H11 Kinase Prevents Myocardial Infarction by Preemptive Preconditioning of the Heart

Christophe Depre, Li Wang, Xiangzhen Sui, Hongyu Qiu, Chull Hong, Nadia Hedhli, Audrey Ginion, Amy Shah, Michel Pelat, Luc Bertrand, Thomas Wagner, Vinciane Gaussin, Stephen F. Vatner

Abstract—Ischemic preconditioning confers powerful protection against myocardial infarction through pre-emptive activation of survival signaling pathways, but it remains difficult to apply to patients with ischemic heart disease, and its effects are transient. Promoting a sustained activation of preconditioning mechanisms in vivo would represent a novel approach of cardioprotection. We tested the role of the protein H11 kinase (H11K), which accumulates by 4- to 6-fold in myocardium of patients with chronic ischemic heart disease and in experimental models of ischemia. This increased expression was quantitatively reproduced in cardiac myocytes using a transgenic (TG) mouse model. After 45 minutes of coronary artery occlusion and reperfusion, hearts from TG mice showed an 82±5% reduction in infarct size compared with wild-type (WT), which was similar to the 84±4% reduction of infarct size observed in WT after a protocol of ischemic preconditioning. Hearts from TG mice showed significant activation of survival kinases participating in preconditioning, including Akt and the 5′AMP-activated protein kinase (AMPK). H11K directly binds to both Akt and AMPK and promotes their nuclear translocation and their association in a multiprotein complex, which results in a stimulation of survival mechanisms in cytosol and nucleus, including inhibition of proapoptotic effectors (glycogen synthase kinase-3β, Bad, and Foxo), activation of antiapoptotic effectors (protein kinase Cε, endothelial and inducible NO synthase isoforms, and heat shock protein 70), increased expression of the hypoxia-inducible factor-1α, and genomic switch to glucose utilization. Therefore, activation of survival pathways by H11K preemptively triggers the antiapoptotic and metabolic response to ischemia and is sufficient to confer cardioprotection in vivo equally potent to preconditioning. (Circ Res. 2006;98:280-288.)

Key Words: Akt ■ apoptosis ■ AMPK ■ cell survival ■ ischemia

The most common form of heart disease is myocardial ischemia, which, if not treated, induces irreversible damage in the form of myocardial infarction. Different survival pathways can be activated that provide cardiac protection against ischemia and thereby decrease infarct size (IS). The most potent mechanism of protection is ischemic preconditioning, a condition in which short episodes of ischemia/reperfusion activate survival kinases that will protect the heart against myocardial infarction during subsequent ischemia. However, preconditioning is difficult to induce in patients, and its protective effects are transient. It would be more practical to activate the expression of a protein that would maintain these beneficial effects continuously. Our hypothesis is that H11 kinase (H11K) is such a candidate and, if overexpressed, could pre-emptively precondition the heart.

H11K is expressed predominantly in heart and skeletal muscle. We found an increased expression of H11K in a swine model of transient ischemia and in patients with prolonged ischemia, along with an array of genes promoting cell survival, suggesting that H11K could participate in the prevention of irreversible ischemic damage. Our previous studies show two potential mechanisms by which H11K can participate in cardiac cell survival. First, H11K activates the serine/threonine kinase Akt/PKB, which can prevent cell death through an inhibition by phosphorylation of proapoptotic effectors, including glycogen synthase kinase-3β (GSK-3β), caspase-9, Bad, and the transcription factor forkhead. Second, H11K promotes glucose metabolism in the heart in vivo. Increased reliance on glucose represents a metabolic survival response to ischemia, which is complementary to the antiapoptotic mechanisms of Akt. The major activator of glucose utilization in the ischemic heart is the 5′AMP-activated protein kinase (AMPK), which promotes cell survival by a switching to anaerobic glucose utilization. Although Akt and AMPK both promote cell survival, they have an opposite effect on cardiac cell growth. The mecha-
nism by which Akt and AMPK shift between growth and survival remains largely unknown, although it could be attributable to a subcellular redistribution of these kinases.10,11

In this study, we used mice with cardiac-specific overexpression of H11K that is quantitatively similar to the increase in H11K protein found in patients with ischemic myocardium to determine whether H11K promotes cardiac cell survival, whether this cardioprotection is related to a specific survival pathway, and whether it relates to the mechanism of ischemic preconditioning. We report that overexpression of H11K provides as powerful protection against myocardial ischemia, as does preconditioning by promoting the activation, subcellular translocation and multiprotein association of survival pathways, including Akt and AMPK.

Materials and Methods

Animal Model
We used 3-month-old mice (bred at UMDNJ) with cardiac-specific expression of hemagglutinin-tagged H11K2 and their control littermates. Ischemia/reperfusion was induced by occlusion of the left anterior descending artery for 20 to 60 minutes, followed by 4-hour or 24-hour reperfusion. Area at risk (AAR) was measured with Alcian blue. IS was measured with triphenyltetrazolium chloride.12

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Tissue Extraction
Tissues were homogenized as described.2 For fractionation, fresh tissue was homogenized in a hypotonic buffer. The homogenate was spun at 100 g for 5 minutes, and the initial pellet was discarded. Fractions were separated by centrifugations at 4°C: 500 g for 5 minutes (nuclei); 20 000 g for 10 minutes (mitochondria); and 100 000 g for 90 minutes (membrane). For immunoprecipitation, protein A-sepharose was incubated overnight at 4°C with 1 mg of antibody and washed in saline.

Immunoblotting and Immunofluorescence
Proteins were denatured, resolved on SDS-PAGE gels, and transferred. Antibodies added at the recommended dilution were detected by chemiluminescence. For immunofluorescence, primary antibodies were incubated overnight on frozen tissue sections.

Cell Culture
Cardiomyocytes were isolated and cultured as described.2,13 Recombinant adenoviruses expressing H11K (5 multiplicities of infection) or the β-galactosidase (β-gal) control were added to the cells for 36 hours.2

Purification of H11K
The H11K coding sequence was subcloned in His-tagged pET-23a-d(+) vector (Invitrogen) and transfected in Escherichia coli. The protein was eluted from a ProBond resin (Amersham) and purified by ion exchange chromatography.

Apoptosis
TUNEL staining was performed in formalin-fixed tissue.4 Positive nuclei from cardiac myocytes were counted in the AAR. Small-size fragmented DNA was extracted (TACS Kit; R & D Systems), separated on 1.5% agarose gel, and visualized with ethidium bromide.

Biochemical Assays
AMPK activity and fructose 2,6-bisphosphate (Fru-2,6-bisP) were measured as described.14,15 Protein kinase Ce (PKCe) activity was measured using the PKC Biotrak assay system (Amersham).

dRNA Microarrays
dRNA synthesis was performed from total RNA with a T7-oligo(24)dT primer. The DNA was transcribed into biotin-labeled RNA and hybridized on mouse microarray (430.2.0 GeneChip; Affymetrix). Data analysis was performed with the Microarray Analysis Suite (Affymetrix). The fold change was averaged from all the samples with a signal above background.

Statistical Analysis
Results are mean±SEM for the number of samples indicated in the legends, and compared using Student’s t test. A P value <0.05 was considered significant.

Results

H11K Promotes Cardiac Cell Survival In Vivo
Transgenic (TG) mice with cardiac-specific overexpression of H11K2 have a 6-fold increase in H11K protein, which corresponds to the increase found in vivo during myocardial ischemia.4 The physiological characteristics of the mice are shown in Table 1 and are characterized by a pattern of compensated hypertrophy. To test whether H11K is cardioprotective, wild-type (WT) and TG mice were submitted to coronary artery ligation for 20 to 60 minutes, followed by full reperfusion for 24 hours (Figure 1a). Both the AAR and the IS were measured. Although the AAR was similar, there was a significant reduction of the IS in TG mice compared with WT (Figure 1a). For example, in mice with 45 minutes of occlusion and 24 hours of reperfusion, the IS as a fraction of the AAR was 55±5% in WT and 10±1% in TG mice, respectively (P<0.01), reflecting an 82% reduction in IS.

We compared this effect with ischemic preconditioning (Figure 1b). Hearts were preconditioned by six episodes of short ischemia/reperfusion before a sustained ischemia of 45 minutes. After preconditioning, the IS/AAR in WT was 9±2%, which is an 84% reduction compared with the nonpreconditioned WT (IS/AAR 55±5%) and is similar to the IS/AAR observed without preconditioning in the TG mouse (IS/AAR 10±1%). When the TG mouse was precon-
ditioned, the IS/AAR was 10±1%, showing no further protection compared with the nonpreconditioned TG mouse (Figure 1b). Therefore, the cardioprotection conferred by H11K is quantitatively comparable to the protection of preconditioning. Similar results were found in mice obtained from a different founder with a similar level of overexpression (data not shown).

To determine whether this protection extends to apoptosis, hearts submitted to ischemia/reperfusion were stained for TUNEL analysis. The percentage of apoptotic myocytes was measured specifically in the noninfarcted AAR (Figure 1c). There was no difference in apoptosis between WT and TG in sham animals (0.03±0.01% versus 0.04±0.01% TUNEL-positive myocytes). After ischemia/reperfusion, the percentage of TUNEL-positive myocytes increased to 1.3±0.2% in WT and only to 0.5±0.2% in TG (P=0.01). Decreased apoptosis in TG was confirmed by a reduction of DNA fragmentation compared with WT (Figure 1d).

Protection by H11K Requires the Phosphatidylinositol 3-Kinase Pathway

We showed previously that the TG mouse is characterized by an increased phosphorylation of Akt, which could be reproduced acutely in isolated cardiac myocytes after overexpression of H11K. The main pathway activating Akt depends on phosphatidylinositol 3-kinase (PI3K), which can be inhibited by wortmannin. As expected, phosphorylation of Akt was significantly increased in TG over WT in absence of wortmannin (Figure 2a). After administration of wortmannin, phospho-Akt was almost undetectable in both groups, whereas the expression of total Akt was comparable among all groups (Figure 2a). Treatment with wortmannin did not affect the expression of H11K in either WT or TG mice (data not shown).

Activation of Akt in TG mice was confirmed by measuring the phosphorylation of its downstream substrates. Activation of Akt promotes the phosphorylation of several downstream effectors, including GSK-3β, Bad, the endothelial isoform of NO synthase (eNOS), and the transcription factor Foxo. Akt inhibits GSK-3β, an activator of cell death, and inhibits the proapoptotic effector Bad. Reciprocally, Akt activates the cytoprotective eNOS. As shown in Figure 2b, the phosphorylation of these three substrates at Akt-specific sites was increased in the TG mouse compared with WT. In the nucleus, Akt phosphorylates the proapoptotic transcription factors of the Foxo family, which is followed by their inhibition. Phosphorylation of Foxo 1α was also increased in TG compared with WT (Figure 2b). Inhibition of Foxo by Akt increases the expression of heat shock proteins (HSPs). Expression of HSP70 and HSP27 was significantly increased in protein extracts from TG mice compared with WT mice (Figure 2c). Subcellular fractionation showed both an accumulation of HSPs in the nuclear fraction and a band shift compatible with increased phosphorylation, which both characterize the active proteins.

Another major mechanism of cardioprotection activated downstream PI3K is PKCe. We tested the potential regulation of PKCe in our model. TG mice were characterized by a translocation of the enzyme from the soluble to the particulate fraction that was blocked by wortmannin, showing that it relies on a PI3K-dependent mechanism (Figure 2d). The activity of PKCe was increased by 40% in TG versus WT (Figure 2e). One protective mechanism of PKCe in the heart is to trigger the expression of the inducible isoform of NOS (iNOS), which is particularly important in activating the
delayed mechanisms of myocardial protection after ischemia/reperfusion. As shown in Figure 2f, expression of iNOS protein was increased 5-fold in TG mice compared with WT. We tested whether activation of the PI3K pathway in TG mice is necessary for the cardioprotection by H11K. When wortmannin-treated hearts were submitted to a protocol of ischemia/reperfusion, the protection against apoptosis found in hearts from TG mice was totally abolished (Figure 2g).

**H11K Overexpression Activates AMPK**

We reported previously that H11K overexpression activates glucose metabolism in hearts from TG mice. Increased reliance on glucose represents a metabolic survival response to ischemia, which is complementary to the antiapoptotic mechanisms described above. The major regulator of glucose utilization in the ischemic heart is AMPK, which is activated by phosphorylation of its α-catalytic subunit. Therefore, we tested whether increased expression of H11K regulates the activity of AMPK. Total and phosphorylated forms of αAMPK subunit were measured in WT and TG mice. As shown in Figure 2h, expression of total αAMPK was similar between both groups, whereas T(P)172 αAMPK was increased 2-fold in TG versus WT mice (P<0.05). This increased phosphorylation resulted in a 2-fold increase in AMPK activity (Figure 2i). We further characterized the activation of AMPK in cell culture. Isolated cardiac myocytes expressing H11K or a β-gal control (β-gal); n=4 per group, except for b. **P<0.01; *P<0.05 vs WT. †P<0.01 vs corresponding Veh. #P<0.01 vs corresponding sham.**
ylation of both Akt and AMPK (Figure 2j) after H11K overexpression in isolated myocytes confirms the specificity of the effects observed in the TG mouse.

**H11K Binds Survival Kinases**

Because H11K belongs to the family of small HSPs, we determined whether H11K interacts with the survival kinases. Immunoprecipitation of Akt and H11K, or AMPK and H11K, and Western blot with the reciprocal antibody showed a signal at the expected size (Figure 3a). The specificity of this interaction was confirmed using the hemagglutinin antibody binding only the TG protein (Figure 3a). To further demonstrate the interaction, a recombinant His-tagged H11K was expressed in *E. coli* and purified by ion exchange chromatography. Coimmunoprecipitation of this purified protein with a Myc-tagged Akt showed a band of the same size as the loading control (Figure 3b). The reciprocal experiment, in which a purified His-tagged Akt was coimmunoprecipitated with a Myc-tagged H11K, also showed a band of the expected size (Figure 3b). No signal was detected after immunoprecipitation with an irrelevant (hemagglutinin) antibody (Figure 3b).

**H11K Promotes the Association of Survival Kinases**

Akt and AMPK have antagonistic effects on cardiac cell growth but they are complementary in promoting cardiac cell survival. We hypothesized that these respective effects could be related to a different subcellular distribution. To test this hypothesis, we first characterized the subcellular localization of H11K. In hearts from WT mice, H11K was found in both the nuclear and cytosolic fractions, whereas the overexpressed protein accumulated predominantly in the nuclear fraction of TG mice (Figure 4a). After normalization, H11K was increased 3-fold in the cytosolic fraction and 6-fold in the nuclear fraction of TG mice compared with WT mice (Figure 4a). These results were confirmed by immunofluorescence. In the WT mouse, the distribution of H11K was found both in the cytosol and at the periphery of the nucleus, whereas H11K in the TG mouse was particularly predominant at the periphery of the nucleus (Figure 4b). Using hearts from WT mice, we determined whether this subcellular distribution is affected by ischemia/reperfusion. As shown in Figure 4c, the protein content increased by 50% in the nuclear fraction after 45 minutes ischemia, and this increase persisted up to 24 hours after reperfusion.

To exclude an artifactual localization attributable to the overexpression of the protein in the TG mouse, we determined whether this distribution applies to other models of ischemia in vivo. We measured the subcellular distribution of H11K in the swine heart submitted to repetitive ischemia, a model in which H11K protein expression increases by 6-fold. In this model, H11K was also found in the nuclear fraction, where its expression markedly increased after six episodes of ischemia/reperfusion (Figure 4d).

Considering the protein interactions described above (Figure 3), we determined next whether this nuclear accumulation of H11K in TG mice would be associated with a redistribution of Akt and AMPK. The expression of Akt in the nuclear fraction was 4- to 5-fold higher in TG mice compared with WT mice (Figure 5a). Similarly, in WT mice, the nuclear/cytosolic ratio of AMPK was 1.7/0.3, whereas this ratio increased to 3.9/0.3 in TG mice (Figure 5a). A subcellular redistribution of Akt was further confirmed by immunofluorescence (Figure 5b). Whereas the protein was detected diffusely in the WT mouse, it was particularly concentrated inside the nucleus of myocytes from the TG mouse (Figure 5b). Immunoprecipitation experiments were repeated separately in cytosolic and nuclear fractions to determine whether the interaction between H11K and the survival kinases is specific for a subcellular compartment. The interaction of H11K with Akt was found in both cytosolic and nuclear fractions but largely predominated in the latter (Figure 5c). Interaction of H11K and AMPK was detected exclusively in the nuclear fraction (Figure 5c). As a result, the TG mice were characterized by an increased interaction between Akt and AMPK specifically in the nucleus (Figure 5d).

**Genomic Pattern of Cell Survival**

Both the AMPK and Akt pathways stabilize the hypoxia-inducible factor-1α (HIF-1α), which is essential in the transcriptional adaptation of the cell to oxygen deprivation. HIF-1α expression was increased by >5-fold in nuclear fractions from TG hearts compared with WT hearts (Figure 5d).
6a). Activation of HIF-1α results in an increased expression of genes encoding enzymes regulating anaerobic metabolism and growth factors. To test this genomic effect, we hybridized the cDNA from hearts of WT and TG mice to mouse-specific microarrays. The TG mouse heart was characterized by an upregulation of multiple genes regulated by HIF-1α and involved in glycogen metabolism, glycolysis, and glucose oxidation (Table 2). The profile includes a marked upregulation of 6-phospho-2-fructokinase (PFK-2), the enzyme producing Fru-2,6-bisP, the most powerful activator of glycolysis in the heart. The biological relevance of this increased expression of PFK-2 was tested by measuring the concentration in Fru-2,6-bisP, which was increased 2-fold in hearts from TG compared with WT (Figure 6b). Expression of other survival genes activated by HIF-1α was also increased (Table 2).

**Discussion**

Increased expression of H11K in the ischemic heart is sufficient to confer protection against necrosis and apoptosis at least as powerful as ischemic preconditioning. The mechanisms include an integrated activation on complementary survival pathways, together with a metabolic switch toward glucose utilization. The molecular pathways involved reca-

![Figure 4. Subcellular distribution of H11K.](http://circres.ahajournals.org/)

To illustrate this, we performed subcellular fractionation of hearts from WT and TG mice (Figure 4a). The localization of H11K was found to be predominantly in the cytosol (Cy) compared with the nucleus (Nu) of WT mice but markedly predominates in the TG heart (arrows). Magnification ×60. c, Nuclear redistribution of H11K in hearts (n=4 per group) from WT mice at the end of ischemia (Isc), and after 2 hours or 24 hours of reperfusion (R) compared with shams (Sh). d, Accumulation of H11K in nuclear fraction of a swine heart after repetitive ischemia. **P<0.01 vs corresponding WT. *P<0.01 vs corresponding sham.

The "gold standard" of cardioprotection is ischemic preconditioning, which, in our study, reduced IS by 84%. IS was reduced by a similar extent (82%) in mice overexpressing H11K, and this protection was not augmented by preconditioning the H11K mouse. Hearts from mice overexpressing a constitutively active Akt show a 50% reduction in IS, whereas a 60% reduction is found in mice overexpressing PKCe. Reciprocally, deletion of AMPK impairs functional recovery and markedly increases apoptosis during postischemic reperfusion. Instead of being part of a specific signaling pathway, H11K redistributes signaling molecules between different compartments, prevents their deactivation and promotes their interactions. The role of H11K is reminiscent of HSP90, which binds Akt, prolongs its half-life, increases its activity, and promotes its membrane localization. Whether other molecules participate in this multiprotein complex by H11K remains to be determined.

Concomitant activation of several survival kinases creates important cross-talk. PKCe further activates Akt-dependent antiapoptotic mechanisms and also activates AMPK,
which will promote the activation of HIF-1α. Increased stability of HIF-1α may also originate from the Akt pathway. In addition, both PKCe and HIF-1α stimulate the expression of iNOS, which is central to the mechanisms of prolonged cardioprotection. Increased production of NO stabilizes the HIF-1α protein and increases PKCe activity. The 5-fold increase of iNOS expression in our model corresponds to the range found in models of preconditioning and does not induce the deleterious effects resulting from a massive production of NO.

The hearts from WT and TG mice are different at baseline because of the increase in heart mass observed in TG mice. Although this might be a confounding variable, we do not think that hypertrophy by itself can explain the differences in IS because ischemic damage is not reduced, and can be even exacerbated, in models of pressure-induced hypertrophy. Reciprocally, adaptive hypertrophy shows a pattern of cardioprotection similar to our model, suggesting that the protection against ischemic damage results from the activation of specific intracellular signaling pathways rather than from hypertrophy itself. However, hypertrophy may activate other signaling pathways affecting cell survival, especially those pathways related to Ca²⁺ metabolism, which remains to be explored in our model. At this point, it also remains unknown whether the same pathways responsible for cell survival are involved in the process of hypertrophy. It was shown before that a nuclear localization of Akt, which promotes cell survival, is not a trigger of hypertrophy. Different mechanisms may therefore regulate growth and survival. Further work will be needed to determine how H11K affects protein turnover (ie, the balance between protein synthesis and degradation).

Activation of a genomic program of cell survival is found during acute and prolonged ischemia. This program is particularly developed in human hibernating myocardium, a condition in which the myocardium submitted to chronic ischemia remains viable and functionally improves on reper-
Our data support a role for the nuclear translocation of survival kinases in the genomic adaptation found in the TG mouse. Akt and AMPK have antagonistic actions on the mammalian target of rapamycin (mTOR) activity, in which both pathways are reciprocally controlled by substrate availability and nutrient sensing mechanisms. However, Akt and AMPK cooperate in the nucleus to activate specific transcription factors, such as HIF-1α. Therefore, by redistributing survival kinases between subcellular compartments, H11K could be a mechanism that turns the antagonism between Akt and AMPK for cell growth into cooperation for cell survival. The subcellular fractionation also shows a slight signal for H11K in the mitochondrial fraction of the TG mouse heart, which might be of importance because H11K is associated with the mitochondria in *Drosophila* and therefore might prevent the activation of the intrinsic pathway of apoptosis.

H11K not only protects the myocardium against apoptosis but also promotes the metabolic switch that characterizes the ischemic heart. Glucose uptake and glycolysis are rapidly activated on ischemia to compensate for the lack of aerobic ATP production. As shown recently, AMPK is essential for this adaptation by promoting the membrane translocation of glucose transporters and by stimulating glycolysis through increased production of Fru-2,6-bisP. Through this metabolic adaptation, AMPK limits apoptosis and irreversible damage, which shows that the metabolic and antiapoptotic mechanisms of cardioprotection are intertwined. This is further supported by the observation that Akt both inhibits proapoptotic effectors and stimulates glucose utilization.

In conclusion, H11K promotes cardiac cell survival by acting on complementary signaling pathways, resulting in a prevention of myocardial infarction equally powerful to ischemic preconditioning, which offers a mechanism of pre-emptive cytoprotection to limit necrosis and apoptosis during ischemia. To enhance the therapeutic potential of H11K, it will be important to minimize potentially adverse effects of chronic H11K stimulation and to optimize the cytoprotective actions reported here. Future experiments of short-term induction will help address that issue.

### Acknowledgments

This work was supported by National Institutes of Health grants HL33065, AG 14121, HL 33107, PO1 HL 69020, and HL 072863, and American Heart Association grant 023001N. L.B. is research associate of the Fonds National de la Recherche Scientifique, Belgium. We are indebted to Dr David Lagunoff for supervising the immunofluorescence experiments.

### References


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**TABLE 2. Upregulation of HIF-1α-Dependent Genes in TG vs WT Mice (n=4 per group)**

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<th>P Value</th>
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UDP indicates uridine diphosphate; FBPase-2, fructose biphosphatase-2; PDH, pyruvate dehydrogenase; VEGF, vascular endothelial growth factor; IGFFB, IGF-binding protein; IGF2, insulin-like growth factor-2.


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Circ Res. 2006;98:280-288; originally published online December 22, 2005;
doi: 10.1161/01.RES.0000201284.45482.e8
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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