 0.1 mmol/L Na_3VO_4, 1 mmol/L NaF, 50 μmol/L phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, and 5 μg/ml leupeptin. The antibodies used include anti-PKN (C-terminal, UPSTATE), anti-PKN (N-terminal, BD Transduction Laboratories), phospho-PRK1(Thr774)/PRK2(Thr816) (Cell Signaling Technology), alpha-sarcomeric actinin (Sigma), actin (Sigma), alpha BC (Stressgen), phospho-alpha BC (Ser59 or Ser45, Stressgen), MAPKAPK-2 (Cell Signaling Technology), phospho-MAPKAPK-2 (Thr334, Cell Signaling Technology), PKCε (Cell Signaling), Akt (Cell Signaling Technology) and phospho-Akt (Ser473, Cell Signaling Technology).

For subcellular fractions, heart homogenates and cardiac myocyte lysates were prepared in Tris extraction buffer containing 20 mmol/L Tris·HCl (pH 7.5), 2 mmol/L Na_3VO_4, 1 mmol/L NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 μmol/LPMSF. The lysate was centrifuged at 3,000 g for 10 min at 4°C. The supernatant was used as the cytosolic fraction. The pellet was resuspended in Tris-Triton buffer containing 10 mM Tris·HCl (pH 7.5), 100 mM

NaCl, 20 mM $Na_4P_2O_7$, 10% glycerol, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 1 mM NaF, 50 μmol/L PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin, then centrifuged at 12,000 g for 20 min at 4°C. The supernatant was used as the cytoskeletal fraction. For preparation of the membrane fraction, the pellet from the cytosolic fraction was resuspended in the tissue sample buffer containing 0.5% NP-40, 0.1% deoxycholate, and 0.1% Brij 35, incubated on ice for 60 min, and recentrifuged at 10,000 rpm for 5 min. The supernatant was used as the membrane fraction [7]. These fractions were analyzed by immunoblot analysis with the use of appropriate antibodies. Densitometric analysis was performed using Scion Image software (Scion).

Proteasome assay

Tissue samples were homogenized in 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, and protease inhibitors. Cell debris was removed by centrifugation for 2 minutes at 12,000 g, and the supernatants were used for assay as described previously [8]. The synthetic fluorogenic peptide substrate III [Suc-LLVY-aminomethylcoumarin (AMC), 25 μM; Calbiochem, San Diego, CA] was used to assay chymotrypsin-like activities. Assays were carried out using 15 μg of cellular protein in a total volume of 200 μl. The assay buffer consisted of 25 mmol/L Tris·HCl (pH 7.5) and 2 mmol/L ATP. After incubation at 37°C for 30 min, the reaction was quenched by addition of 0.3 ml ethanol. A 2 ml aliquot of H_2O was then added, and the fluorescence intensity of the samples was evaluated using a luminescence spectrometer. The excitation and emission wavelengths are 350 and 440 nm, respectively, for AMC products. Peptidase activities were measured in the absence and presence (10 μmol/L) of the proteasome-specific inhibitor, MG-132, and the difference between the two rates was attributed to the proteasome.

Supplemental Figures

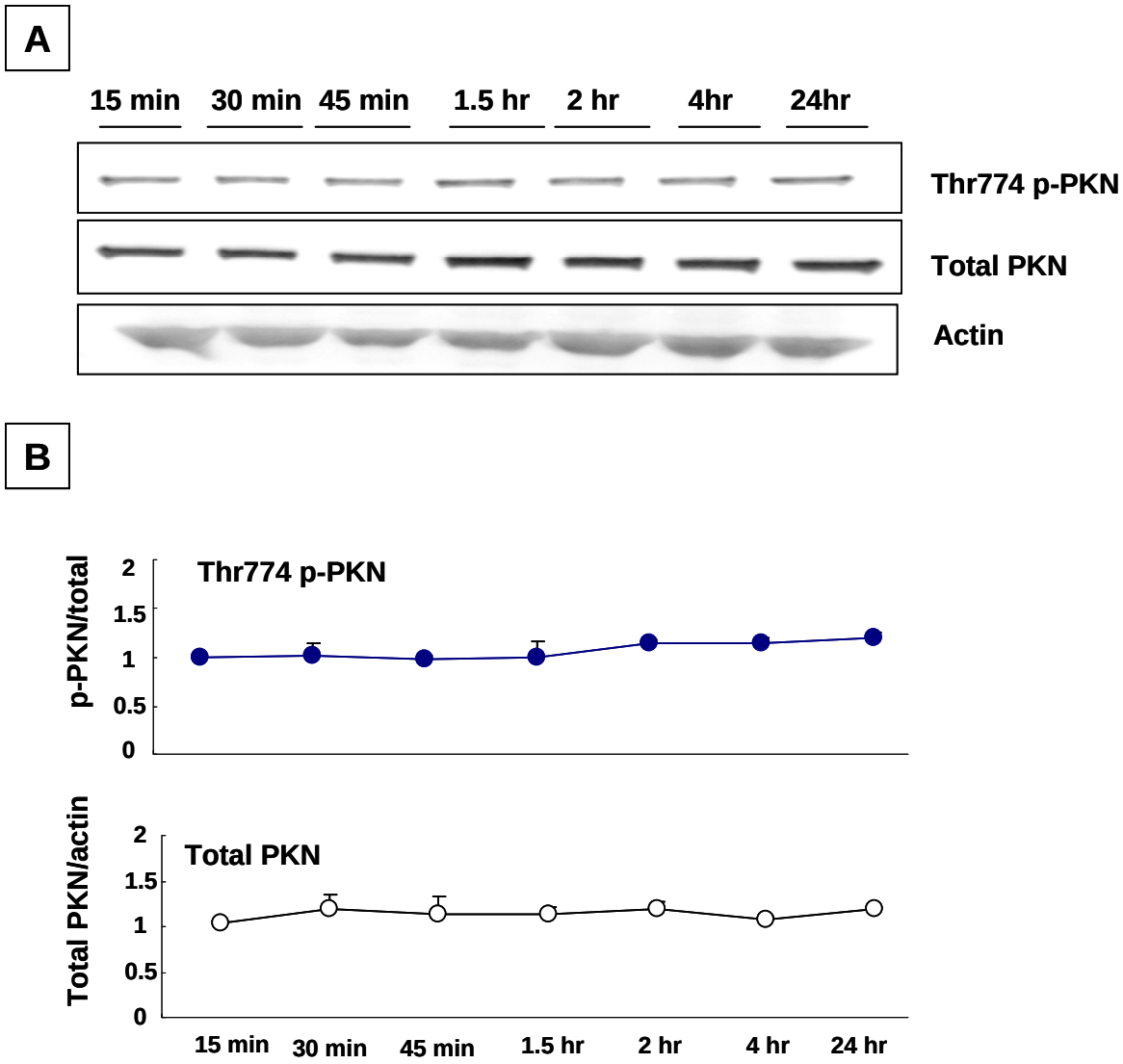


Figure I Effects of sham operation on PKN expression and phosphorylation in mouse hearts

(A) Time course of PKN Thr 774 phosphorylation is shown. (B) The level of PKN expression (lower) and Thr 774 phosphorylation (upper) was determined by densitometric analysis. The mean total PKN/actin or phosphorylated PKN/total PKN in 15min sham-operated mouse hearts was set as 1. n=3 each.

Supplemental Figure I

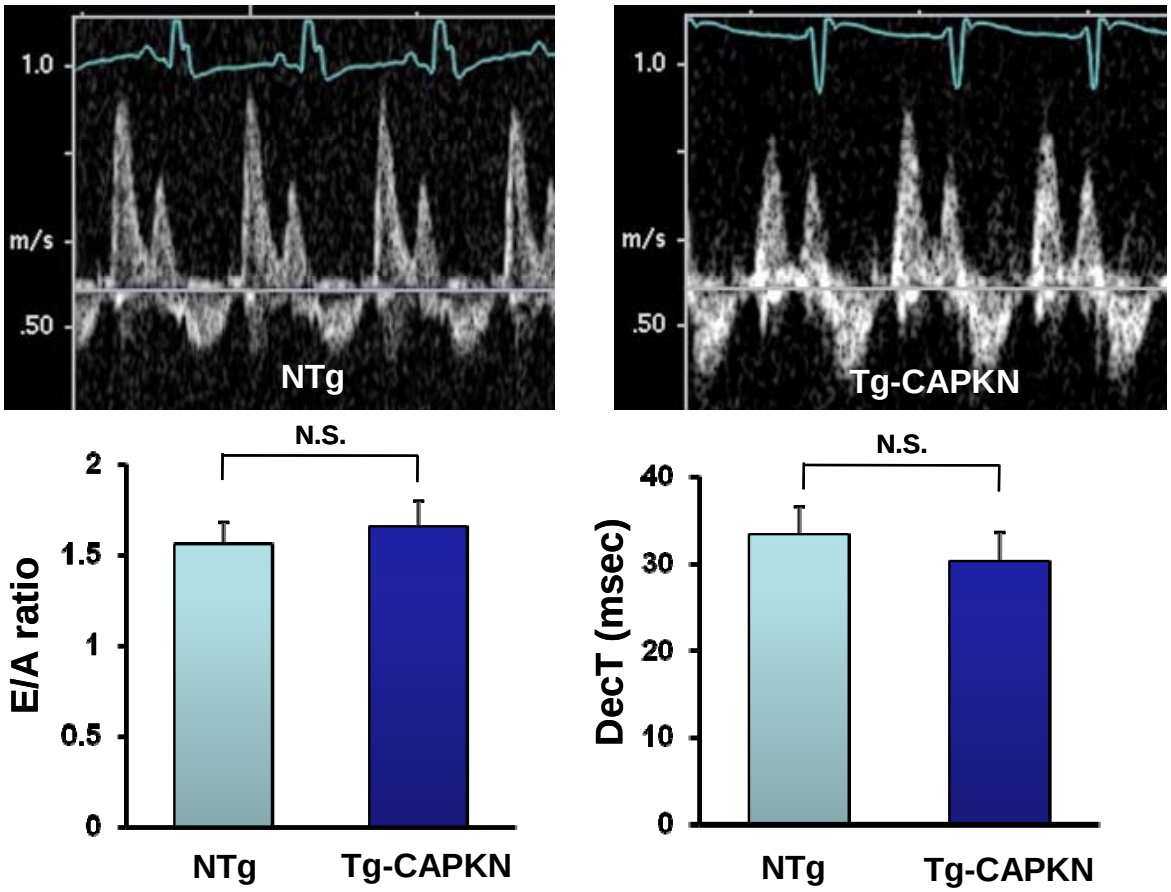


Figure II Diastolic function is not impaired in Tg-CAPKN mouse hearts.
Doppler analysis was performed in Tg-CAPKN mice using echocardiography. E/A ratio and deceleration time was comparable between Tg-CAPKN and NTg mice.

Supplemental Figure II

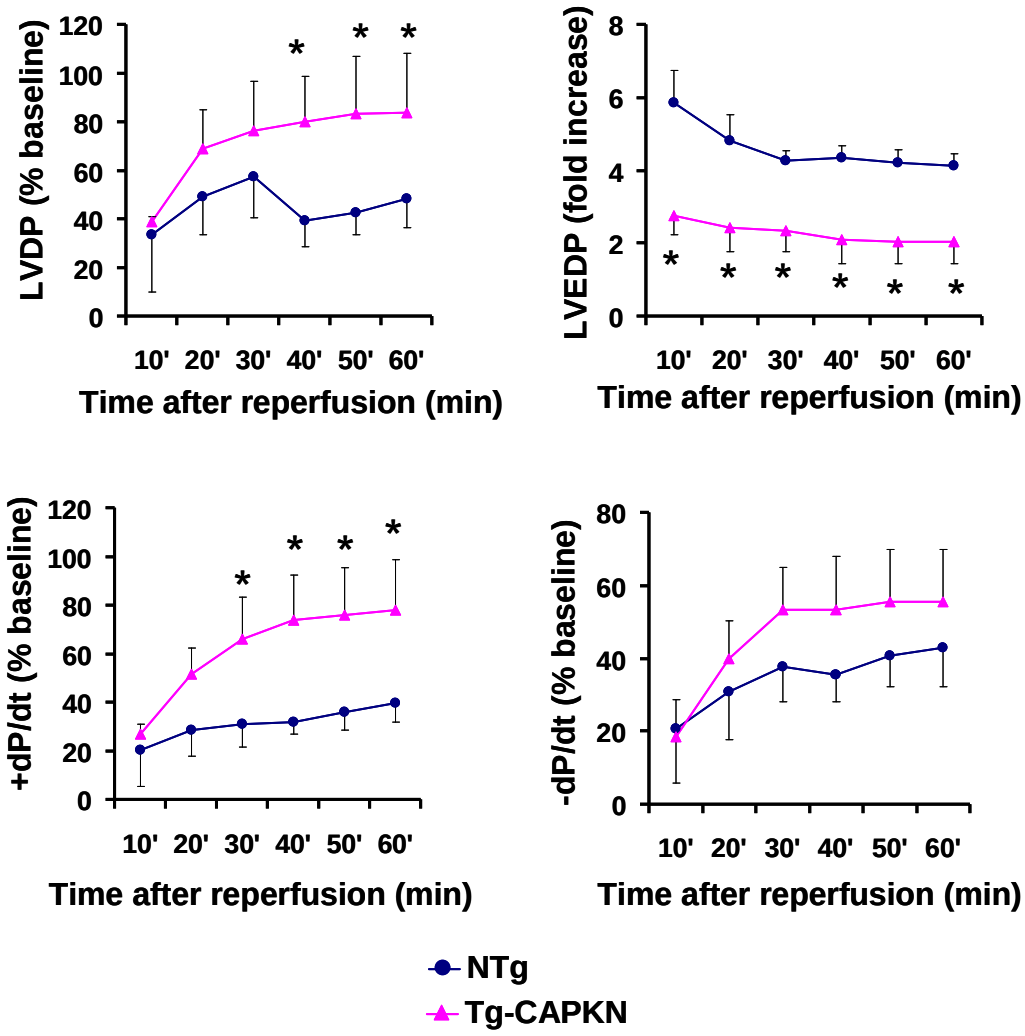


Figure III LV function is better in Tg-CAPKN mouse hearts after I/R
Mouse hearts were perfused with the Langendorff apparatus, and subjected to 45 min of global ischemia and reperfusion. LV developed pressure (LVDP), LV end-diastolic pressure (LVEDP), and LV positive and negative dP/dt were measured. n=3 each. * $p<0.05$ vs NTg.

Supplemental Figure III

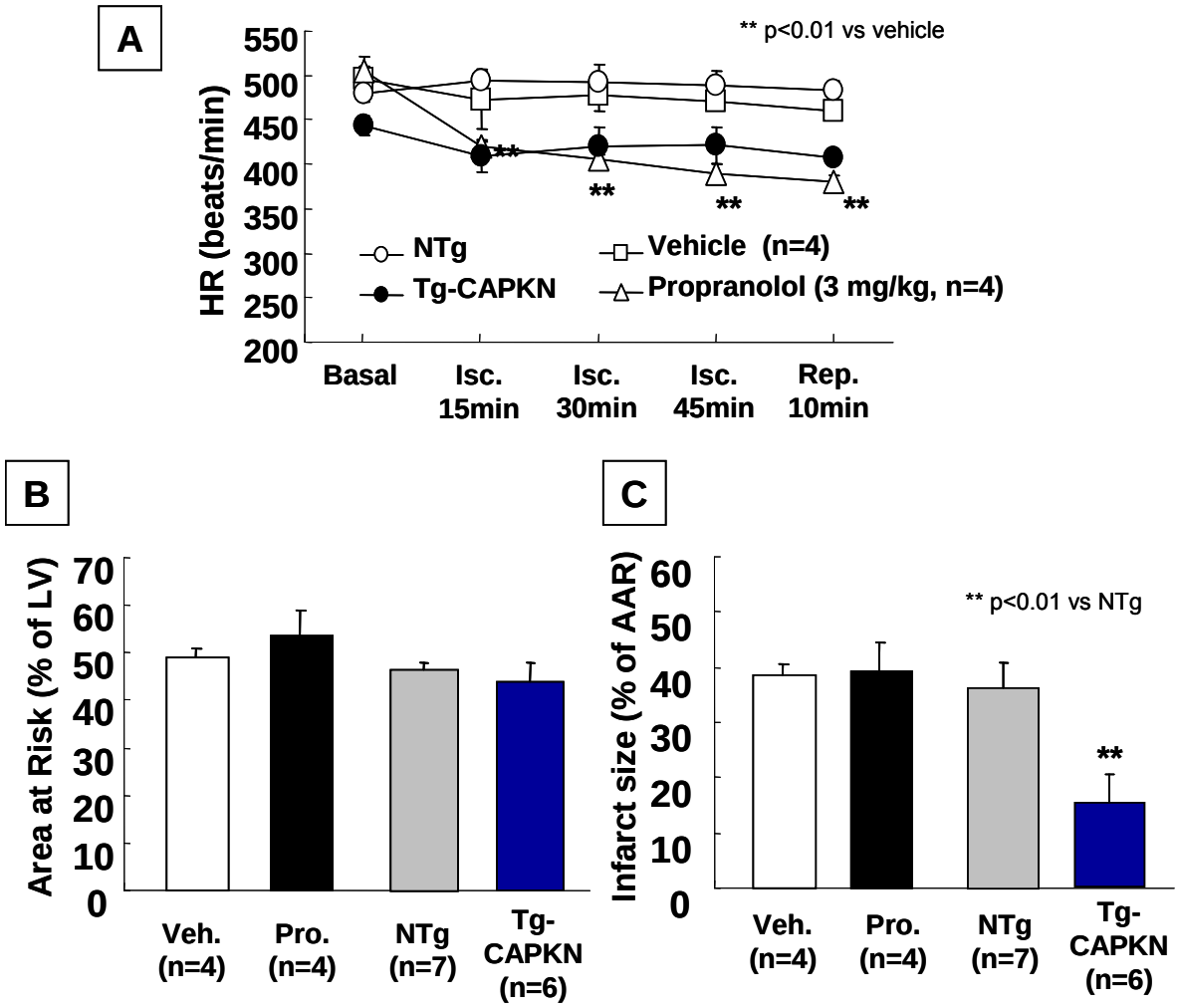


Figure IV Propranolol reduced heart rate, but did not affect Ischemia-reperfusion injury

Mice were subjected to 45 minutes of ischemia and 24 hours of reperfusion. Five min before ischemia, propranolol (Pro.) at a dose of 3 mg/kg or vehicle (Veh.) alone was injected intraperitoneally into some NTg mice. (A) Time course of heart rate during ischemia and reperfusion is shown. (B, C) The effect of propranolol injection upon AAR and the size of MI was evaluated. AAR/LV (%) (B) and MI area /AAR (%) (C) are shown. Note that propranolol reduced heart rate in NTg mice to the level of Tg-CAPKN mice, but it did not reduce the MI size to the level of that in Tg-CAPKN.

Supplemental Figure IV

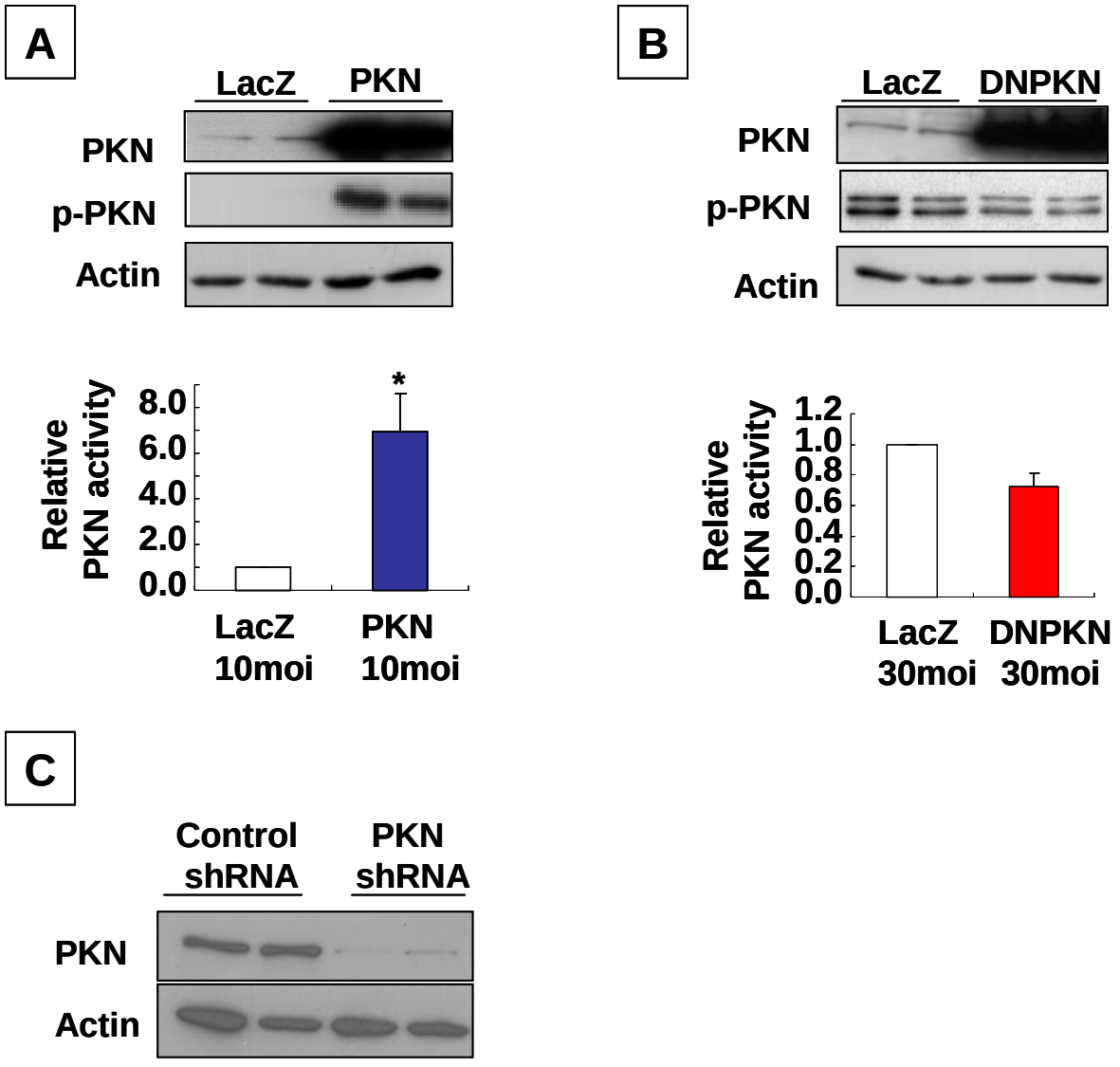
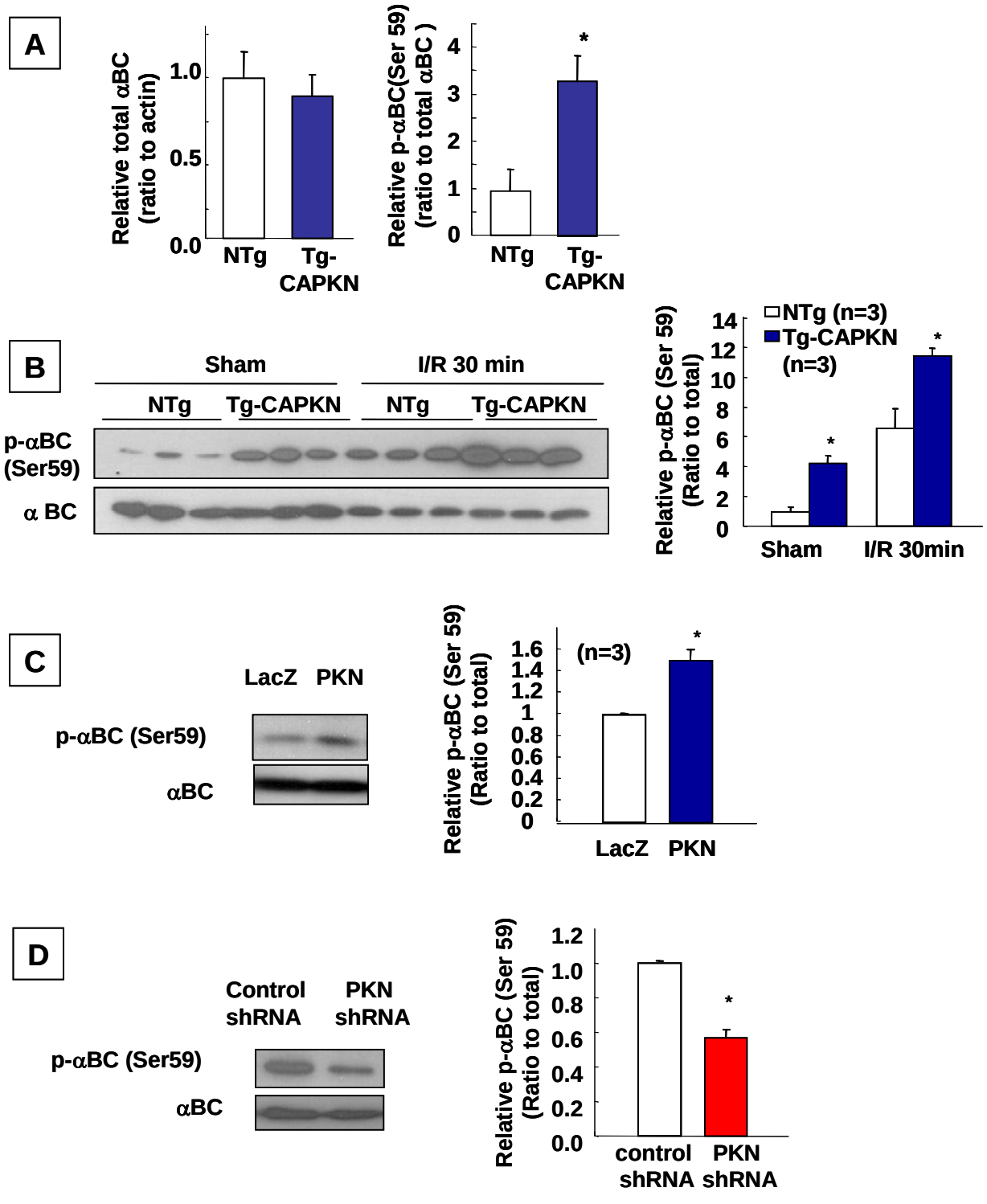


Figure V Expression and kinase activity of PKN in cardiac myocytes
Cultured cardiac myocytes were transduced with Ad-PKN (10 moi), Ad-DNPKN (10 moi), Ad-LacZ (10 moi), Ad-sh-PKN (30 moi)or Ad-sh-scramble (30 moi). Forty-eight hours after transduction, either immunoblot analyses with anti-PKN, anti-phospho (Thr774) or anti-actin antibody or immune complex kinase assays were conducted. (A) The effect of Ad-PKN transduction on PKN expression, PKN phosphorylation and PKN activity in cardiac myocytes. *p<0.05 vs. LacZ (B) The effect of Ad-DNPKN expression upon PKN expression and PKN activity is shown. Note that a gel picture with a longer exposure is shown for p-PKN in order to demonstrate the effect of DNPKN upon p-PKN at baseline. (C) The effect of Ad-sh-PKN upon expression of PKN is shown. In bar graphs, the level of PKN activity in LacZ transduced myocytes was expressed as 1.

Supplemental Figure V



Supplemental Figure VI

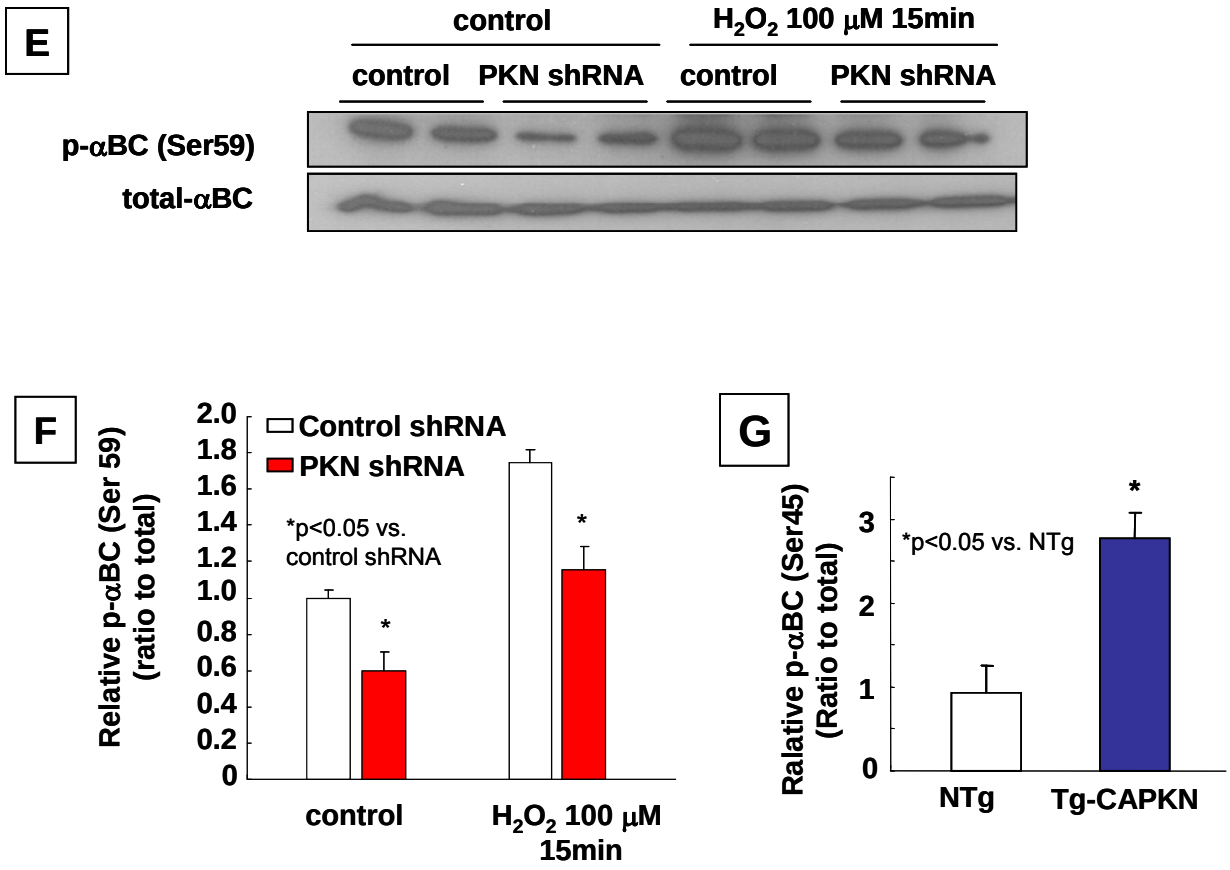


Figure VI PKN causes Serine 59 and Serine 45 phosphorylation of alpha B crystallin
Immunoblot analysis of alpha B crystallin (αBC) and Serine 59 phosphorylated alpha B crystallin (p-αBC (Ser59)) and the results of densitometric analyses (A-F) are shown. (A) The results of densitometric analysis of alpha B crystallin (αBC) and p-αBC (Ser59) in Tg-CAPKN mice are shown. Expression of total αBC normalized with actin or p-αBC in NTg was set as 1. n=4. * p<0.05 vs NTg. (B) p-αBC (Ser59) in Tg-CAPKN and NTg hearts with or without I/R. * p<0.05 vs NTg (C) p-αBC (Ser59) in cultured cardiac myocytes transduced with Ad-PKN. * p<0.05 vs LacZ. (D) p-αBC (Ser59) in cultured cardiac myocytes transduced with Ad-sh-PKN. * p<0.05 vs. control shRNA. (E-F) Myocytes were transduced with either Ad-sh-scramble or Ad-sh-PKN and then treated with or without H_2O_2. Immunoblot analysis of αBC and p-αBC (Ser59), E) and the results of densitometric analyses (F) are shown. (G) The results of densitometric analysis Serine 45 phosphorylated αBC (p-αBC (Ser45)) in Tg-CAPKN mice are shown.

Supplemental Figure VI contiuned

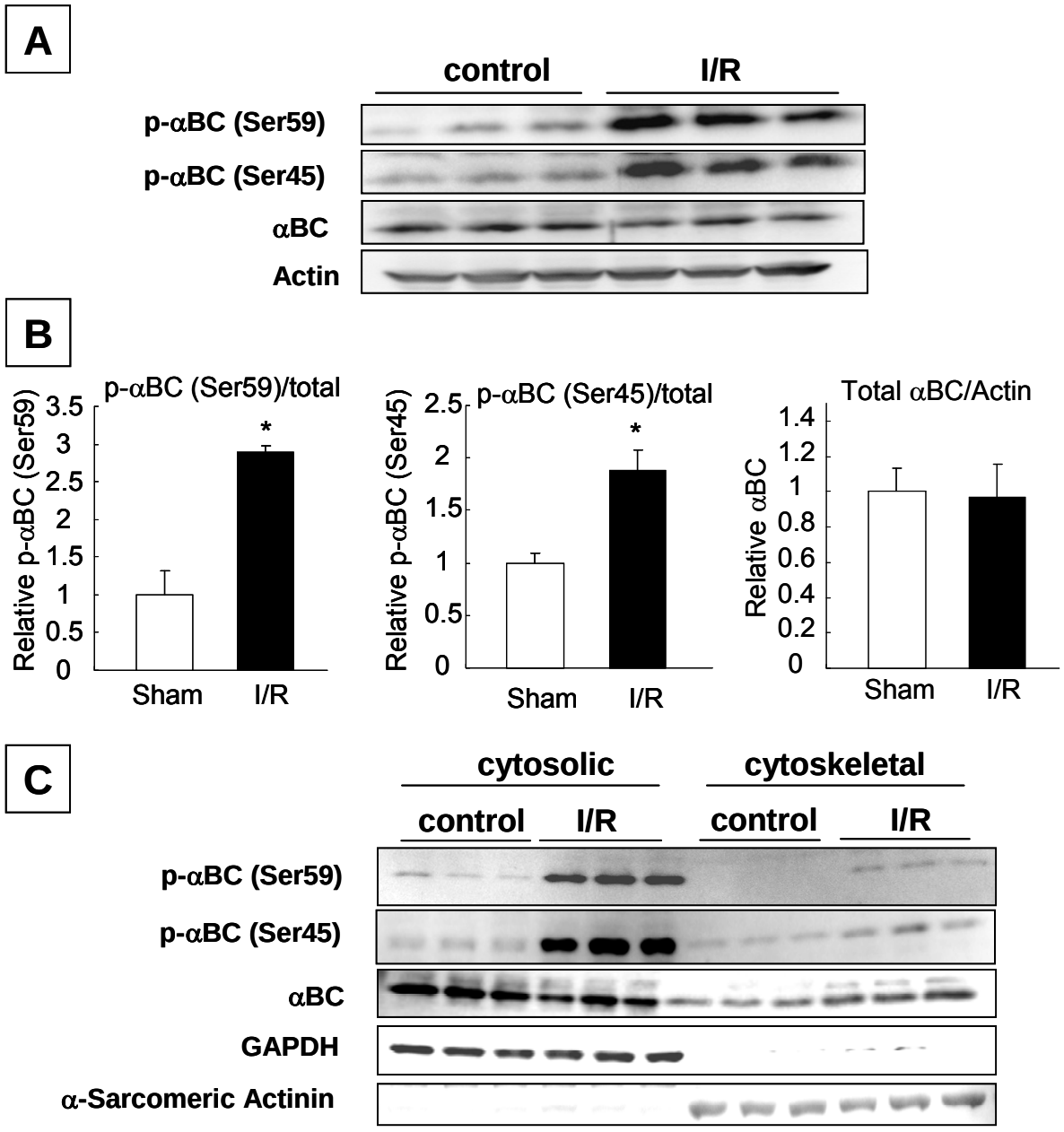


Figure VII I/R causes Serine 59 and 45 phosphorylation and translocation of alpha B crystallin in vivo.

A) Phosphorylation of alpha B crystallin (αBC) in the heart subjected to I/R is shown. B) The results of densitometric analysis of αBC and Ser59 and Ser45 phosphorylated p-αBC. Expression of total αBC normalized with actin or p-αBC normalized with total αBC in sham-operated mouse hearts was set as 1. n=3 each. C） Cytoskeletal and cytosolic fractions were prepared from the hearts subjected to 45 min ischemia followed by 1 hr of reperfusion as described in the methods. Subcellular localization of p-αBC (total, Ser45 or Ser59) in the heart subjected to 45 min ischemia followed by 1 hr reperfusion was evaluated by immunoblots.

Supplemental Figure VII

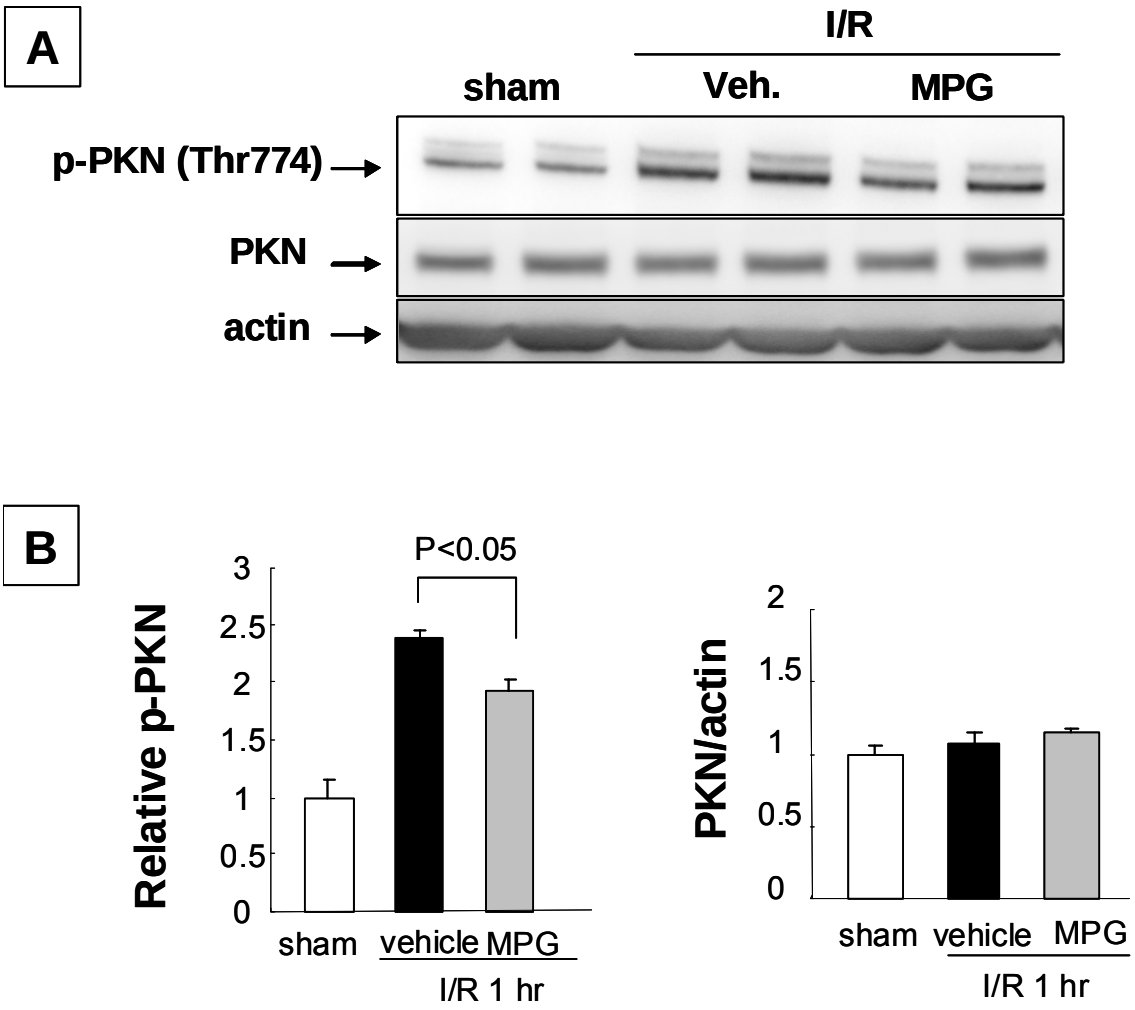


Figure VIII Effect of MPG, an anti-oxidant, on the phosphorylation of PKN in the mouse hearts subjected to I/R

Mice were subjected to 45 minutes of ischemia and 1 hours of reperfusion. Fifteen minutes before ischemia, MPG at the dose of 100 mg/kg/hr or vehicle alone was infused intravenously throughout the experiment in some NTg mice. (A) Immunoblot analysis of phospho-PKN in the heart subjected to I/R with or without MPG treatment. (B) The level of PKN Thr 774 phosphorylation was determined by densitometric analysis. The mean total PKN/actin or phosphorylated PKN/total PKN in sham operated mouse hearts was set as 1. n=4 each.

Supplemental Figure VIII

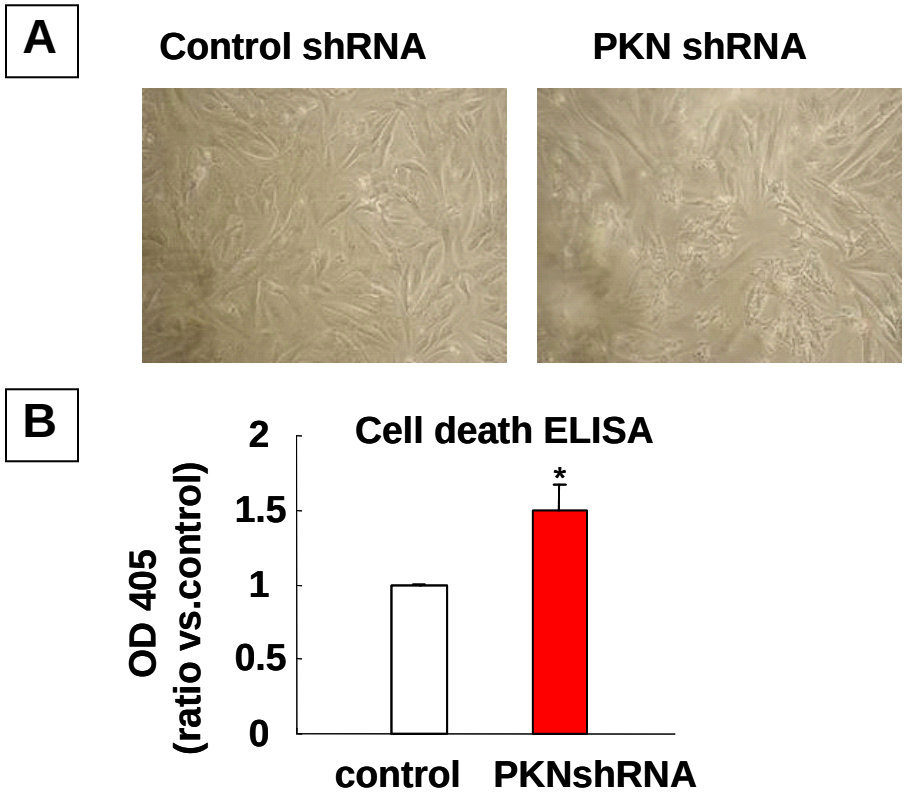


Figure IX Downregulation of endogenous PKN induces apoptosis in cultured cardiac myocytes

Cardiac myocytes were transduced with Ad-sh-scramble (control) or Ad-sh-PKN and cultured for 96 hours. DNA fragmentation was evaluated by Cell Death ELISA assay. (A) Phase contrast microscopic images are shown. Downregulation of PKN induces shrinkage in cardiac myocytes. (B) The effect of PKN downregulation upon cytoplasmic accumulation of mono- and oligonucleosomes determined by Cell Death ELISA assays. N=4 *p<0.05

Supplemental Figure IX

PKC epsilon

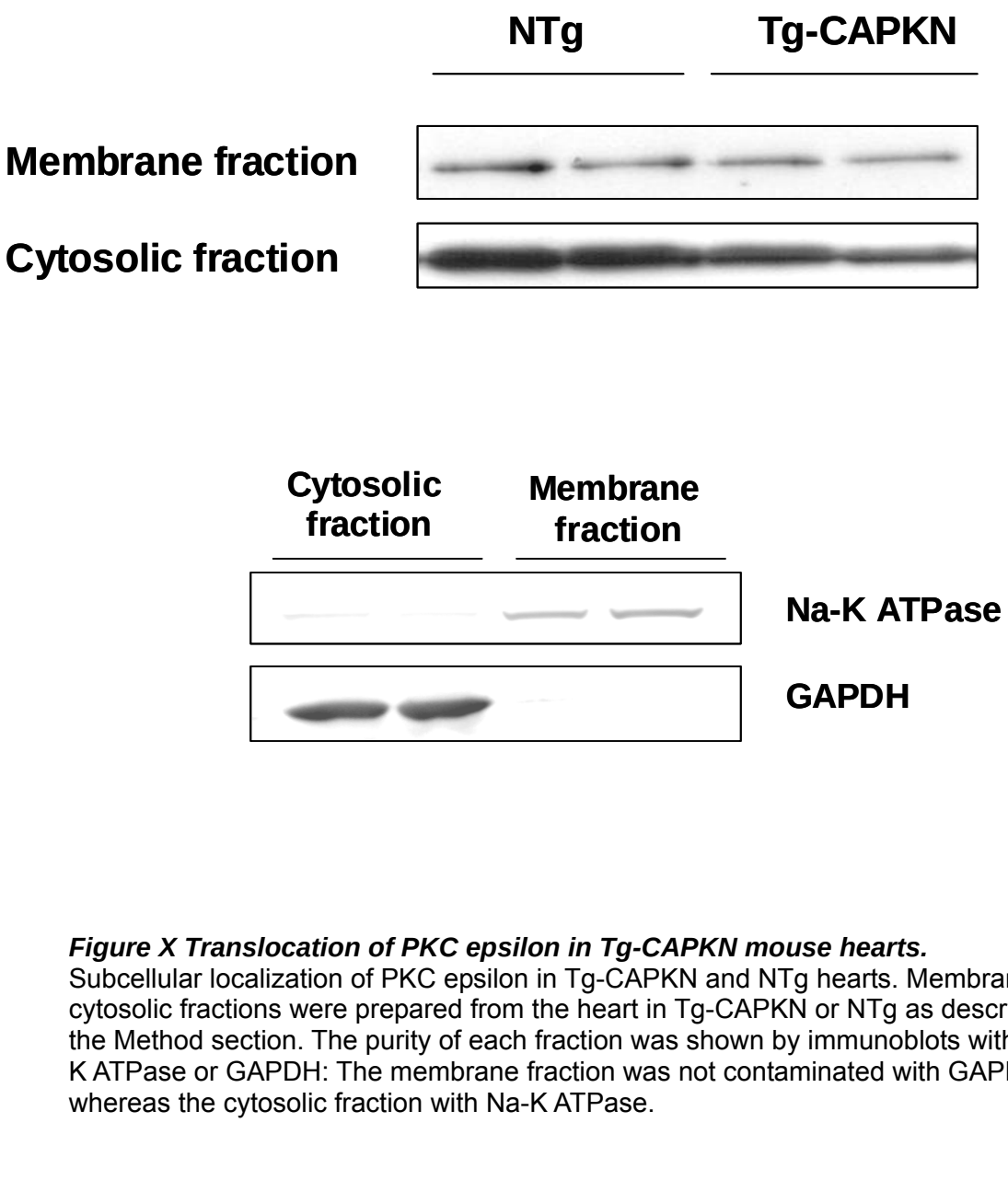


Figure X Translocation of PKC epsilon in Tg-CAPKN mouse hearts.
Subcellular localization of PKC epsilon in Tg-CAPKN and NTg hearts. Membrane and cytosolic fractions were prepared from the heart in Tg-CAPKN or NTg as described in the Method section. The purity of each fraction was shown by immunoblots with anti-Na-K ATPase or GAPDH: The membrane fraction was not contaminated with GAPDH whereas the cytosolic fraction with Na-K ATPase.

Supplemental Figure X

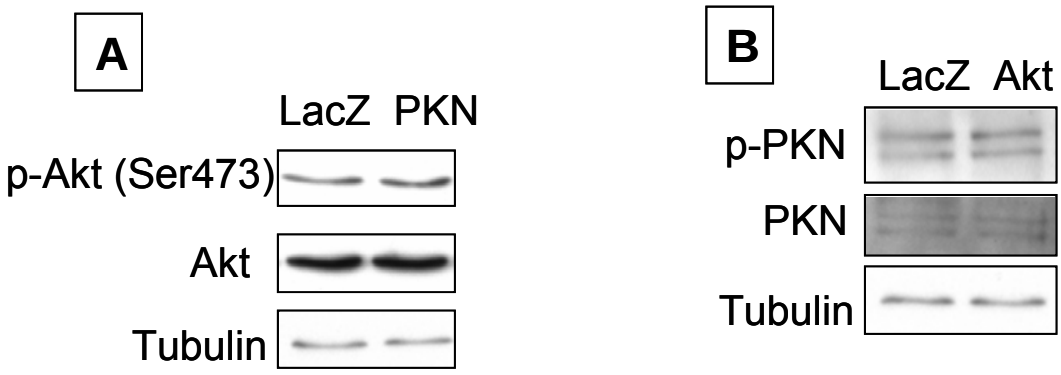


Figure XI Expression and phosphorylation of Akt in vivo and in vitro
Cultured cardiac myocytes were transduced with Ad-PKN, Ad-LacZ, or Ad-Akt (30 MOI). Forty-eight hours after transduction, either immunoblot analyses with anti-PKN, anti-phospho (Thr 774) PKN, anti-Akt, ani-phospho (Ser473)Akt or anti-actin antibody were conducted. (A) The effect of Ad-PKN transduction on Akt expression and phosphorylation in cardiac myocytes is shown. (B) The effect of Ad-Akt transduction on PKN expression and phosphorylation in cardiac myocytes is shown.

Supplemental Figure XI

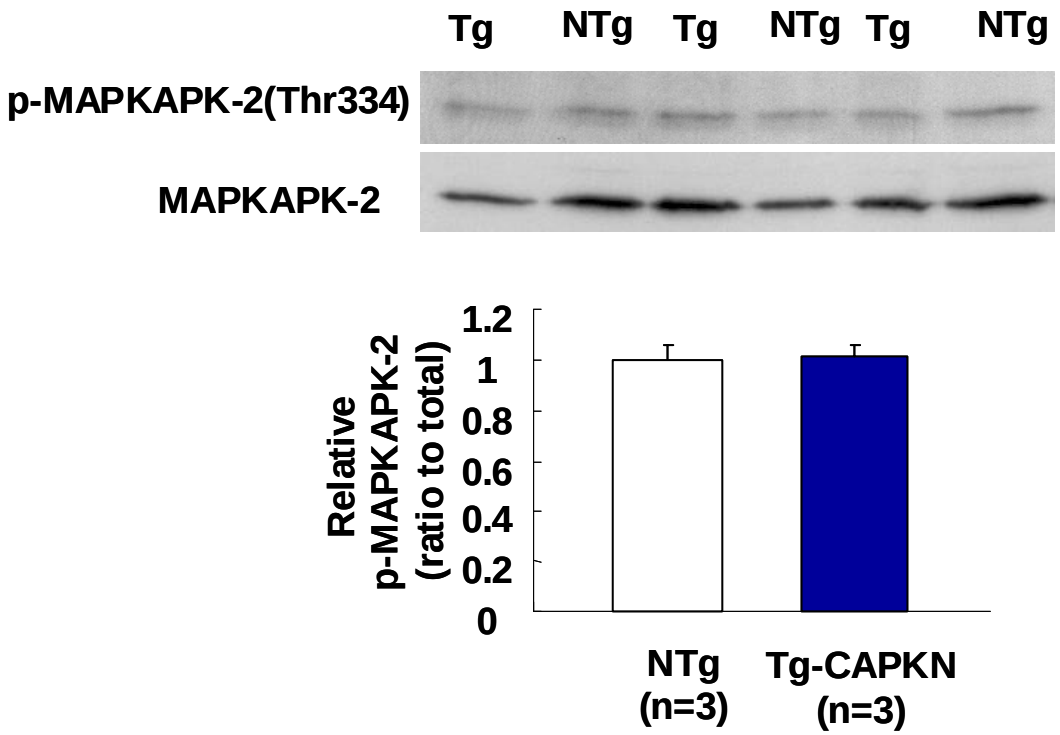


Figure XII Phosphorylation of MAPKAPK-2 in Tg-CAPKN mouse hearts.
Immunoblot analyses with anti-Thr334 phosphorylated MAPKAPK-2 in Tg-CAPKN or WT mouse hearts. In the bar graph, the level pf p-MAPKAPK2/total MAPKAPK-2 in NTg was set as 1.

Supplemental Figure XII

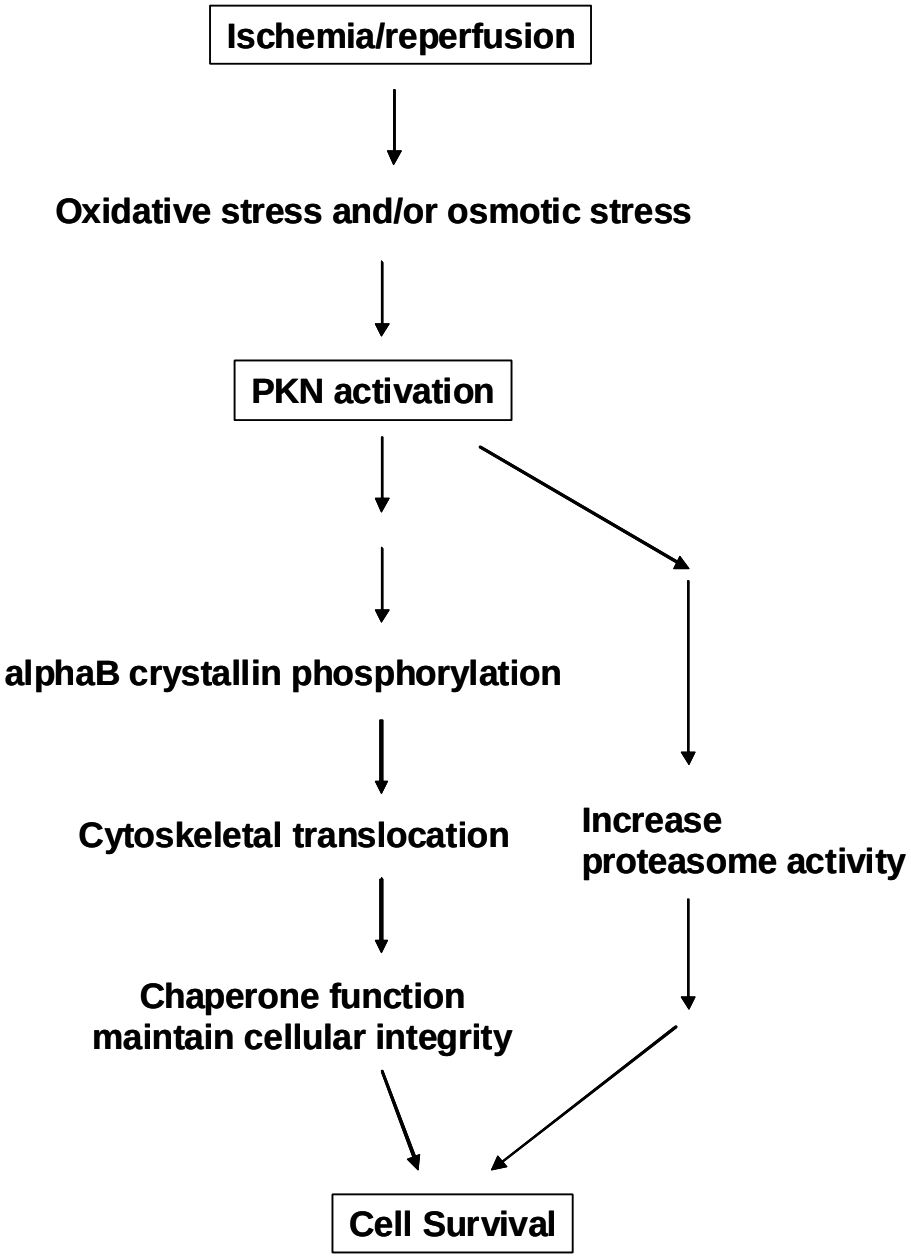


Figure XIII Schematic representation of our hypothesis regarding the role of PKN in mediating cell death and survival of cardiac myocytes during ischemia and reperfusion in the heart

Supplemental Figure XIII

Supplemental Tables

Table I Postmortem pathologic measurements of Tg-CAPKN mice

Group	NTg	Tg-CAPKN	NTg	Tg-CAPKN
Age (months)	**3**	**3**	**6**	**6**
N	**7**	**8**	**7**	**6**
BW (g)	**28 ± 1**	**29 ± 1**	**31 ± 1**	**35 ± 2**
HW (mg)	**111 ± 6**	**150 ± 7***	**123 ± 5**	**172 ± 8***
LV (mg)	**78 ± 3**	**102 ± 3***	**81 ± 3**	**123 ± 5***
RV (mg)	**19 ± 1**	**21 ± 1**	**24 ± 1**	**26 ± 1**
Atrium (mg)	**8 ± 1**	**8 ± 1**	**11 ± 1**	**14 ± 1**
Lung (mg)	**156 ± 8**	**146 ± 7**	**153 ± 5**	**158 ± 8**
HW/BW (mg/g)	**4.00 ± 0.12**	**5.14 ± 0.11***	**3.96 ± 0.10**	**5.04 ± 0.21***
LV/BW (mg/g)	**2.80 ± 0.05**	**3.52 ± 0.10***	**2.61± 0.07**	**3.61 ± 0.17***
RV/BW (mg/g)	**0.70 ± 0.04**	**0.73 ± 0.03**	**0.78 ± 0.04**	**0.76 ± 0.03**
Atrium/BW (mg/g)	**0.27 ± 0.02**	**0.28 ± 0.02**	**0.36 ± 0.02**	**0.42 ± 0.02**
Lung/BW (mg/g)	**5.72 ± 0.46**	**5.05 ± 0.25**	**4.91 ± 0.14**	**4.61 ± 0.25**

Values are means±SEM. *p<0.05, **p<0.01 vs. NTg

Supplemental Table I

Table II Echocardiographic Analysis of Tg-CAPKN Mice

Group	NTg	Tg-CAPKN	NTg	Tg-CAPKN
Age (months)	**3**	**3**	**6**	**6**
N	**8**	**8**	**7**	**6**
DSEP WT (mm)	**0.75 ± 0.04**	**1.06 ± 0.03***	**0.76 ± 0.03**	**1.21 ± 0.05***
LVEDD (mm)	**3.81 ± 0.19**	**3.78 ± 0.11**	**3.79 ± 0.08**	**3.65 ± 0.11**
DPW WT (mm)	**0.72 ± 0.05**	**1.01 ± 0.05***	**0.76 ± 0.03**	**1.21 ± 0.05***
SSEP WT (mm)	**1.18 ± 0.07**	**1.51 ± 0.08***	**1.15 ± 0.02**	**1.56 ± 0.05***
LVESD (mm)	**2.50 ± 0.12**	**2.48 ± 0.07**	**2.47 ± 0.06**	**2.52 ± 0.13**
SPW WT (mm)	**1.06 ± 0.07**	**1.34 ± 0.09***	**0.94 ± 0.03**	**1.43 ± 0.04***
EF (%)	**72 ± 4**	**72 ± 2**	**72 ± 1**	**72 ± 2**
FS (%)	**34 ± 1**	**34 ± 1**	**35 ± 1**	**31 ± 2**
HR (beats/min)	**429 ± 33**	**346 ± 29***	**429 ± 13**	**372 ± 10***

Values are means±SEM. *p<0.05, **p<0.01 vs. NTg

Supplemental Table II

Table III Postmortem pathologic measurements of Tg-DNPKN mice

Group	NTg	Tg-DNPKN
Age (months)	**3**	**3**
N	**7**	**8**
BW (g)	**29 ± 2**	**29 ± 3**
HW (mg)	**122 ± 8**	**117 ± 8**
LV (mg)	**85 ± 4**	**81 ± 6**
RV (mg)	**19 ± 1**	**19 ± 2**
Atrium (mg)	**10 ± 1**	**10 ± 1**
Lung (mg)	**134 ± 4**	**131 ± 7**
HW/BW (mg/g)	**4.20 ± 0.28**	**4.11 ± 0.22**
LV/BW (mg/g)	**2.93 ± 0.16**	**2.84 ± 0.12**
RV/BW (mg/g)	**0.68 ± 0.04**	**0.67 ± 0.06**
Atrium/BW (mg/g)	**0.33 ± 0.04**	**0.34 ± 0.05**
Lung/BW (mg/g)	**4.63 ± 0.37**	**4.59 ± 0.59**

Values are means±SEM.

Supplemental Table III

Table IV Echocardiographic Analysis of Tg-DNPKN Mice

Group	NTg	Tg-DNPKN
Age (months)	**3**	**3**
N	**6**	**6**
DSEP WT (mm)	**0.90 ± 0.09**	**0.84 ± 0.05**
LVEDD (mm)	**3.67 ± 0.29**	**3.65 ± 0.11**
DPW WT (mm)	**0.85 ± 0.12**	**0.84 ± 0.06**
SSEP WT (mm)	**1.43 ± 0.15**	**1.36 ± 0.06**
LVESD (mm)	**2.36 ± 0.25**	**2.32 ± 0.12**
SPW WT (mm)	**1.12 ± 0.10**	**1.19 ± 0.09**
EF (%)	**74 ± 4**	**74 ± 5**
FS (%)	**36 ± 3**	**36 ± 4**
HR (beats/min)	**415 ± 13**	**406 ± 36**

Values are means±SEM.

Supplemental Table IV

Reference for Supplement

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