Activation of PKN Mediates Survival of Cardiac Myocytes in the Heart During Ischemia/Reperfusion

Hiromitsu Takagi, Chiao-Po Hsu, Katsuya Kajimoto, Dan Shao, Yanfei Yang, Yasuhiro Maejima, Peiyong Zhai, Ghassan Yehia, Chikaoi Yamada, Daniela Zablocki, Junichi Sadoshima

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 minutes of ischemia and 24 hours of reperfusion was significantly smaller in transgenic mice with cardiac-specific overexpression of constitutively active (CA) PKN (Tg-CAPKN) than in nontransgenic (NTg) mice (15±5 versus 38±5%, P<0.01). The number of TUNEL-positive nuclei was significantly lower in Tg-CAPKN (0.3±0.2 versus 1.0±0.2%, P<0.05). Both MI/AAR (63±9 versus 45±8%, P<0.05) and the number of TUNEL-positive cells (7.9±1.0 versus 1.3±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 α B crystallin and increased cardiac proteasome activity. The infarct reducing effect in Tg-CAPKN mice was partially inhibited by epoxomicin, a proteasome inhibitor.

Conclusions: PKN is activated by I/R and inhibits apoptosis of cardiac myocytes, thereby protecting the heart from I/R injury. PKN mediates phosphorylation of α B crystallin and stimulation of proteasome activity, which, in part, mediates the protective effect of PKN in the heart. (Circ Res. 2010;107:642-649.)

Key Words: ischemia/reperfusion ■ PKN ■ α B crystallin ■ proteasome

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. 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. 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 120 kDa. PKN consists of a catalytic domain highly homologous to that of protein kinase (PK)C in the carboxyl-terminal region and a regulatory domain in the amino-terminal region. 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, and it has been shown to be involved in the pathogenesis of Alzheimer’s disease and amyotrophic lateral sclerosis. Interestingly, PKN is cleaved by caspase 3 and a truncated and kinase-active form of PKN has been found in ischemia/reperfusion (I/R) models of the rat retina and brain, suggesting that PKN may modulate cell survival or death during I/R. Transgenic (Tg) mice overexpressing a constitutively active (CA) form of PKN in mammary epithelium exhibit impaired tight junction sealing and increased apoptosis, resulting in precocious involution in the mammary gland.

PKN activates atrial natriuretic factor gene expression in cardiac myocytes. 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.
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.

**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% IGEPAI CA-630, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L βglycerophosphate, 0.1 mmol/L Na3VO4, 1 mmol/L NaF, 50 μmol/L phe-nylmethylsulfonyl fluoride, 5 μg/mL aprotinin, and 5 μg/mL leupeptin. PKN was immunoprecipitated with PKN antibody (BD Transduction Laboratories) at 4°C for 120 minutes. Protein G Sepharose beads were added and incubated at 4°C for 50 minutes. After washing 3 times, the precipitates were incubated for 30 minutes 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 PKCα peptide (AMFPTMNRRG-I) 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 (amino acids 561 to 942) and DNPKN (full-length K644D3) were generated on an FVB background using the U6 promoter. The loop sequence is underlined. A recombinant hairpin-forming oligo 5'-GATTGACATCATCGGCCATGTCAGGAACAATGGGGTATGATGTCATAC-3’ from the rat PKN cDNA and its antisense and the hairpin-forming oligo 5'-ACGTGGCATCTCCTCCTCCATACCATTATCGAGATATGGTGAGAGTAGATCAGC-3’ from the rat αB crystallin (BC) cDNA and its antisense with Apal and HindIII 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 means±SEM. Differences between exper-imental groups were evaluated for statistical significance using Student t test for unpaired data or 1-way ANOVA, followed by Dunnett’s post test when appropriate. Probability values of <0.05 were considered to be statistically significant.

**Results**

**PKN Is Activated by I/R in the Heart**

We examined whether PKN was 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 to 30 minutes and declined after 45 minutes (Figure 1A and 1B). After reperfusion, PKN phosphorylation was observed again, and this phosphorylation was sustained for >24 hours (Figure 1A and 1B). In the hearts of sham-operated mice, expression and phosphorylation of PKN were unchanged over the entire experimental period (Online Figure 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 αMHC promoter. In Tg-CAPKN mouse hearts, phosphorylation of endogenous PKN was increased 5-fold compared to in nontransgenic (NTg) hearts (Figure 2A). Heart weight/body weight (HW/BW) and left ventricular (LV) weight/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 (Figure 2B and 2C). 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 Figure II). In Tg-CAPKN mice, the heart rate was slower by 10% compared to NTg mice (Online Table II). Taken together, these data indicate that Tg-CAPKN mice showed LV 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 was protective against I/R injury.

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AAR</td>
<td>area at risk</td>
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<tr>
<td>αBC</td>
<td>α B crystallin</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>CA</td>
<td>constitutively active</td>
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<tr>
<td>DN</td>
<td>dominant negative</td>
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<tr>
<td>HW</td>
<td>heart weight</td>
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<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
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<tr>
<td>LV</td>
<td>left ventricular</td>
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<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MPG</td>
<td>N-(2-mercaptopropionyl)-glycine</td>
</tr>
<tr>
<td>NTg</td>
<td>nontransgenic</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricle</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
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<td>Tg</td>
<td>transgenic</td>
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Tg-CAPKN and NTg mice at 3 months old were subjected to 45 minutes 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 versus 37.5 ± 4.9%, P < 0.01; Figure 3A through 3C). There were also fewer TUNEL-positive cells in the AAR in Tg-CAPKN than in NTg mice (0.3 ± 0.2 versus 1.0 ± 0.2%, P < 0.05; Figure 3D and 3E). 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 minutes of global ischemia (Online Figure 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% to 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 β-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 Figure 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 (data not shown; see also Figure 6E). In the DNPKN heart, MI/AAR (62.5 ± 9.3 versus 45.2 ± 8.4%, P < 0.05) and the number of TUNEL-positive cells (7.9 ± 1.0 versus 1.3 ± 0.9%, P < 0.05) were both greater than in NTg mice (Figure 4A through 4F), 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 short hairpin (sh)RNA-PKN (Ad-sh-PKN) (Online Figure 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 (H₂O₂). Treatment with H₂O₂ induced PKN Thr774 phosphorylation in cardiac myocytes (Figure 5A). Transduction of Ad-PKN reduced (Figure 5B), whereas that of Ad-DNPKN or Ad-sh-PKN enhanced (Figure 5C and 5D), cell death. 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 αBC Into the Cytoskeletal Fraction
αBC, a member of the small heat shock protein family of molecular chaperones, has a cell-protective effect against stresses. PKN upregulates αBC gene expression in HeLa S3 cells. To address the molecular mechanism mediating the cell-protective effect of PKN in cardiac myocytes, we evaluated expression of total and phosphorylated forms of αBC. The level of total αBC was not different between Tg-CAPKN and NTg hearts (Figure 6A; Figure VI, A). However, the level...
BC phosphorylated at Ser59 was significantly increased in Tg-CAPKN mice (Figure 6A; Online Figure VI, A and B). In neonatal rat cardiac myocytes, transduction with Ad-PKN increased, whereas that with Ad-sh-PKN reduced, Ser59 phosphorylation of BC (Online Figure VI, C and D). Treatment with H2O2 caused an increase in Ser59 phosphorylation of BC, which was partially inhibited by shRNA PKN (Online Figure VI, E and F). BC translocates to the cytoskeletal fraction and interacts with cytoskeletal proteins in response to stress, thereby playing a protective role in the heart. Translocation of BC from the cytosol to the cytoskeletal fraction was also increased in Tg-CAPKN mice (Figure 6B). In addition to Ser59 phosphorylation, Ser45 phosphorylation of BC and its translocation to the cytoskeletal fraction were also increased in Tg-CAPKN mice (Figure 6C and 6D; Online Figure VI). In hearts subjected to I/R, both Ser45 and Ser59 phosphorylation of BC and its translocation to the cytoskeletal fraction were increased (Online Figure VII). The increase in BC phosphorylation at both Ser45 and Ser59 in response to I/R was markedly inhibited in Tg-DNPKN mice (Figure 6E). These results suggest that PKN plays an important role in mediating phosphorylation of BC, which may in turn mediate protective effects in the heart during I/R. To address the role of BC in mediating the cell-protective effect of PKN, we knocked down BC by shRNA and examined the cell viability under H2O2 treatment in cultured cardiac myocytes. Ad-sh-BC inhibited the cell-protective effect of PKN, indicating that BC mediates the cell-protective effect of PKN (Figure 6F).

PKN Increases Proteasome Activity

Phosphorylation of BC is important for its chaperone activity, 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 (Figure 7A). Similarly, cardiac myocytes transduced with Ad-PKN exhibited greater chymotrypsin-like activities than Ad-LacZ–transduced myocytes (data not shown). In hearts subjected to I/R, chymotrypsin-like activity was decreased, an effect that was significantly attenuated in Tg-CAPKN hearts (Figure 7B). Furthermore, the decrease in chymotrypsin-like activity in response to I/R was significantly enhanced in Tg-DNPKN hearts (Figure 7C). To test the functional significance of the elevated proteasome activity in Tg-CAPKN hearts, the effect of epoxomicin, a proteasome inhibitor, on the cardioprotective effect of CAPKN was evaluated. Epoxomicin partially attenuated the infarct-reducing effect in Tg-CAPKN mice (Figure 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.

Figure 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 images of wheat germ agglutinin staining of the heart from Tg-CAPKN and NTg mice. C, Myocyte cross-sectional area was determined from wheat germ agglutinin staining. The mean myocyte cross-sectional area in NTg mice was set as 1. *P<0.05, **P<0.01 vs NTg.

Figure 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 and 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 and 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.
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 αβC(proteasome pathway).

In our study, Thr774 phosphorylation of PKN, which is essential for its activation, was observed in hearts subjected to I/R. In yeast, Pkc1, whose amino-terminal regulatory region is highly homologous to that of PKN, is activated by cell wall stress, including hypotonic stress.17 Pkc1 is also important for cell viability in the adaptive response to oxidative stress18,19 or nitrosative stress.19 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 Figure 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 (K.K. and J.S., manuscript in preparation). Thus, we speculate that osmotic stress may also mediate PKN activation in response to I/R. Interestingly, RhoA, a known activator of PKN,3 is activated by reactive oxygen species.22 Oxygen radicals can stimulate PKC directly by oxidative modification of its regulatory domain.23 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 Figure 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. Because 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 on other forms of cell death remains to be elucidated.

Because 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, because 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/H9255 into the membrane fraction (Online Figure X) nor Akt phosphorylation, common mediators of ischemic preconditioning, was induced by overexpression of PKN in cardiac myocytes in vitro (Online Figure XI). Thus, it is likely that the cardioprotective effects of CAPKN are mediated by PKC/H9255- and Akt-independent mechanisms. Development of small molecules that specifically stimulate PKN would be relevant clinically, considering the fact that stimulators of PKC/H9255 have thus far shown promising results in their preclinical studies.24

Tg-CAPKN mice showed mild LV hypertrophy. PKN is involved in actin reorganization in vascular smooth muscle cells25 and stimulates atrial natriuretic factor gene expression in cardiac myocytes,10 integral features of cardiac hypertrophy. PKN has also been shown to interact directly with α-actinin.26 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, because 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 >1 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 on 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 contractual 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...
cardiac myocytes in response to H₂O₂, indicating that αBC mediates the cell-protective effect of PKN. Phosphorylation of αBC at the Ser59 residue contributes to cytoprotection in the heart, primarily because of its association with cytoskeletal elements, where the chaperone stabilizes myofilament, thereby maintaining cellular integrity. Phosphorylation of αBC at either Ser59 or Ser45 stimulates its chaperone activity, 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. 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. 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 αBC and stimulation of ubiquitin-proteasome activity, which, in part, mediates the protective effect of PKN in the heart (Online Figure XIII). Thus, stimulation of PKN may represent a novel strategy for protecting the heart from I/R injury.

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Disclosures

None.

References


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 αB crystallin (αBC) 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 LV 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 αBC 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.

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Activation of PKN Mediates Survival of Cardiac Myocytes in the Heart During Ischemia/Reperfusion

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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 previously¹. 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. pBHGloxΔE1,3Cre (Microbix), including the ΔE1 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. 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 from 15 min before ischemia throughout the experiment, as described previously.

**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. 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, CaCl₂ 2.0 mmol/L, MgSO₄ 1.2 mmol/L, KH₂PO₄ 1.2 mmol/L, NaHCO₃ 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 Na₃VO₄, 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 Na₃VO₄, 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 Na₄P₂O₇, 10% glycerol, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, 2 mM Na₂VO₄, 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. 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. 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 H₂O 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 Figure I

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.
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 H₂O₂. 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
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, anti-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 of p-MAPKAPK2/total MAPKAPK-2 in NTg was set as 1.

Supplemental Figure XII
Ischemia/reperfusion

Oxidative stress and/or osmotic stress

PKN activation

alphaB crystallin phosphorylation

Cytoskeletal translocation

Increase proteasome activity

Chaperone function maintain cellular integrity

Cell Survival

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

<table>
<thead>
<tr>
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<td>BW (g)</td>
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<td>78 ± 3</td>
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<td>Lung/BW (mg/g)</td>
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Values are means±SEM. *p<0.05, **p<0.01 vs. NTg
Table II Echocardiographic Analysis of Tg-CAPKN Mice

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<td>DSEP WT (mm)</td>
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<td>LVEDD (mm)</td>
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Values are means±SEM. *p<0.05, **p<0.01 vs. NTg

Supplemental Table II
Table III Postmortem pathologic measurements of Tg-DNPKN mice

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Values are means±SEM.

Supplemental Table III
Table IV Echocardiographic Analysis of Tg-DNPKN Mice

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<td>DSEP WT (mm)</td>
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Values are means±SEM.
Reference for Supplement


