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Activation of PKN mediates survival of cardiac myocytes in the heart during ischemia/reperfusion

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Abstract

Rationale—The function of PKN, a stress-activated protein kinase, in the heart is poorly understood.

Objective—We investigated the functional role of PKN during myocardial ischemia/reperfusion (I/R).

Methods and Results—PKN is phosphorylated at Thr774 in hearts subjected to ischemia and reperfusion. Myocardial infarction/area at risk (MI/AAR) produced by 45 min ischemia and 24 hours reperfusion was significantly smaller in transgenic mice with cardiac specific overexpression of constitutively active (CA) PKN (Tg-CAPKN) than in non-transgenic (NTg) mice (15 ± 5 vs $38 \pm 5\%$, $p < 0.01$). The number of TUNEL positive nuclei was significantly lower in Tg-CAPKN (0.3 ± 0.2 vs $1.0 \pm 0.2\%$, $p < 0.05$). Both MI/AAR (63 ± 9 vs $45 \pm 8\%$, $p < 0.05$) and the number of TUNEL positive cells (7.9 ± 1.0 vs $1.3 \pm 0.9\%$, $p < 0.05$) were greater in transgenic mice with cardiac specific overexpression of dominant negative PKN (Tg-DNPKN) than in NTg mice. Thr774 phosphorylation of PKN was also observed in response to H₂O₂ in cultured cardiac myocytes. Stimulation of PKN prevented, whereas inhibition of PKN aggravated cell death induced by H₂O₂, suggesting that the cell protective effect of PKN is cell-autonomous in cardiac myocytes. PKN induced phosphorylation of alpha B crystallin and increased cardiac proteasome activity. The infarct reducing effect in Tg-CAPKN mice was partially inhibited by epoxomicin, a proteasome inhibitor.

Conclusion—PKN is activated by I/R and inhibits apoptosis of cardiac myocytes, thereby protecting the heart from I/R injury. PKN mediates phosphorylation of alpha B crystallin and stimulation of proteasome activity, which in part mediates the protective effect of PKN in the heart.

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Disclosures
None

Keywords

Ischemia/reperfusion; PKN; alpha B crystallin; proteasome

Introduction

Although myocardial ischemia and reperfusion induce lethal injury in the heart, the heart and the cardiac myocytes therein have powerful endogenous mechanisms to protect themselves from energy deficiency, oxidative stress, protein aggregation and organelle malfunction, thereby minimizing myocardial injury 1. Ischemic preconditioning is one of the strongest of these endogenous mechanisms of cardioprotection, and pharmacological intervention mimicking the ischemic preconditioning effect is considered to be a promising modality for the treatment of ischemic heart disease 1. However, other endogenous mechanisms of cardioprotection activated during ischemia and reperfusion remain to be elucidated.

PKN, also known as protein kinase C-related kinase 1 (PRK1), is a serine (Ser)/threonine (Thr) kinase, with a molecular mass of 120KDa 2. PKN consists of a catalytic domain highly homologous to that of protein kinase C (PKC) in the carboxyl-terminal region, and a regulatory domain in the amino-terminal region 2. PKN localizes primarily in the cytosolic fraction and possesses a wide variety of functions, such as cytoskeletal regulation, cell adhesion, vesicle transport, and cell cycle regulation 3, and it has been shown to be involved in the pathogenesis of Alzheimer's disease 4 and amyotrophic lateral sclerosis 5. Interestingly, PKN is cleaved by caspase 3 6, and a truncated and kinase-active form of PKN has been found in ischemia/reperfusion (I/R) models of the rat retina 7 and brain 8, suggesting that PKN may modulate cell survival or death during I/R. Transgenic (Tg) mice overexpressing a constitutively activated (CA) form of PKN in mammary epithelium exhibit impaired tight junction sealing and increased apoptosis, resulting in precocious involution in the mammary gland 9.

PKN activates atrial natriuretic factor (ANF) gene expression in cardiac myocytes 10. However, the *in vivo* function of PKN is poorly understood in the heart. Thus, a major goal in this study was to elucidate the function of PKN in the heart *in vivo*. Our preliminary studies indicated that PKN is activated by stress, such as hypotonic stress and I/R. Therefore, we examined the role of PKN in regulating cell survival and death and myocardial injury in response to I/R, and the underlying molecular mechanisms mediating the action of PKN in cardiac myocytes. Our results suggest that activation of PKN is an endogenous mechanism of cardioprotection against I/R injury.

Materials and Methods

An expanded Methods section is available in the Online Data Supplement at <http://circres.ahajournals.org>. All animal protocols were approved by the review board of the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

PKN kinase assay

Cardiac myocyte lysates were prepared in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% IGEPAL CA-630, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L beta-glycerophosphate, 0.1 mmol/L Na₃VO₄, 1 mmol/L NaF, 50 μmol/L phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, and 5 μg/ml leupeptin. PKN was immunoprecipitated with PKN antibody (BD Transduction Laboratories) at 4°C for 120

min. Protein G Sepharose beads were added and incubated at 4°C for 50 min. After washing three times, the precipitates were incubated for 30 min at 30°C with reaction buffer containing 20 mmol/L Tris-HCl (pH7.5), 4 mmol/L MgCl₂, 20 μmol/L ATP, and 0.8 μCi [γ -³²P]ATP, and with 40 μmol/L PKC δ peptide (AMFPTMNRSGSIKQAKI) as a peptide substrate. Incorporation of ³²P into the substrate was measured by scintillation counting.

Generation of Tg-CAPKN and Tg-DNPKN mice

Transgenic mice with cardiac specific overexpression of CAPKN 6 (amino acids 561-942) and DNPKN (full length K644D 3) were generated on an FVB background using the α -myosin heavy chain promoter (courtesy, Dr. J Robbins, Children's Hospital, Cincinnati, OH).

Construction of short hairpin RNA (shRNA) adenoviral expression vectors

The U6 RNA polymerase III promoter and the polylinker region were subcloned into the adenoviral shuttle vector pDC311 (Microbix). The hairpin-forming oligo, 5'-GATTGACATCATCCGCATGTTCAAGAGACATGCGGATGATGTCAATC-3' from the rat PKN cDNA, and its antisense, and the hairpin-forming oligo, 5'-ACGTGGATCCTCTCACCATTATTCAAGAGATAATGGTGAGAGGATCCACGT-3' from the rat alpha B crystallin (BC) cDNA, and its antisense with ApaI and Hind III overhangs were synthesized, annealed, and subcloned distal to the U6 promoter. The loop sequence is underlined. A recombinant adenovirus was generated as described 11.

Statistics

All data are expressed as mean \pm SEM. Differences between experimental groups were evaluated for statistical significance using Student's t-test for unpaired data or one-way ANOVA followed by Dunnett's post-test when appropriate. P values < 0.05 were considered to be statistically significant.

Results

PKN is activated by I/R in the heart

We examined whether PKN is activated in the heart under stress conditions, using a myocardial I/R model. In hearts subjected to ischemia, Thr774 phosphorylation of PKN was observed after 15–30 min, and declined after 45 min (Fig. 1AB). After reperfusion, PKN phosphorylation was observed again, and this phosphorylation was sustained for more than 24 hours (Fig. 1AB). In the hearts of sham-operated mice, expression and phosphorylation of PKN were unchanged over the entire experimental period (Online Fig. I).

CAPKN transgenic mice show cardiac hypertrophy without cardiac dysfunction

To elucidate the role of PKN activation in the *in vivo* heart, we generated mice with cardiac specific expression of constitutively active PKN (Tg-CAPKN), using the α MHC promoter. In Tg-CAPKN mouse hearts, phosphorylation of endogenous PKN was increased 5 fold compared to in non-transgenic (NTg) hearts (Fig. 2A). Heart weight/body weight (HW/BW) and left ventricular weight/BW (LVW/BW) were significantly higher in Tg-CAPKN than in NTg mice at both 3 and 6 months old (Online Table I). Lung weight/BW was not different between Tg-CAPKN and NTg mice (Online Table I). LV cardiac myocyte cross sectional area was significantly greater in Tg-CAPKN mice than in NTg mice (Fig. 2BC). The echocardiographic parameters in Tg-CAPKN mice were normal except that the wall thickness was significantly greater in Tg-CAPKN mice than in NTg mice at both the ages of 3 and 6 months (Online Table II). The end-diastolic diameter and end-systolic diameter were similar in both Tg-CAPKN and NTg mice, and the fractional shortening and the ejection

fraction were also comparable (Online Table II). Doppler analysis showed that the E/A ratio and deceleration time, indexes of diastolic function, were comparable in Tg-CAPKN and NTg mice (Online Fig. II). In Tg-CAPKN mice, the heart rate (HR) was slower by 10% compared to NTg mice (Online Table II). Taken together, these data indicate that Tg-CAPKN mice showed left ventricular hypertrophy without LV dysfunction.

We also generated transgenic mice with cardiac specific overexpression of dominant negative (DN) PKN (Tg-DNPKN). The baseline cardiac phenotype of Tg-DNPKN was normal (Online Tables III and IV).

I/R injury is attenuated in Tg-CAPKN mice

Using the gain of function animal model, we examined whether activation of PKN is protective against I/R injury. Tg-CAPKN and NTg mice at 3 months old were subjected to 45 min of ischemia and 24 hours of reperfusion. The area at risk (AAR) was similar in Tg-CAPKN and NTg mice. The infarct size/AAR (MI/AAR) was significantly smaller in Tg-CAPKN than in NTg mice (15.1 ± 5.2 vs $37.5 \pm 4.9\%$, $p < 0.01$, Fig. 3A–C). There were also fewer TUNEL positive cells in the AAR in Tg-CAPKN than in NTg mice (0.3 ± 0.2 vs $1.0 \pm 0.2\%$, $p < 0.05$, Fig. 3DE). Furthermore, in Langendorff-perfused heart preparations, LV developed pressure and LV dP/dt were higher, and LV end-diastolic pressure was lower in Tg-CAPKN mice during the recovery phase after 45 min of global ischemia (Online Fig. III). These results suggest that activation of PKN is protective against I/R injury.

It should be noted that the heart rate was slower by 10–15% in Tg-CAPKN mice than in NTg mice. To test the possibility that the reduced heart rate alone could be the mechanism of cardioprotection in the Tg-CAPKN heart, we reduced the heart rate in the NTg mice to the same level as that in Tg-CAPKN mice using propranolol, a beta blocker, during the ischemia and early reperfusion periods. Although intraperitoneal injection of propranolol (3 mg/kg) reduced the heart rate in NTg to a similar level as that in Tg-CAPKN mice, MI/AAR was still significantly smaller in Tg-CAPKN than in NTg (Online Fig. IV), suggesting that lowering the heart rate alone in NTg mice is not sufficient to achieve the infarct reducing effect observed in Tg-CAPKN mice.

I/R injury is enhanced in Tg-DNPKN mice

To elucidate the role of endogenous PKN in mediating protection against I/R injury, we also conducted I/R in Tg-DNPKN mice. Activation of PKN in response to I/R was abolished in Tg-DNPKN (not shown, See also Fig. 6E). In the DNPKN heart, MI/AAR (62.5 ± 9.3 vs $45.2 \pm 8.4\%$, $p < 0.05$) and the number of TUNEL positive cells (7.9 ± 1.0 vs $1.3 \pm 0.9\%$, $p < 0.05$) were both greater than in NTg mice (Fig. 4A–F), indicating that activation of endogenous PKN is protective during I/R.

Activation of PKN attenuates, whereas inactivation of PKN enhances death of cardiac myocytes in response to hydrogen peroxide stimulation *in vitro*

To evaluate whether the protective effect of PKN is cell-autonomous in cardiac myocytes, we conducted *in vitro* experiments, using cultured cardiac myocytes and adenovirus vectors harboring PKN (Ad-PKN), DNPKN (Ad-DNPKN) or shRNA-PKN (Ad-sh-PKN) (Online Fig. V). Oxidative stress is one of the most important causes of I/R injury in the heart. To evaluate the role of PKN in mediating cell survival and death in response to oxidative stress, cardiac myocytes were transduced with Ad-PKN, Ad-DNPKN or Ad-sh-PKN, and then treated with hydrogen peroxide (H_2O_2). Treatment with H_2O_2 induced PKN Thr774 phosphorylation in cardiac myocytes (Fig. 5A). Transduction of Ad-PKN reduced (Fig. 5B), while that of Ad-DNPKN or Ad-sh-PKN enhanced cell death (Fig. 5CD). These results

suggest that activation of endogenous PKN during oxidative stress is protective, and that the cell protective effect of PKN is cell-autonomous.

Activation of PKN causes phosphorylation and translocation of alpha BC into the cytoskeletal fraction

Alpha BC, a member of the small heat shock protein family of molecular chaperones, has a cell protective effect against stresses 12. PKN upregulates alpha BC gene expression in HeLa S3 cells 13. To address the molecular mechanism mediating the cell protective effect of PKN in cardiac myocytes, we evaluated expression of total and phosphorylated forms of alpha BC. The level of total alpha BC was not different between Tg-CAPKN and NTg hearts (Fig 6A, Online Fig. VI A). However, the level of alpha BC phosphorylated at Ser59 was significantly increased in Tg-CAPKN mice (Fig. 6A, Online Fig. VI AB). In neonatal rat cardiac myocytes, transduction with Ad-PKN increased, whereas that with Ad-sh-PKN reduced Ser59 phosphorylation of alpha BC (Online Fig. VI CD). Treatment with H₂O₂ caused an increase in Ser59 phosphorylation of alpha BC, which was partially inhibited by shRNA PKN (Online Fig. VI EF). Alpha BC translocates to the cytoskeletal fraction and interacts with cytoskeletal proteins in response to stress 14, thereby playing a protective role in the heart 15. Translocation of alpha BC from the cytosol to the cytoskeletal fraction was also increased in Tg-CAPKN mice (Fig. 6B). In addition to Ser59 phosphorylation, Ser45 phosphorylation of alpha BC and its translocation to the cytoskeletal fraction were also increased in Tg-CAPKN mice (Fig. 6CD, Online Fig. VI G). In hearts subjected to I/R, both Ser45 and Ser59 phosphorylation of alpha BC and its translocation to the cytoskeletal fraction were increased (Online Fig. VI I). The increase in alpha BC phosphorylation at both Ser45 and Ser59 in response to I/R was markedly inhibited in Tg-DNPKN mice (Fig. 6E). These results suggest that PKN plays an important role in mediating phosphorylation of alpha BC, which may in turn mediate protective effects in the heart during I/R. To address the role of alpha BC in mediating the cell protective effect of PKN, we knocked down alpha BC by shRNA, and examined the cell viability under H₂O₂ treatment in cultured cardiac myocytes. Ad-sh-alpha BC inhibited the cell protective effect of PKN, indicating that alpha BC mediates the cell protective effect of PKN (Fig. 6F).

PKN increases proteasome activity

Phosphorylation of alpha BC is important for its chaperone activity 16, which in turn plays an important role in regulating protein quality control via the ubiquitin-proteasome system. We therefore evaluated proteasome activity in Tg-CAPKN mice and cardiac myocytes treated with Ad-PKN. The proteasome activity, as evaluated by chymotrypsin-like activity, was significantly elevated in Tg-CAPKN hearts compared to NTg hearts (Fig. 7A). Similarly, cardiac myocytes transduced with Ad-PKN exhibited greater chymotrypsin-like activities than Ad-LacZ transduced myocytes (not shown). In hearts subjected to I/R, chymotrypsin-like activity was decreased, an effect which was significantly attenuated in Tg-CAPKN hearts (Fig. 7B). Furthermore, the decrease in chymotrypsin-like activity in response to I/R was significantly enhanced in Tg-DNPKN hearts (Fig. 7C). In order to test the functional significance of the elevated proteasome activity in Tg-CAPKN hearts, the effect of epoxomicin, a proteasome inhibitor, upon the cardioprotective effect of CAPKN was evaluated. Epoxomicin partially attenuated the infarct-reducing effect in Tg-CAPKN mice (Fig. 7D). These results are consistent with the notion that the elevated proteasome activity in Tg-CAPKN may in part mediate the protective effect against I/R.

Discussion

Our main findings in this work are 1) that PKN is activated by I/R in the heart *in vivo* and by oxidative stress in cardiac myocytes *in vitro*, 2) that activation of PKN causes cardiac

hypertrophy without LV dysfunction, 3) that activation of PKN plays a cell protective role in cardiac myocytes during I/R *in vivo* and in response to oxidative stress *in vitro*, and 4) that PKN mediates its protective effects against I/R in part through activation of the alpha BC/ proteasome pathway.

In our study, Thr774 phosphorylation of PKN, which is essential for its activation, was observed in hearts subjected to I/R *in vivo*. In yeast, Pkc1, whose amino-terminal regulatory region is highly homologous to that of PKN, is activated by cell wall stress, including hypotonic stress¹⁷. Pkc1 is also important for cell viability in the adaptive response to oxidative stress^{18, 19} or nitrosative stress¹⁹. I/R causes both oxidative and osmotic stress, both of which affect survival and death of cardiac myocytes in the heart^{20, 21}. PKN is phosphorylated by H₂O₂ in cardiac myocytes *in vitro*, and PKN phosphorylation caused by I/R is inhibited in the presence of MPG, an antioxidant, in the heart *in vivo* (Online Fig. VIII), suggesting that oxidative stress plays an important role in mediating I/R-induced PKN activation. Furthermore, we found previously that hypotonic stress causes activation of PKN *in vitro* (manuscript in preparation by KK and JS). Thus, we speculate that osmotic stress may also mediate PKN activation in response to I/R. Interestingly, RhoA, a known activator of PKN³, is activated by reactive oxygen species²². Oxygen radicals can stimulate PKC directly by oxidative modification of its regulatory domain²³. The role of RhoA and oxidative posttranslational modification of PKN in mediating activation of PKN during I/R remains to be elucidated.

Our findings suggest that activation of PKN in cardiac myocytes is necessary and sufficient for cardioprotection during I/R *in vivo* and in response to H₂O₂ *in vitro*. Although LV function in Tg-DNPKN mice was comparable to that in NTg mice at 3 months of age, nearly complete downregulation of PKN by Ad-sh-PKN induces cell death in cardiac myocytes *in vitro* (Online Fig. IX), suggesting that a low level of PKN is required for survival of cardiac myocytes. Thus, all lines of experimental evidence presented in this work support the notion that PKN promotes survival of cardiac myocytes. Since PKN reduces the number of TUNEL positive myocytes in the ischemic area after I/R, we speculate that the protective effect of PKN is mediated through suppression of apoptosis, but its effect upon other forms of cell death remains to be elucidated.

Since suppression of myocardial injury during I/R was exacerbated when activation of PKN was inhibited in Tg-DNPKN mice, activation of PKN during I/R protects the heart. However, since Tg-CAPKN mice have stronger and more persistent activation of PKN, the cardioprotective effect observed in Tg-CAPKN may also be mediated in part by preconditioning effects. Our preliminary results suggest that PKN is also activated by preconditioning (data not shown). Whether or not activation of endogenous PKN can achieve protection against prolonged ischemia or late preconditioning remains to be elucidated. Neither translocation of PKC ϵ into the membrane fraction (Online Fig. X) nor Akt phosphorylation, common mediators of ischemic preconditioning, was induced by overexpression of PKN in cardiac myocytes *in vitro* (Online Fig. XI). Thus, it is likely that the cardioprotective effects of CAPKN are mediated by PKC ϵ - and Akt-independent mechanisms. Development of small molecules that specifically stimulate PKN would be relevant clinically, considering the fact that stimulators of PKC ϵ have thus far shown promising results in their preclinical studies²⁴.

Tg-CAPKN mice showed mild LV hypertrophy. PKN is involved in actin reorganization in vascular smooth muscle cells²⁵ and stimulates ANF gene expression in cardiac myocytes¹⁰, integral features of cardiac hypertrophy. PKN has also been shown to interact directly with alpha-actinin²⁶. Thus, activation of PKN by stress could stimulate cardiac hypertrophy *in vivo*. It should be noted that whether or not PKN is a physiological mediator of cardiac

hypertrophy requires further evaluation with loss of function models of PKN. The presence of hypertrophy alone may secondarily affect the cardioprotective effect of PKN against I/R. However, since even short term activation or inactivation of PKN affects cell survival in a cell autonomous manner, the protective effect of PKN may be at least in part hypertrophy-independent.

Thus far, we have found that LV function in Tg-CAPKN mice is well maintained, even after over a year of follow up (data not shown). Together with the cardioprotective effect of PKN during I/R, we speculate that cardiac hypertrophy induced by PKN may be compensatory/physiological. However, this notion would require further testing by applying long-term hypertrophic stimulation, such as pressure overload, to Tg-CAPKN mice. In addition, the reduced heart rate could have resulted in a compensatory increase in ejection fraction, an index of LV contractility, in Tg-CAPKN mice. Thus, the effect of PKN upon the intrinsic contractility of cardiac myocytes should be evaluated.

As noted above, PKN physically interacts with RhoA 3, which positively regulates PKN. Transgenic mice with cardiac specific overexpression of constitutively active RhoA exhibit early lethality, and those with overexpression of wild type RhoA exhibit atrial enlargement, LV dilation and contractile failure, with a markedly reduced heart rate 27. The phenotype of Tg-CAPKN mice appears to be quite different from either of the above, suggesting that PKN may not be a major effector of RhoA in the heart. It should be noted, however, that Tg-CAPKN mice do show mild bradycardia. Since RhoA regulates the function of ion channels, such as Kir2.1 28 and L type calcium channel 29, PKN may participate in the regulation of ion channels by RhoA, thereby controlling heart rate.

Alpha BC, a member of the small heat shock protein and the molecular chaperone families, has protective effects against stresses 12, 30, 31. Whereas some PKC isoforms phosphorylate alpha BC 15, PKN upregulates mRNA expression of alpha BC through heat shock factor-1 in HeLa S3 cells 13. Interestingly, although total expression of alpha BC did not differ between Tg-CAPKN and NTg hearts, both alpha BC phosphorylation at Ser59 and Ser45 and its expression in the cytoskeletal fraction were increased in Tg-CAPKN mice. Furthermore, I/R-induced increases in alpha BC phosphorylation at Ser59 and Ser45 were inhibited in Tg-DNPKN mice. These results are consistent with the notion that PKN plays an important role in mediating phosphorylation of alpha BC in response to I/R in the heart. Although phosphorylation of alpha BC at Ser59 can be mediated by activation of MAPKAPK-2 32, MAPKAPK-2 is not activated in Tg-CAPKN mice (Online Fig. XII).

Although the specific role of PKN-induced Ser59 and Ser45 phosphorylation of alpha BC during I/R remains to be clarified, knockdown of alpha BC inhibited the protective effect of PKN in cardiac myocytes in response to H₂O₂, indicating that alpha BC mediates the cell protective effect of PKN. Phosphorylation of alpha BC at the Ser59 residue contributes to cytoprotection in the heart 12, 32, primarily due to its association with cytoskeletal elements, where the chaperone stabilizes myofilament, thereby maintaining cellular integrity 33, 34. Phosphorylation of alpha BC at either Ser59 or Ser45 stimulates its chaperone activity 16, which in turn plays an important role in mediating cellular protein quality control through modulation of the ubiquitin-proteasome system and autophagy. These mechanisms of protein degradation act as defense mechanisms against unfolded proteins, and are essential for cellular function and survival 35. It has been shown that I/R decreases the proteasome activity but ischemic preconditioning improves the proteasomal activity after I/R, and that increased proteasome activity is involved in the cardioprotective effect of preconditioning 36. Interestingly, Tg-CAPKN mice showed enhanced proteasome activity both at baseline and after I/R, and epoxomicin treatment partially reversed the cardioprotective effect of PKN against I/R injury. Furthermore, decreases in the proteasome

activity during I/R were significantly enhanced in the Tg-DNPKN heart. Thus, these results suggest that the increased proteasome activity, in part, mediates the cardioprotective effect of PKN.

In conclusion, PKN is activated by I/R and activation of PKN plays a cell protective role in the heart *in vivo*. Furthermore, PKN mediates phosphorylation of alpha BC and stimulation of ubiquitin-proteasome activity, which in part mediates the protective effect of PKN in the heart (Online Fig. XIII). Thus, stimulation of PKN may represent a novel strategy for protecting the heart from I/R injury.

Novelty and Significance

What Is Known?

- PKN is a serine/threonine kinase with a catalytic domain homologous to protein kinase C.
- PKN is activated by ischemia/reperfusion (I/R) in the retina and the brain in rats.

What New Information Does This Article Contribute?

- PKN is activated by ischemia (I) and reperfusion (R) in the heart and protects the heart against I/R injury.
- PKN phosphorylates alpha B crystallin (α BC) and stimulates proteasome activity, which plays an essential role in mediating the protective effect of PKN against I/R injury.

I/R in the heart causes myocardial injury and cardiac arrhythmia, which, in turn, induce left ventricular dysfunction and/or cardiac death. Identifying novel molecular mechanisms protecting the heart from I/R injury may lead to the development of new treatment for acute myocardial infarction. Here we show that PKN, a serine/threonine kinase, is activated by both ischemia and reperfusion in the mouse heart. Activation of PKN promotes phosphorylation of α BC and stimulates ubiquitin-proteasome activity in the heart, which in turn plays an essential role in mediating the protective effect of PKN against myocardial I/R injury. Our study identifies PKN as a novel endogenous mediator of cardioprotection against I/R injury. PKN is unique in that chaperone-mediated activation of the proteasome plays an important role in mediating its cardioprotective action. We propose that stimulation of endogenous PKN could be a novel therapeutic strategy for limiting myocardial injury caused by acute I/R.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

Tg	transgenic
NTg	non-transgenic
CA	constitutively active
DN	dominant negative
LV	left ventricle
RV	right ventricle
BW	body weight
shRNA	short hairpin RNA
Ser	serine
Thr	threonine
I/R	ischemia/reperfusion
AAR	area at risk
MI	myocardial infarction
alpha BC	alpha B crystallin
MPG	N-(2-mercaptopropionyl)-glycine

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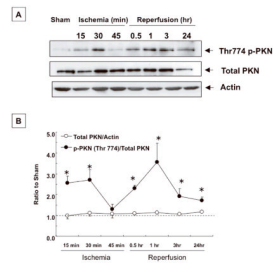


Fig. 1. PKN is activated in hearts subjected to I/R

Effects of I/R on PKN Thr774 phosphorylation. (A) Time course of PKN Thr774 phosphorylation. (B) The level of PKN Thr774 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 (dotted line). n=3-4. * p<0.05 vs. sham

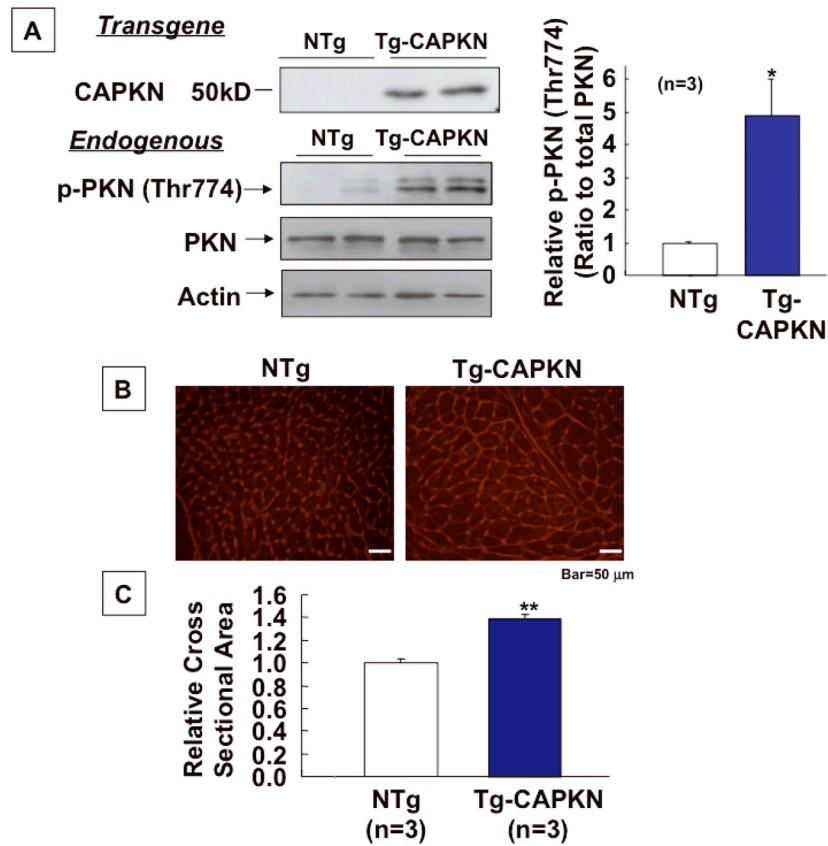


Fig. 2. Development of cardiac hypertrophy in Tg-CAPKN

Baseline characterization of Tg-CAPKN mice at 3 months of age. (A) Representative immunoblots of heart homogenates with anti-PKN and anti-phospho (Thr774) PKN antibodies. Densitometric analysis was performed to determine the relative phospho-PKN level. CAPKN is N-terminally truncated and does not exist in NTg. (B) Representative pictures of WGA staining of the heart from Tg-CAPKN and NTg mice. (C) Myocyte cross sectional area was determined from WGA staining. The mean myocyte cross sectional area in NTg mice was set as 1. * $p < 0.05$, ** $p < 0.01$ vs NTg.

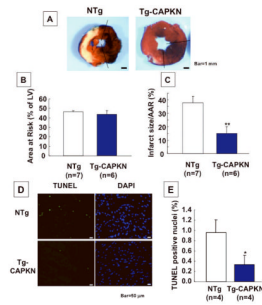


Fig. 3. I/R injury was attenuated in CA-PKN Tg mice

Tg-CAPKN and NTg mice at 3 months old were subjected to I/R. (A) Gross appearance of LV tissue sections after Alcian blue (1%) and TTC (1%) staining in Tg-CAPKN and NTg mice subjected to I/R. (B, C) The effect of I/R on the extent of LV MI in Tg-CAPKN and NTg mice. AAR/LV size (%) (B) and MI area/AAR (%) (C) are shown. (D, E) LV tissue sections were subjected to TUNEL and DAPI staining. (D) Representative staining with TUNEL and DAPI are shown. (E) The number of TUNEL-positive nuclei was expressed as a percentage of total nuclei detected by DAPI staining. *p<0.05, **p<0.01 vs NTg.

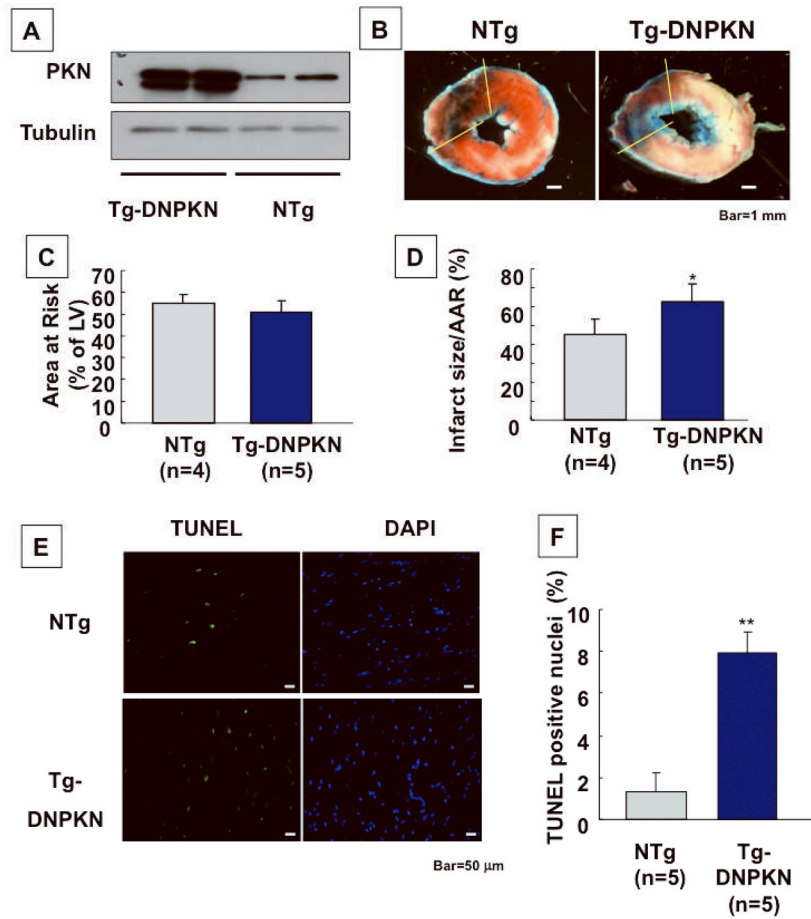


Fig. 4. I/R injury was exacerbated in DN-PKN Tg mice

Tg-DNPKN and NTg mice were subjected to I/R. (A) Expression of transgene (DNPKN, PKN K664D) is shown. (B) Gross appearance of LV tissue sections after Alcian blue and TTC staining in Tg-DNPKN and NTg mice subjected to I/R. (C, D) The quantitative analyses of AAR/LV size (%) (C) and MI area/AAR (%) (D) are shown. (E, F) LV tissue sections were subjected to TUNEL and DAPI staining. (E) Representative stainings with TUNEL and DAPI. (F) The number of TUNEL-positive nuclei was expressed as a percentage of total nuclei detected by DAPI staining. * $p < 0.05$, ** $p < 0.01$ vs NTg.

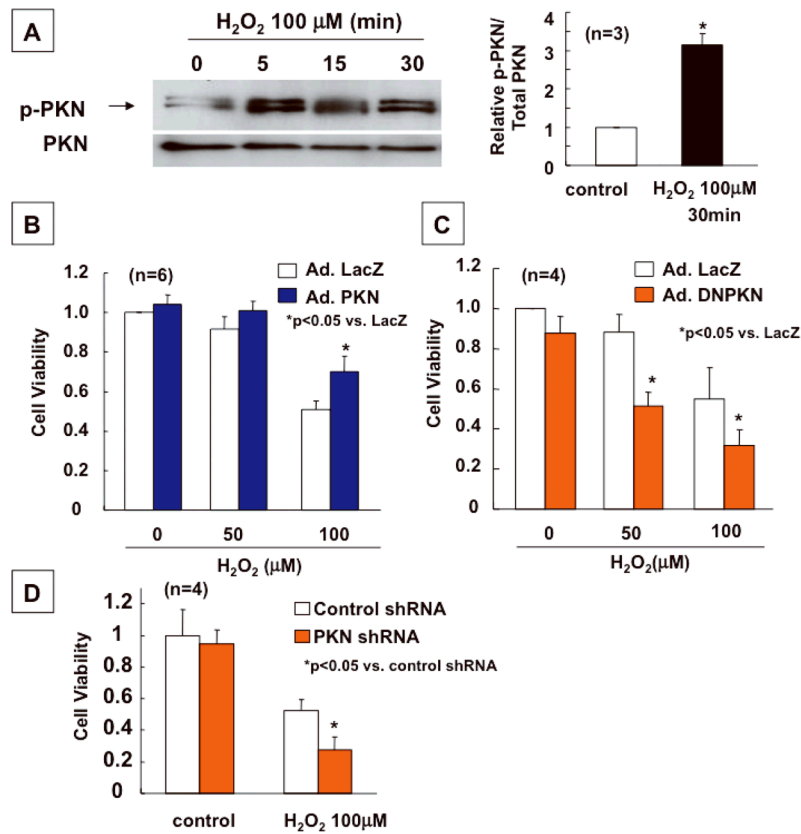


Fig. 5. Transduction of PKN protects, whereas knockdown of PKN enhances cell death induced by H₂O₂

Cardiac myocytes were transduced with Ad-PKN, Ad-DNPKN or Ad-sh-PKN. (A) The effect of H₂O₂ (100 μM) upon activity of PKN, as indicated by Thr774 phosphorylation of PKN, was evaluated. Immunoblots with anti-PKN and anti-phospho-PKN antibodies are shown. The result of densitometric analyses is shown. Phosphorylated PKN/total PKN without H₂O₂ was set as 1. * p<0.05 vs control. (B, C) Myocytes were treated with the indicated doses of H₂O₂ for 8 hours. Cell viability was determined by the Cell Titer Blue assay. Cell viability after the H₂O₂ treatment in cardiac myocytes transduced with Ad-PKN (B) or Ad-DNPKN (C) are shown. (D) Cell viability after H₂O₂ treatment in cardiac myocytes transduced with Ad-sh-PKN is shown. In B–D, cell viability in cardiac myocytes transduced with Ad-LacZ in the absence of H₂O₂ treatment was set as 1.

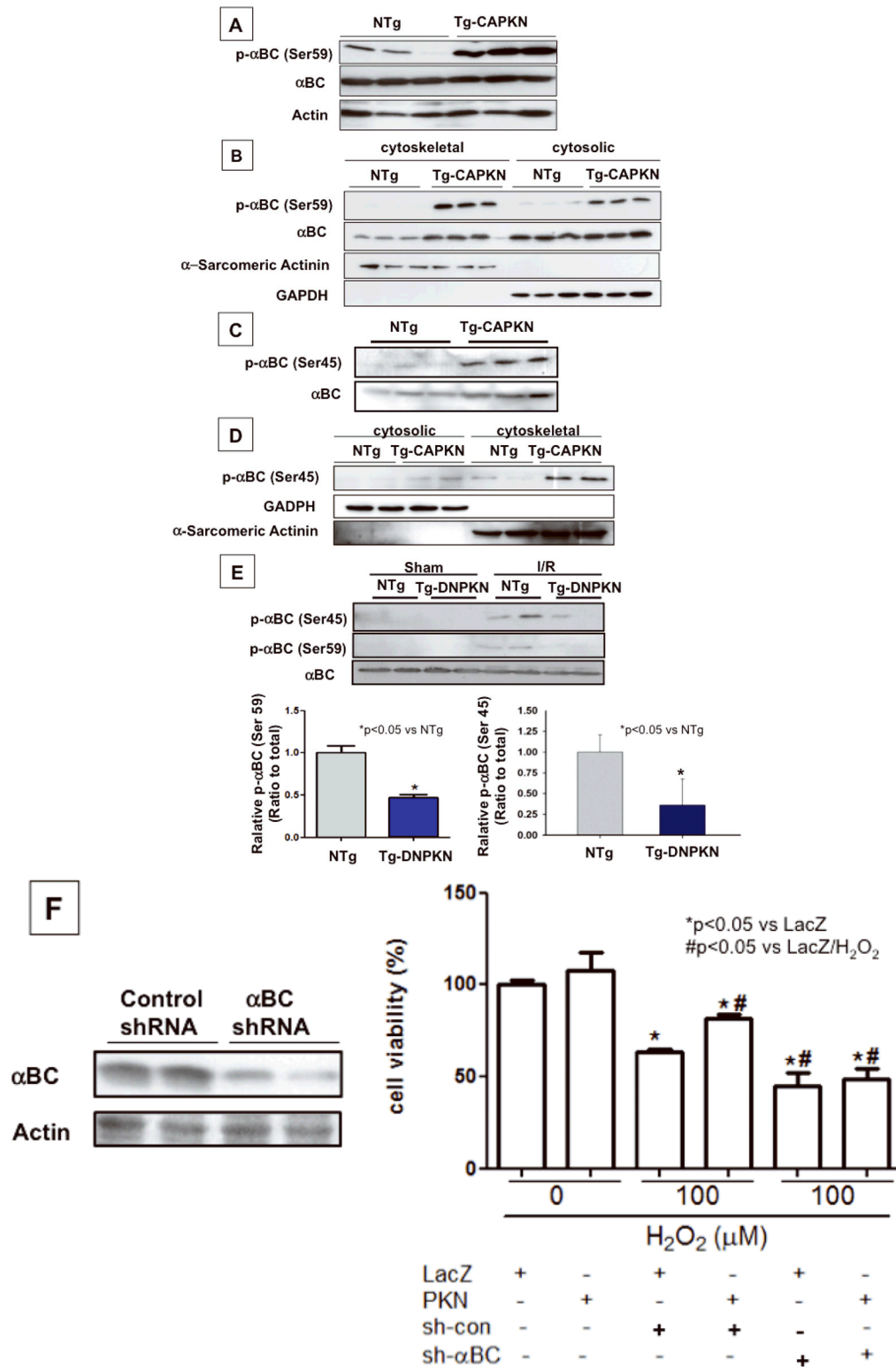


Fig. 6. Alpha B crystallin mediates protective effect of PKN
 Immunoblot analyses of alpha B crystallin (αBC) and Serine 59 and Serine 45 phosphorylated alpha B crystallin (p-αBC (Ser59 or Ser45)) (A) p-αBC (Ser59) in Tg-

CAPKN and NTg hearts. (B) Subcellular localization of α BC and p- α BC (Ser59) in Tg-CAPKN and NTg hearts. Cytoskeletal and cytosolic fractions were prepared from the heart in Tg-CAPKN or NTg. The purity of each fraction was analyzed by immunoblots with anti- α -sarcomeric actinin or GAPDH. (C) Immunoblot analyses of α BC and Serine 45 phosphorylated α BC (p- α BC (Ser45)). (D) Subcellular localization of p- α BC (Ser45) in Tg-CAPKN and NTg hearts. (E) NTg and Tg-DNPKN mice were subjected to either sham operation or 45 min ischemia/2 hour reperfusion (I/R). Total heart homogenates were subjected to immunoblot analyses. The effect of I/R upon Ser45 and Ser59 phosphorylation of α BC and the role of PKN. * $p < 0.05$ vs NTg. (F) Effect of α BC knockdown on the protective effect of PKN in cardiac myocytes. Immunoblot analysis of α BC expression. (Left). Myocytes were transduced with either Ad-LacZ or Ad-PKN in combination with Ad-sh-scramble or Ad-sh- α BC and then treated H_2O_2 (Right).

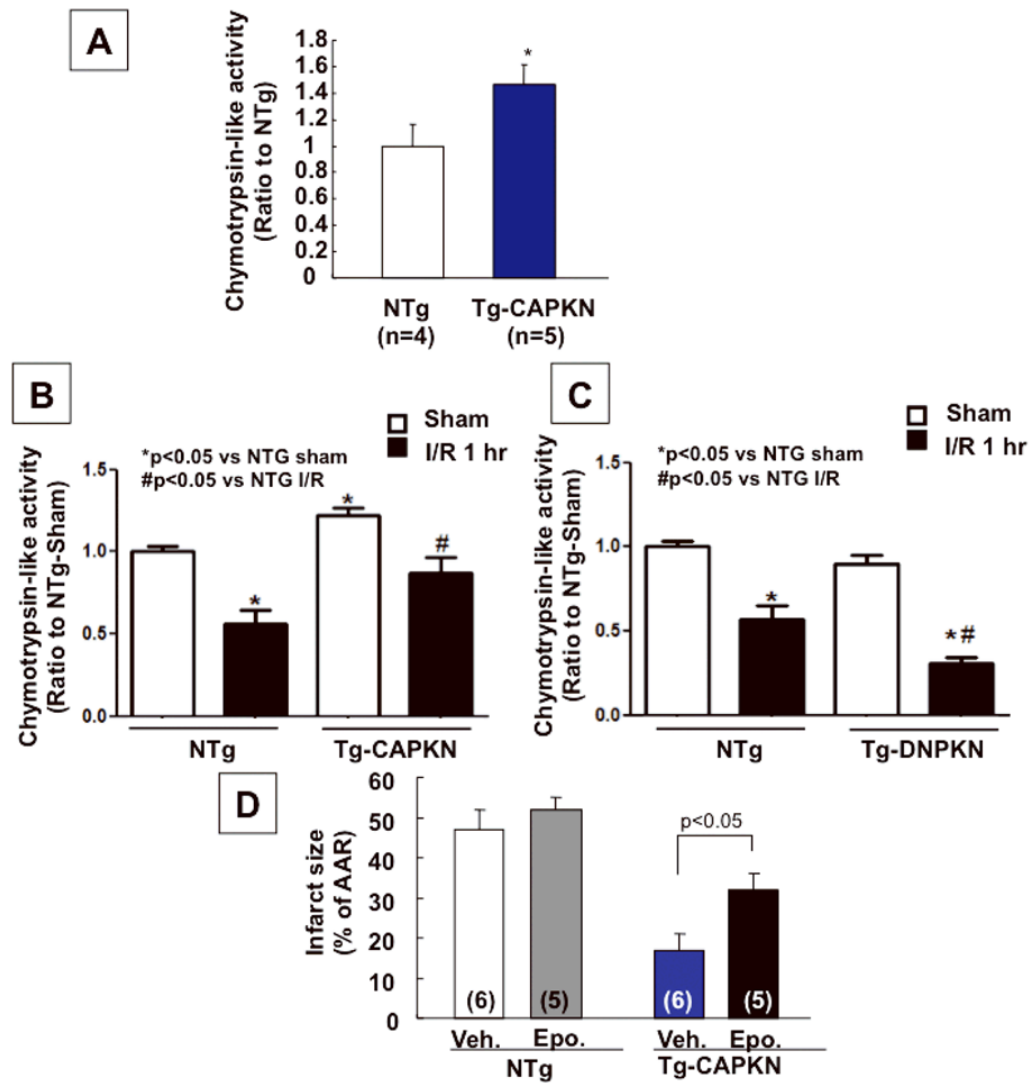


Fig. 7. Proteasome is activated in Tg-CAPKN mouse hearts

Chymotrypsin-like peptidylglutamyl-peptide hydrolase activity of proteasomes present in the cytoplasmic milieu was evaluated with the fluorogenic peptide LLVY-MCA. (A) Chymotrypsin-like activity in either Tg-CAPKN or NTg hearts was evaluated. The mean proteasome activity in NTg was set as 1. * $p < 0.05$ vs. NTg. (B and C) Chymotrypsin-like activity in the heart subjected to I/R was measured in Tg-CAPKN (B) or Tg-DNPKN mice (C) and compared with that in NTg mice. The mean proteasome activity in NTg-Sham hearts was set as 1. N=4. (D) Epoxomicin (Epo. 0.5 mg/kg) or vehicle (Veh.) was injected intraperitoneally in Tg-CAPKN or NTg mice one day before and 1 hour before coronary occlusion. Mice were then subjected to I/R. Infarct size (% of AAR) are shown. The values of AAR were not different between all groups (not shown).