Kallikrein/Kinin Protects against Myocardial Apoptosis after Ischemia/Reperfusion via Akt-Glycogen Synthase Kinase-3 and Akt-Bad 14-3-3 Signaling Pathways*

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Our previous study has shown that human tissue kallikrein protected against ischemia/reperfusion-induced myocardial injury. In the present study, we investigated the protective role of local kallikrein gene delivery in ischemia/reperfusion-induced cardiomyocyte apoptosis and its signaling mechanisms in promoting cardiomyocyte survival. Adenovirus carrying the human tissue kallikrein gene was delivered locally into the heart using a catheter-based technique. Expression and localization of recombinant human kallikrein in rat myocardium after gene transfer were determined immunohistochemically. Kallikrein gene delivery markedly reduced reperfusion-induced cardiomyocyte apoptosis identified by both in situ nick end-labeling and DNA fragmentation. Delivery of the kallikrein gene increased phosphorylation of Src, Akt, glycogen synthase kinase (GSK)-3β, and Bad(Ser-136) but reduced caspase-3 activation in rat myocardium after reperfusion. The protective effect of kallikrein on apoptosis and its signaling mediators was blocked by icatibant and dominant-negative Akt, indicating a kinin B2 receptor-Akt-mediated event. Similarly, kinin or transduction of kallikrein in cultured cardiomyocytes promoted cell viability and attenuated apoptosis induced by hypoxia/reoxygenation. The effect of kallikrein on cardiomyocyte survival was blocked by dominant-negative Akt and a constitutively active mutant of GSK-3β, but it was facilitated by constitutively active Akt, catalytically inactive GSK-3β, lithium, and caspase-3 inhibitor. Moreover, kallikrein-promoted Bad14-3-3 complex formation and inhibited Akt-GSK-3β-dependent activation of caspase-3, whereas caspase-3 administration caused reduction of the Bad14-3-3 complex, indicating an interaction between Akt-GSK-caspase-3 and Akt-Bad14-3-3 signaling pathways. In conclusion, kallikrein/kinin protects against cardiomyocyte apoptosis in vivo and in vitro via Akt-Bad14-3-3 and Akt-GSK-3β-caspase-3 signaling pathways.

It has been widely accepted that apoptosis and necrosis are the major contributors of cardiomyocyte dysfunction associated with acute ischemia and reperfusion (I/R). Because the capacity of cell proliferation in cardiomyocytes is limited, even a small loss of cardiomyocytes resulting from apoptosis is likely to cause reduced cardiac function and the development of heart failure. Significant increases in myocardial apoptosis have been documented in patients with ischemic cardiomyopathy and terminal stage heart failure (1). Growing evidence from in vitro and in vivo studies indicate that inhibition of cardiomyocyte apoptosis would minimize cardiac injury induced by myocardial I/R (2, 3). Akt signaling is an important mediator of survival in response to growth factors. Akt promotes the survival of cardiomyocytes in vitro in addition to protection against acute I/R-induced injury in the mouse heart (4, 5). Similarly, our recent study showed that kallikrein gene delivery protects against cerebral ischemia injury and apoptosis along with activation of Akt signaling (6). Activation of Akt triggers downstream signaling pathways such as GSK-3 phosphorylation in a variety of cell lines (7). GSK activity is inactivated by Akt-induced phosphorylation at serine 21 (α isofrom) and serine 9 (β isofrom), leading to inhibition of apoptosis in insulin-mediated signal transduction (8, 9). GSK-mediated signaling events in cardiomyocyte apoptosis are not clear. Apoptosis can be induced by overexpression of catalytically active GSK-3 and prevented by dominant-negative GSK-3 (10). Under ischemic preconditioning, GSK-3β phosphorylation increases through a PI 3-kinase-Akt-dependent pathway, suggesting that inhibition of GSK-3β is protective in myocardial ischemia (11). Moreover, several studies reported that GSK-3β exerted its effect via activation of caspase-3 (8, 12, 13). Taken together, these studies support a role for GSK-3β in regulating cardiomyocyte apoptosis. Akt has also been shown to play an important role in regulating Bcl-2 family members and thus cell fate. Akt phosphorylates Bad, thereby inhibiting its proapoptotic function, which may partly account for its antiapoptotic effect (8, 14). Bad is known to bind to Bcl-XL in mitochondria and promote apoptosis, whereas phosphorylation of Bad by Akt results in binding to 14-3-3 proteins and promotes the survival of neurons and fibroblasts (8, 15–17). These studies suggest a potential role of Akt-Bad 14-3-3 signaling in promoting cell survival. However, the relation-

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1 The abbreviations used are: I/R, ischemia/reperfusion; Ad, adenoviral; CMV, cytomegalovirus; DN, dominant-negative; GSK, glycogen synthase kinase; GSK-KM, adenoviral vector expressing kinase mutant GSK; GSK-S9A, adenoviral vector expressing constitutively active GSK; H/R, hypoxia/reoxygenation; KM, catalytically inactive; m.o.i., multiplicity of infection; MOPS, 4-morpholinepropanesulfonic acid; Myr, constitutively inactive; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; TK, tissue kallikrein; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.
ship between GSK-3β-caspase-3 and Bad-14-3-3 in cardiomyocyte apoptosis has not been examined. We hypothesized that interactions of the proapoptotic members of Bcl-2 family with 14-3-3 may contribute greatly to the regulation of cardiomyocyte fate after I/R injury and that GSK-3β-caspase-3 facilitates these signaling effects.

The tissue kallikrein/kinin system has been implicated in protection against cardiac remodeling (18–21). Our previous study showed that systemic delivery of the kallikrein gene inhibited I/R-induced myocardial injury, which was accompanied by increased kinin and cGMP levels (22). Similarly, adrenomedullin gene transfer also increased cardiac cGMP levels after I/R (23). cGMP is an indicator of nitric oxide formation, and these results indicate that the nitric oxide-cGMP signaling cascade may serve as the common signaling cascade for cardioprotective stimuli in the injured myocardium. In this study, we employed a catheter-based gene delivery technique to investigate the role and signaling mechanisms by which kallikrein/kinin inhibits myocardial apoptosis in an acute I/R rat model and in primary cardiomyocytes. Our results show that kallikrein/kinin protects against cardiomyocyte apoptosis via activation of Akt-Bad-14-3-3 and Akt-GSK-3β-caspase-3 signaling pathways. We also observed a novel mechanism in which caspase-3 directly disrupts the Bad-14-3-3 complex in cardiomyocytes, establishing a link between these two pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal anti-α-actinin sarcomeric, lithium chloride, and bisbenzimide (Hoechst 33342) were purchased from Sigma. Z-VAD (OMe)-CH_{2}F was purchased from Enzyme System Products (Livermore, CA). Recombinant caspase-3 was from purchased Calbiochem. Hoe140 was a gift from Hoechst Marion Russell Co. (Frankfurt, Germany). LY294002, anti-c-Src, anti-v-Src, anti-Akt, anti-phospho-Akt, anti-Bad, anti-phospho-Bad (Ser-136), anti-phospho-GSK3β (Ser-9), anti-GSK3β, anti-cleaved caspase-3, and anti-Bcl-xL were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-14-3-3-β was from Chemicon (Temecula, CA).

**Preparation of Replication-deficient Adenoviral Vectors**—Adenoviral vector harboring the human tissue kallikrein cDNA (Ad.CMV-TK) under the control of the cytomegalovirus (CMV) enhancer/promoter or adenoviral vector alone (Ad.Null) were constructed and prepared as described previously (23). Adenoviruses containing the dominant-negative mutant of Akt (Ad.DN-Akt), constitutively active Akt (Ad.Myr-Akt), catalytically inactive GSK-3β (Ad.GSK3β-KM) and constitutively active mutant of GSK-3β (Ad.GSK3β-9A) were kindly provided by Dr. Kenneth Walsh, St. Elizabeth’s Medical Center in Boston.

**Catheter-based Gene Delivery in Rat Myocardium and Immunohistochemical Evidence**—Male CD-1 Wistar rats (male, 250–290 g, Harlan, Indianapolis, IN) were employed in this study. The study complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Bethesda, MD). Five days before coronary occlusion, an adenoviral gene was delivered using a catheter-based strategy as described previously (24, 25). Briefly, rats were anesthetized, intubated, and mechanically ventilated before surgery. The chest was entered via a left intercostal approach. A 24-gauge catheter (BD Biosciences) containing 300 μl of virus solution (2 × 10¹⁰ plaque-forming units/ml in PBS) was advanced from the apex of the left ventricle to the aortic root. The aorta and pulmonary trunk were clamped distal to the site of the catheter, and the solution was injected. The clamp was maintained for 20 s. After injection, the exposed heart was monitored for 5 min for resumption of normal sinus rhythm. The chest incision was then closed after removal of air and blood, and the animals were allowed to recover. Expression and localization of TK in rat ventricles after gene delivery were identified immunohistochemically by antibody to TK. Kallikrein levels in rat heart were determined using an enzyme-linked immunosorbent assay specific for human tissue kallikrein for detection of active kallikrein. TK standard ranges from 0.4 to 25 ng/ml.

**Two Hemodynamics Measurements**—To determine the hemodynamic effects of TK, we measured hemodynamic parameters before and 1 and 5 days after adenovirus solution was injected. Rats were under-ventilated and mechanically ventilated. A thoracotomy was performed via the fourth intercostal space, and the heart was exposed. An electrocardiographic monitor was then connected. A 6.0-polypropylene suture (Ethicon, Somerville, NJ) was passed loosely around the left anterior descending coronary artery near its origin. Once hemodynamics were stabilized, left anterior descending occlusion was performed by tightening the suture loop for 30 min. Acute myocardial ischemia was deemed successful on the basis of regional cyanosis of the myocardial papillary muscle accompanied by elevation of the ST segment on electrocardiogram. The loop was then loosened and reperfusion was identified on the basis of return of the original color, accompanied by obvious ST segment change. At the end of reperfusion period, the ischemic regions were then removed for further analysis.

Animals were randomly divided into eight groups. In the sham groups, the chest was opened and injected with saline (n = 5), Ad.Null (n = 7), or Ad.CMV-TK (n = 6). The I/R-injured rats were divided into five groups. Control group (n = 6) was also injected with saline, the second group received Ad.Null (n = 6), and the third group received Ad.CMV-TK (n = 7) delivery. The fourth group received Ad.CMV-TK together with administration of kinin B2 antagonist, icatibant, delivered intraperitoneally by an osmotic minipump (Alza, Palo Alto, CA) at 1 μmol/kg of body weight/day. The fifth group was injected with Ad.DN-Akt delivered via the same catheter (n = 6).

**Detection of Apoptosis in Situ and DNA Laddering**—DNA fragmentation was determined using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay in 4-μm-thick paraffin-embedded sections as described previously (22). The procedure was
Cardioprotection of Kallikrein via Akt Signaling Pathways

Fresh ischemic and nonischemic myocardium (100 mg) was minced and homogenized in 600 μl of lysis buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS, pH 7.4). Tissues were digested with 100 μg/ml proteinase K (Invitrogen) at 55 °C overnight followed by centrifugation at 13,000 × g for 15 min. After incubation, tissues were precipitated and centrifuged at 13,000 × g for 5 min. Supernatants containing DNA were precipitated with isopropyl alcohol. After centrifugation at 13,000 × g for 5 min, the resulting DNA pellets were washed with 75% ethanol and dissolved in DNA hydration solution and measured at 260 nm by spectrophotometry. 10 μg of DNA was loaded onto 1.2% agarose gel containing 0.5 μg/ml ethidium bromide. DNA electrophoresis was carried out at 80 V for 1.5 h. DNA ladders, an indicator of tissue apoptotic nucleosomal DNA fragmentation, were visualized under ultraviolet light and photographed.

Primary Cardiomyocyte Culture and Hypoxia/Reoxygenation (H/R)—Cardiomyocytes were isolated from the hearts of 2–3-day-old Wistar rats. The ventricles were cut into four equal parts and digested enzymatically through multiple rounds in 0.05% pancreatin (Sigma) and 84 units/ml collagenase (Sigma) in a balanced salt solution. The cells were centrifuged at 800 rpm for 10 min at 4 °C and resuspended in F-12 Nutrient Mixture (Invitrogen). Afterward, the cells were differentially plated for 2 h to remove contaminating nonmyocytes. Enriched cardiomyocyte fractions were then cultured in Dulbecco’s modified Eagle’s medium and F-12 (Invitrogen) medium with 10% fetal bovine serum. Cardiomyocyte origin was confirmed immunocytochemically using antibody to α-actinin sarcomeric (Sigma).

Cultured cells were grown for 18 h at 37 °C before the experiments. Cells were transduced with Ad.CMV-TK, Ad.Null, Ad.Akt, Ad.P-Akt, Ad.GSK3β-TK, or Ad.mRNA-TK at m.o.i. 50 for 12 h followed by 12-h hypoxia (95% N₂ and 5% CO₂) and 24-h reoxygenation (95% O₂ and 5% CO₂). Prior to H/R, myocytes were also treated with GSK-3β inhibitor 20 mM LiCl for 30 min or caspase-3 inhibitor 100 μM Z-VAD for 60-min. Expression and localization of human kallikrein in cardiomyocytes after Ad.CMV-TK transduction were identified immunocytochemically using a rabbit anti-kallikrein antibody. Apoptotic cardiomyocytes were identified in cells (fixed in 4% paraformaldehyde) by Hoechst 33342 staining (27, 28). The positive cells were determined by counting 500–800 cardiac myocytes in six randomly chosen fields. Cell viability was determined by trypan blue eliminating assay.

GSK-3β Kinase and Caspase-3 Activity Assay—Control and injured ventricular tissues were pulverized under liquid nitrogen and homogenized in ice-cold lysis buffer as described previously (29). For GSK-3β activity assay, 10 μg of protein from cardiac tissue or cell extracts was incubated at 37 °C for 30 min with the reaction buffer (8 mM MOPS, 0.2 mM EDTA, 10 mM magnesium acetate), 62.5 μM GSK-3β substrate peptide (Upstate Biotechnology, Inc., Lake Placid, NY) and 1 μCi/10 μl [γ-32P]ATP (PerkinElmer Life Sciences). Samples were transferred to P-81 paper and washed three times with 0.75% phosphoric acid followed by a final rinse with acetone. Radioactivity was measured in a scintillation counter. Caspase-3 activity in lysates was determined using a fluorometric caspase-3 assay kit (Oncogene, San Diego, CA) according to the manufacturer’s instructions. Reaction was monitored by a blue to green shift in fluorescence upon cleavage of the 7-amino-4-trifluoromethylcoumarin. Samples were read with a fluorescence reader (PerkinElmer Life Sciences).

Immunoprecipitation and Western Blot Analysis—Heart tissues were homogenized, and cultured cardiomyocytes were harvested in the extraction buffer (10 mmol/liter Tris, pH 7.4, 100 mmol/liter NaCl, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 2% nonidet NP-40, protease inhibitor mixture including 104 mmol/liter benzamidesulfonyl fluoride, 0.08 mmol/liter aprotinin, 2.1 mmol/liter leupeptin, 3.6 mmol/liter bestatin, 1.5 mmol/liter pepstatin A, and 1.4 mmol/liter leucylaminobutyrate (Sigma). The protein concentration was determined by micro-Lowry. Aliquots was separated on SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with T-PBS (1× PBS, 0.3% Tween 20) containing 5% dry milk and incubated with primary antibody (1:1,000) overnight at 4 °C. After three washes with T-PBS, the membrane was incubated with secondary antibody for 1 h at room temperature. The primary antibodies used were anti-c-Sac, anti-v-Sac, anti-Akt, anti-phospho-Akt (Ser-473) antibody, anti-Bad, anti-phospho-Bad (Ser-136), anti-phospho-GSK-3β(Ser-9), anti-GSK-3β, anti-cleaved caspase-3 at 4 °C overnight. The secondary antibodies were anti-rabbit IgG/horseradish peroxidase conjugate or anti-mouse IgG/horseradish peroxidase conjugate (1:2,500 dilution, Promega). Bound antibodies were visualized by double-blinded conditions. The ratio of TUNEL-positive cardiomyocytes to the total number of cardiomyocytes was calculated.

performed using an in situ cell death detection kit (Roche Applied Science) according to the manufacturer’s instructions. TUNEL-positive cardiomyocytes in the ischemic myocardium were carefully distinguished from TUNEL-positive noncardiomyocytes and evaluated under
ECL chemiluminescence (PerkinElmer Life Sciences). For determination of Bad-Bcl-xL or Bad-14-3-3, cell lysates were immunoprecipitated with anti-Bad antibody overnight at 4 °C followed by incubation with protein A-agarose Beads (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were then separated by SDS-PAGE and immunoblotted with antibodies to 14-3-3, cell lysates were immunoprecipitated with anti-Bad antibody overnight at 4 °C followed by incubation with protein A-agarose Beads (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were then separated by SDS-PAGE and immunoblotted with antibodies to 14-3-3 (Chemicon, Temecula, CA) or Bcl-xL (Cell Signaling).

Statistical Analysis—Data were expressed as the mean ± S.E. and were compared between experimental groups with the use of one-way analysis of variance followed by Fisher’s protected least significant difference. Probability values of \( p < 0.05 \) were considered statistically significant.

RESULTS

Expression of Human Tissue Kallikrein and Hemodynamics Parameters—Using a catheter-based strategy, we delivered Ad.CMV-TK locally into the rat left ventricle. Five days after kallikrein gene delivery, expression and localization of recombinant human tissue kallikrein were identified immunohistochemically in the left ventricle (Fig. 1). No specific staining was found in the left ventricle injected with or without control adenovirus, Ad.Null. Immunoreactive human tissue kallikrein was detectable in rats injected with control virus. These results demonstrate that human tissue kallikrein was expressed in rat heart after local gene delivery. Table 1 shows that the hemodynamic parameters are comparable prior to and at 1 day and 5 days after gene delivery, indicating that local kallikrein gene transfer had no effect on the hemodynamic parameters during the experiment.

Kallikrein/Kinin Attenuates Cardiomyocyte Apoptosis after I/R—Apoptotic cardiomyocytes were detected by TUNEL staining in the myocardium of I/R. The ratio of TUNEL-positive cardiomyocytes to total number of cardiomyocytes in the Ad.CMV-TK group was significantly reduced compared with the Ad.Null group (28.4 ± 5.8% versus 41.3 ± 6.9%, \( n = 6–7 \), *, \( p < 0.01 \)). However, the protective effect of kallikrein was blocked by icatibant (28.4 ± 5.8% versus 42.7 ± 6%, \( n = 6–7 \), \( p < 0.01 \)) (Fig. 2A). The effect of kallikrein on apoptosis was confirmed further by DNA laddering (Fig. 2B). DNA fragmentation was not visualized in the myocardium of sham-operated rats, whereas I/R markedly increased DNA laddering in rat myocardium with or without Ad.Null delivery. Kallikrein gene transfer abrogated I/R-induced DNA fragmentation, whereas the effect of kallikrein was reversed by both icatibant and dominant-negative Akt (Ad.DN-Akt). These findings indicate that kallikrein/kinin protects against I/R-induced cardiomyocyte apoptosis via the kinin B2 receptor-Akt signaling pathway.

Kallikrein/Kinin Activates Akt Signaling Cascade in the Ischemic Myocardium—We next examined the effect of kallikrein gene transfer on the Akt signaling cascade in the ischemic heart by Western blot analysis (Fig. 3A). Kallikrein gene transfer resulted in increased phosphorylation of Src, Akt, GSK-3β, and Bad (Ser-136) in the I/R-injured myocardium compared with the control with or without injection of Ad.Null. However, kallikrein gene transfer had no effect on total Src, Akt, GSK-3β, and Bad levels. Furthermore, kallikrein did not lead to phosphorylation of Bad at Ser-112 or Ser-155 (data not shown). Cleaved caspase-3, which is a downstream proapoptotic signal, was reduced markedly after kallikrein gene delivery. The effects of kallikrein on these signaling effectors were blocked by icatibant and Ad.DN-Akt. These findings indicate that kallikrein/kinin protects against I/R-induced cardiomyocyte apoptosis via the kinin B2 receptor-Akt signaling pathway.

Kinin/kinin activates Akt signaling cascade in the ischemic myocardium—We next examined the effect of kallikrein gene transfer on the Akt signaling cascade in the ischemic heart by Western blot analysis (Fig. 2A). Kallikrein gene transfer resulted in increased phosphorylation of Src, Akt, GSK-3β, and Bad (Ser-136) in the I/R-injured myocardium compared with the control with or without injection of Ad.Null. However, kallikrein gene transfer had no effect on total Src, Akt, GSK-3β, and Bad levels. Furthermore, kallikrein did not lead to phosphorylation of Bad at Ser-112 or Ser-155 (data not shown). Cleaved caspase-3, which is a downstream proapoptotic signal, was reduced markedly after kallikrein gene delivery. The effects of kallikrein on these signaling effectors were blocked by icatibant and Ad.DN-Akt. These findings indicate that kallikrein/kinin protects against I/R-induced cardiomyocyte apoptosis via the kinin B2 receptor-Akt signaling pathway.
injection of Ad.Null. These combined data indicate that activation of Akt and Bad(Ser-136) and inactivation of GSK-3β and caspase-3 may mediate the protective effect of tissue kallikrein in I/R-induced myocardial apoptosis.

**Effect of Kallikrein/Kinin on Apoptosis in Primary Cardiomyocytes**—Based on the results from the *in vivo* studies, we further determined the role and cellular mechanisms mediated by kallikrein/kinin in protection against H/R-induced apoptosis in cultured neonatal cardiomyocytes. To evaluate infection efficiency for the *in vitro* experiments, adenovirus containing the green fluorescence protein gene at a m.o.i. of 50 was transduced into cultured cardiomyocytes. More than 80–90% transfection efficiency was identified by homogeneous green fluorescence in cardiomyocytes (data not shown). Similarly, successful transfection of Ad.CMV-TK was confirmed by immunocytochemistry for human tissue kallikrein at a m.o.i. of 50 with high efficiency.
and low cytotoxicity (Fig. 4A). TUNEL and Hoechst staining showed that H/R treatment effectively induced cardiomyocyte apoptosis compared with normoxia treatment. Similar to in vivo studies, transduction of Ad.CMV-TK resulted in significant reduction of apoptotic myocytes compared with the H/R control (16.7 ± 3.7% versus 24.2 ± 2.8% by TUNEL staining; 21.3 ± 3.9% versus 36.5 ± 4.3% by Hoechst staining; n = 6, p < 0.01) (Fig. 4, A, C, and D). To confirm the role of kallikrein/kinin in protection against cardiomyocyte apoptosis, we further examined the effect of kinin on H/R-induced apoptosis in cultured cardiomyocytes. Kinin (0.1 μM) significantly reduced apoptotic myocytes (17.3 ± 3.2% versus 24.2 ± 2.8% by TUNEL; 23.8 ± 3.6% versus 36.5 ± 4.3% by Hoechst, n = 6, p < 0.01), as did 1 μM kinin (12.2 ± 2.9% versus 24.2 ± 2.8% by TUNEL; 18.6 ± 4.0% versus 36.5 ± 4.3% by Hoechst, n = 6, p < 0.01) (Fig. 4, B, C, and D). These results indicate that kallikrein/kinin protects against H/R-induced cardiomyocyte apoptosis.

Effect of Kallikrein on Akt-GSK-3-Caspase-3 and Akt-Bad Signaling Pathways in Primary Cardiomyocytes—We further investigated the effect of kallikrein on Akt-mediated signaling pathways in neonatal cardiomyocytes. Fig. 5 shows that transduction of Ad.CMV-TK in H/R-injured cardiomyocytes resulted in increased phosphorylation of Akt and Bad(Ser-136). However, these effects were blocked by LY294002, a PI 3-kinase inhibitor, compared with the H/R control with or without Ad.Null transduction. Kallikrein also increased GSK-3β phosphorylation and reduced caspase-3 activation, whereas Ad.Null transduction had no effect on GSK-3β phosphorylation or caspase-3 activation under normoxic or H/R conditions. The GSK-3β activity inhibitor, LiCl, had no effect on GSK-3β phosphorylation but reduced cleaved caspase-3 levels in kallikrein-treated cardiomyocytes. LY294002 abolished the effects of kallikrein on GSK-3β phosphorylation and caspase-3 inactivation, whereas inhibition of caspase-3 by Z-VAD had no effect on GSK-3β phosphorylation. These data indicate that kallikrein inhibits cardiomyocyte apoptosis via activation of PI-3K-Akt-Bad(Ser-136) and inhibition of caspase-3 in an Akt-GSK-3β-dependent manner.

Akt Signaling and Cardiomyocyte Survival—To analyze the end point of Akt signaling pathways, we determined the effects of kallikrein gene transduction on these signal events related to cardiomyocyte apoptosis and cell survival. Kallikrein gene transduction significantly reduced cardiomyocyte apoptosis induced by H/R (16.7 ± 3.7% versus 24.2 ± 3.4%, n = 6, p < 0.01) (Fig. 6A). There was no difference between H/R-treated cardiomyocytes with or without transduction with Ad.Null. The protective effect of kallikrein was blocked by cotransduction with Ad.DN-Akt (25.6 ± 3.4% versus 16.7 ± 3.7%, n = 6, p < 0.01), whereas cotransduction with Ad.Myr-Akt reduced the number of apoptotic myocytes compared with H/R control (14.5 ± 3.2% versus 24.2 ± 3.4%, n = 6, p < 0.01). These results indicate that Akt plays a pivotal role in the protective effects of kallikrein on apoptosis. Furthermore, cotransduction of dominant-negative GSK-3β, Ad.GSK3β-KM, or intervention with LiCl in kallikrein-transduced myocytes resulted in reduction of apoptosis, whereas cotransduction of constitutively active
GSK-3β (Ad.GSK3β-S9A) reversed the kallikrein effect (27.9 ± 3.5% versus 16.7 ± 3.7%, n = 6, p < 0.01). Similarly, Z-VAD also reduced cardiomyocyte apoptosis (16.7 ± 3.7% versus 24.2 ± 3.4%, n = 6, p < 0.01), indicating the proapoptotic effect of caspase-3 after injury. These results support the notion that inhibition of GSK-3β and caspase-3 activities facilitates the protective effect of kallikrein on apoptosis.

The role of these signaling events in mediating the effects of kallikrein was investigated further by cell viability assay (Fig. 6B). Kallikrein significantly increased cardiomyocyte viability compared with the H/R control (76.6 ± 5.2% versus 59.7 ± 5.2%, n = 6, p < 0.01). This effect was blocked by cotransduction with Ad.DN-Akt (52.6 ± 9% versus 76.6 ± 5.2%, n = 6, p < 0.01), whereas cotransduction with Ad.Myr-Akt resulted in increased viable cells compared with the H/R control (80.4 ± 6.0% versus 59.7 ± 5.2%, n = 6, p < 0.01). Constitutively active Akt, dominant-negative GSK3β-KM, LiCl, and Z-VAD increased cell viability, whereas Ad.GSK3β-S9A reversed the effects of kallikrein (54.9 ± 18.8% versus 80.4 ± 6.0%, n = 6, p < 0.01). The above interventions have no effect on apoptosis and viability among all groups under normoxia condition (Fig. 6B and A). The results obtained from both cell viability and apoptotic assays indicate that the protective effect of kallikrein in cardiomyocytes is mediated by activation of Akt, leading to inhibition of the GSK-3-caspase-3 signaling pathway.

**Interaction of GSK-3-Caspase-3 and Bad 14-3-3 Signaling Pathways.—** To elucidate molecular mechanisms mediated by kallikrein in protection against apoptosis, we examined the binding of Bad with 14-3-3 and Bcl-xL by analyzing Bad-14-3-3 and Bad-Bcl-xL complex levels in H/R-injured cardiomyocytes. Bad-14-3-3 complex levels were reduced significantly with or without Ad.Null transduction after H/R treatment compared with normoxic conditions, whereas kallikrein gene transduction restored the Bad-14-3-3 levels to those of the normoxic group (Fig. 7A). Conversely, kallikrein transduction significantly reduced Bad-Bcl-xL complex formation in the cardiomyocytes after H/R treatment. Kallikrein effects on Bad-14-3-3 and Bad-Bcl-xL complex levels were reversed by LY294002, indicating a PI 3-kinase/Akt-dependent event. These results indicate that kallikrein protects against cardiomyocyte apoptosis by activation of PI 3-kinase/Akt and Bad phosphorylation, leading to increased Bad-14-3-3 complex formation and decreased Bad-Bcl-xL complex levels.

We further examined a potential interaction of caspase-3 with Bad-14-3-3 and Bad-Bcl-xL complex. Fig. 7B shows that the addition of recombinant caspase-3 resulted in reduced Bad-14-3-3 complex, but increased Bad-Bcl-xL complex under...
normoxic conditions. However, the effects were reversed by Z-VAD and kallikrein. These results indicate that caspase-3 could exert its proapoptotic effects by disrupting the Bad-14-3-3 complex and facilitating Bad-Bcl-xL complex formation.

**DISCUSSION**

The present study demonstrates that kallikrein/kinin protects against acute I/R-induced cardiomyocyte apoptosis via both Akt-GSK-3β-caspase-3 and Akt-Bad-14-3-3 signaling pathways in an acute I/R rat model and in cultured cardiomyocytes. Our results show that catheter-based delivery of the kallikrein gene resulted in efficient expression of recombinant human tissue kallikrein in rat myocardium, which was accompanied by activation of Src, Akt, and Bad(Ser-36) and inhibition of GSK-3β and caspase-3 activities. In primary cardiomyocytes, kallikrein protected against apoptosis via activation of Akt-GSK-3β and Akt-Bad pathways. Significantly, our results showed that: 1) caspase-3 promotes apoptosis in a GSK-3β-dependent manner; 2) interaction of Bad and 14-3-3 plays a crucial role in inhibiting apoptosis; and 3) caspase-3 could disrupt the Bad-14-3-3 complex to promote cell death. These studies reveal a link between the interaction of Akt-GSK3-caspase-3 and Akt-Bad-14-3-3 signaling pathways. Our findings provide new and important insights into the signaling mechanism mediated by kallikrein/kinin in protection against cardiomyocyte apoptosis.

This is the first study to demonstrate that kallikrein/kinin inhibits GSK-3-caspase-3 signaling by Akt in the I/R myocardium. Akt is a key effector of PI-3-kinase in the survival pathway against apoptosis. Phosphorylation of GSK-3β by Akt results in its inactivation (30, 31). Inactivation of GSK-3β has been shown to be a negative regulator of cardiomyocyte hypertrophy induced by angiotensin II and endothelin-1 in vitro (29). Moreover, inhibition of GSK-3β has been shown to reduce injury after cerebral ischemia (31) and cardiac preconditioning (11). In addition, studies in human neuroblastoma cell lines showed that increased nuclear GSK-3β levels precede activation of the caspase cascade, suggesting a role of GSK in regulating caspase activation (12, 13). Recent studies also reported that inactivation of GSK-3β blocked cytochrome c release and caspase-3 activation in SH-SY5Y and HeLa cells (32, 33). In this study, we further demonstrated that inhibition of GSK-3β with lithium or catalytically inactive GSK-3β reduced caspase-3 activation, whereas the caspase-3 inhibitor Z-VAD suppressed caspase-3 activities but had no influence on GSK-3β activity. These results indicate that caspase-3 is a downstream target of GSK-3β and that GSK-3β-caspase-3 signaling is an important pathway in the initiation of apoptosis caused by acute myocardial I/R.

It has been established that the proapoptotic factor Bad forms a complex with Bcl-xL in mitochondria and that dissociation of the Bad-Bcl-xL complex in the cytosol promotes cell survival (34). Our results show that kallikrein gene transfer results in phosphorylation of Bad, leading to dissociation of Bad-Bcl-xL and thus decreased Bad-Bcl-xL complex formation and increased Bcl-xL levels. Phosphorylation of Bad provides an important link between extracellular survival factors and the intrinsic cell death pathway. The primary role of 14-3-3 proteins is to inhibit apoptosis; they bind to phosphorylated Bad, which prevents the formation of the Bad-Bcl-xL complex (35). Phosphorylation of different residues of the Bad proteins appears to have distinct consequences. It is not clear whether a different role exists between Bad(Ser-112) and Bad(Ser-136) in mediating 14-3-3 binding for inhibition of Bad. However, our results demonstrate for the first time that kallikrein gene transfer promotes the interaction of Bad with 14-3-3 proteins primarily by phosphorylation of Bad at the Ser-136 residue in I/R myocardium. This is consistent with a previous study that Bad (Ser-136) is a more potent and efficacious inducer of cell death than Bad(Ser-112) in Bad-regulated apoptosis in COS7 cell lines (36). Our results suggest that Bad(Ser-136) and Bad-
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(Ser-112) may represent acceptors of 14-3-3-dependent and 14-3-3-independent survival transduction signals, respectively.

Although there are several isoforms of 14-3-3 proteins, the interaction of specific isoform with target signals to inhibit cardiomyocyte apoptosis is not clear. We investigated the role of the isoform 14-3-3σ in Akt cascade because it has been documented that hypoxia selectively up-regulates 14-3-3σ, indicating an adaptive mechanism to hypoxia-induced apoptosis in myocardium (37). Our data showed that 14-3-3σ exerted an antiapoptotic effect by binding to phospho-Bad (136), which may prevent its translocation to mitochondria.

We also showed that lithium, a GSK-3 inhibitor, had no effect on Bad phosphorylation, indicating no interaction between GSK-3σ and Bad. However, we found that recombinant caspase-3 protein treatment can reduce the Bad-14-3-3 complex in primary cardiomyocytes. However, this effect was blocked by caspase-3 inhibitor, suggesting that Bad-14-3-3σ could be disrupted by activated caspase-3. This finding represents a novel cellular mechanism by which kallikrein protects against cell death: reduction of caspase-3 levels prevents it from disrupting the Bad-14-3-3 complex. In the apoptotic signaling cascade, the resultant release of Bad leads to its translocation to the mitochondrial outer membrane, where Bad heterodimerizes with Bcl-xL. In fact, one recent study reported that caspase-3 could cleave 14-3-3σ at Asp-238 in yeast EGY48 cells (38). However, it has yet to be investigated at which site caspase-3 cleaves the Bad-14-3-3σ complex in cardiomyocytes.

In summary, our results demonstrates that crucial signaling effects in regulating cardiomyocyte apoptosis mediated by TK after acute I/R injury include activation of Src, Akt, Bad(Ser112), and inactivation of GSK-3σ after acute I/R injury include activation of Src, Akt, Bad(Ser112), and inactivation of GSK-3σ.

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REFERENCES

1. Di Napoli, P., Taccardi, A. A., Grilli, A., Felaço, M., Balbone, A., Angelucci, D., Gallina, S., Calafiore, A. M., De Caterina, R., and Barsotti, A. (2003) Am. J. Heart. J. 146, 1105–1111

2. Suzuki, K., Murtaza, B., Smolenzak, R. T., Sammut, I. A., Suzuki, N., Kaneda, Y., and Yacoub, M. H. (2001) Circulation 104, 1308–1313

3. Codorelli, G., Roncari, R., Ross, J. Jr., Pisani, A., Stassi, G., Todaro, M., Trocha, S., Drusco, A., Gu, Y., Russo, M. A., Frati, G., Jones, S. P., Fefer, D. J., Napoli, C., and Croce, C. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9977–9982

4. Zou, Y., Zhu, W., Sakamoto, M., Qin, Y., Akazawa, H., Toko, H., Mizukami, M., Takeda, N., Minamino, T., Takano, H., Nagai, T., Nakai, A., and Komuro, I. (2003) Circulation 108, 3024–3030

5. Bell, R. M., and Yellon, D. M. (2003) J. Mol. Cell. Cardiol. 35, 185–193

6. Xia, C. F., Yin, H., Bortolong, C. V., Chao, L., and Chao, J. (2004) Hypertension 43, 452–459

7. Roberts, M. S., Woods, A. J., Dale, T. C., Van Der Sluijs, P., and Norman, J. C. (2004) Mol. Cell. Biol. 24, 1505–1515

8. Sztica, A. N., Movseyan, V. A., Leu, P. M., IV, and Faden, A. I. (2003) Mol. Cell. Neurosci. 22, 365–382

9. Loberg, R. D., Vesely, E., and Brosius, F. C., III (2002) J. Biol. Chem. 277, 41607–41673

10. Kim, H. S., Skurk, C., Thomas, S. R., Bialik, A., Suhara, T., Kureishi, Y., Birnbaum, M., Keaney, J. F., Jr., and Walsh, K. (2002) J. Biol. Chem. 277, 41888–41896

11. Tong, H., Imahashi, K., Steenbergen, C., and Murphy, E. (2002) Circ. Res. 90, 377–379

12. Xia, C. F., Yin, H., Borlongan, C. V., and Chao, J. (2004) Hypertension 43, 452–459

13. Loberg, R. D., Vesely, E., and Brosius, F. C., III (2002) J. Biol. Chem. 277, 44701–44708

14. Kim, T. D., Bijur, G. N., and Jope, R. S. (2001) Brain Res. 919, 106–114

15. Shimamura, H., Terada, Y., Okado, T., Tanaka, H., Inoshita, S., and Sasaki, S. (2003) J. Am. Soc. Nephrol. 14, 1427–1434

16. Chiang, C. W., Kanies, C., Fang, W. B., Parkhurst, C., Xie, M., Henry, T., and Yang, E. (2003) Mol. Cell. Biol. 23, 6350–6362

17. Shindo, S., Schindler, C. K., Quan-Lan, J., Saugstad, J. A., Taki, W., Simon, R. P., and Henshall, D. C. (2003) J. Neurochem. 86, 460–469

18. Agata, J., Chao, L., and Chao, J. (2002) Hypertension 40, 653–659

19. Chao, J., Zhang, J. J., Lin, K. F., and Chao, L. (1998) Hum. Gene Ther. 9, 21–31

20. Silva, J. A., Jr., Araujo, R. C., Baltatu, O., Oliveira, S. M., Tschop, C., Fink, E., Hoffmann, S., Plem, R., Chao, K. X., Chao, L., Chao, J., Ganten, D., Picq, J. P., and Henshall, D. C. (2003) J. Mol. Cell. Cardiol. 33, 1493–1504

21. Nozato, T., Ito, H., Watanabe, M., Ono, Y., Adachi, S., Tanaka, H., Hirao, M., Sunanori, M., and Marum, F. (2001) J. Mol. Cell. Cardiol. 33, 1493–1504

22. Dobrzensky, E., Montanari, D., Agata, J., Zhu, J., Chao, J., and Chao, L. (2002) Am. J. Physiol. 283, E1291–E1298

23. Nebigil, C. G., Etienne, N., Messadegq, N., and Maroteaux, L. (2003) FASEB J. 17, 1373–1375

24. Craig, R., Wagner, M., McCardle, T., Craig, A. G., and Glembotski, C. C. (2001) J. Biol. Chem. 276, 27577–27582

25. Haq, S., Zou, H., Zhang, S., and Chao, J. (2002) Hypertension 40, 59–66

26. Chiang, C. W., Kanies, C., Fang, W. B., Parkhurst, C., Xie, M., Henry, T., and Yang, E. (2003) Mol. Cell. Biol. 23, 6350–6362

27. Shinoda, S., Schindler, C. K., Quan-Lan, J., Saugstad, J. A., Taki, W., Simon, R. P., and Henshall, D. C. (2003) J. Neurochem. 86, 460–469

28. Li, Z., Liao, X., and Chao, J. (2002) J. Biol. Chem. 277, H9295–H9305

29. Li, Z., Liao, X., and Chao, J. (2002) J. Biol. Chem. 277, H9295–H9305

30. Pap, M., and Cooper, G. M. (2002) Mol. Cell. Biol. 22, 578–586

31. Wang, J. M., Hayashi, T., Zhang, W. R., Nakai, A., and Sesti, G. (2001) J. Biol. Chem. 276, 22–24

32. Zhao, S., Hwang, H., and Chao, J. (2003) J. Biol. Chem. 278, 1325–1331

33. Yang, C. C., Lin, H. P., Chen, C. S., Yang, Y. T., Tseng, P. H., Rangnekar, R. P., and Henshall, D. C. (2003) Circ. Res. 93, 1373–1375

34. Masters, S. C., Yang, H., Datta, S. R., Greenberg, M. E., and Fu, H. (2001) J. Biol. Chem. 276, I308–I313

35. Federici, M., Hirali, M., Ranalli, M., Marselli, L., Porzio, O., Lauro, R., Marchetti, P., Melino, G., and Sesti, G. (2001) FASEB J. 15, 22–24

36. Fedor, S., Xiao, Y., Li, G., Kasian, C. A., and Zhang, L. (2003) Am. J. Physiol. 285, H983–H990

37. Kim, J., Kim, D. Y., La, M., Kim, D., Meadows, G. G., and Joe, C. O. (2003) J. Biol. Chem. 278, 19347–19351
