Kallikrein Gene Delivery Attenuates Myocardial Infarction and Apoptosis After Myocardial Ischemia and Reperfusion

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Abstract—The tissue kallikrein-kinin system is present in the heart, and kinin has been shown to have cardioprotective effects. In this study, we investigated the potential role of tissue kallikrein in myocardial ischemia/reperfusion injury through adenovirus-mediated human kallikrein gene delivery. One week after gene delivery, the rats were subjected to a 30-minute coronary occlusion followed by a 2-hour reperfusion. Kallikrein gene delivery caused significant decreases in the ratio of infarct size to ischemic area at risk (from 69.6% to 44.5%, n=10 and 8, P<0.01) and in the incidence of ventricular fibrillation (from 64.3% to 16.7%, n=14 and 24, P<0.01) compared with the group injected with control adenovirus. Kallikrein gene delivery also attenuated programmed cell death in the ischemic area compared with the control area as assessed with the terminal deoxynucleotidyl transferase–mediated nick end labeling assay (n=6, P<0.01). Icatibant, a specific bradykinin B_{2} receptor antagonist, abolished these kallikrein-mediated beneficial effects. The expression of human tissue kallikrein mRNA was identified in rat heart, kidney, lung, liver, and adrenal gland. After kallikrein gene delivery, cardiac kinin and cGMP levels were significantly elevated compared with the control (29.6±12.7 versus 6.1±2.1 pg/mg protein, n=7, P<0.01; 1.30±0.6 versus 0.86±0.09 pmol/mg protein, n=5, P<0.05). These results indicate that kallikrein gene delivery protects against myocardial infarction, ventricular arrhythmias, and apoptosis in ischemia/reperfusion injury via kinin-cGMP signal pathway. The successful application of this technology may have potential therapeutic value in the treatment of coronary artery diseases. (Hypertension. 2000;35:25-31.)

Key Words: kallikrein genes myocardial infarction arrhythmia guanosine kinins

Oclusion of the coronary artery causes cardiomyocyte dysfunction and eventual tissue necrosis. Reperfusion relieves ischemia by providing cells with metabolites and oxygen, thereby preventing extensive tissue damage. Although reperfusion salvages the myocardium, it also initiates a series of events that result in ventricular arrhythmia and accelerate myocardial apoptosis and necrosis.1 This consequence greatly limits the benefits of thrombolytic or angioplasty therapy. It is well known that the inhibition of ACE has cardioprotective effects, such as improvement in cardiac function under the condition of heart failure, attenuation of hypertensive cardiac hypertrophy, reduction in myocardial infarct size, and prevention of reperfusion injury.2–6 ACE is the same enzyme as kininase II, a kinin-degrading enzyme. Because icatibant (Hoe 140), a bradykinin B_{2} receptor antagonist, abolishes the protective effects of ACE inhibition on cardiac function, these beneficial effects act in part via the kallikrein-kinin system.7–9 This notion is further supported by the results of a study that show the administration of bradykinin reduces infarct size.8 Ischemic occlusion/reperfusion can induce apoptosis in the myocardium, whereas inhibition of caspase activity has been shown to attenuate both ischemia/reperfusion injury and apoptosis in cardiomyocytes.10,11 However, the potential role of the tissue kallikrein-kinin system in myocardioocyte apoptosis has not been investigated.

The tissue kallikrein-kinin system components, including tissue kallikrein, kininogen, kinin, and bradykinin B_{2} receptor, have been identified in the heart.12–14 Tissue kallikrein cleaves kininogen substrate to release vasoactive kinin peptide via limited proteolysis.15,16 Kinin is then degraded by kininase I or II. Intact kinins bind to B_{1} receptors, whereas the metabolites of kinin, such as Des-Arg^{9}-bradykinin and Des-Arg^{10}-Lys-bradykinin, bind to B_{2} receptors.16 The binding of kinins to their respective receptors activates second messengers in target tissues and triggers biological effects such as vasodilation and vasoconstriction. Through the use of somatic gene delivery approaches, we showed that the expression of recombinant human tissue kallikrein resulted in blood pressure reduction, attenuation of renal damage, and cardiac hypertrophy in genetic and experimentally induced hypertensive rat models.17,18 In this study, we examined the potential roles of the tissue kallikrein-kinin system in ischemia/reperfusion injury through the delivery of the human tissue kallikrein gene and show that adenovirus-mediated kal-
likrein gene delivery significantly attenuated myocardial infarction and apoptosis after ischemia/reperfusion injury in rats.

Methods
Preparation of Replication-Deficient Adenoviral Vector Ad.CMV-cHK
The adenoviral vector containing human tissue kallikrein cDNA under the control of the cytomegalovirus (CMV) enhancer/promoter was prepared as previously described.\textsuperscript{17} Adenovirus harboring the β-galactosidase gene under the control of the CMV enhancer/promoter (Ad.CMV-LacZ) was also prepared as previously described.\textsuperscript{17}

Animal Treatment
Wistar male rats weighing 250 to 280 g (Sprague-Dawley; Harlan) were used in this study. Rats were housed in an air-conditioned room with a 12-hour light/dark cycle, received a standard rat chow (0.4% sodium chloride), and drank tap water. All procedures complied with the standards for the care and use of animal subjects as stated in the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Resources, National Academy of Sciences). One week before surgery, the rats were randomly divided into 5 groups. The first group was injected with saline via the jugular vein (control, n = 23); the second group was injected with Ad.CMV-LacZ (n = 14), and the third group was injected with Ad.CMV-cHK (n = 24) at a dose of $1 \times 10^{10}$ plaque-forming units/rat. The fourth and fifth groups were injected with Ad.CMV-chk (n = 10) or saline (n = 10), respectively, together with icatibant administration (2 mg/kg) via the jugular vein at 15 minutes before coronary occlusion.

Surgical Preparation
Rats were anesthetized with sodium pentobarbital (50 mg/kg IP) and then incubated and ventilated with room air through the use of a respirator (model 683; Harvard Apparatus). A thoracotomy was performed through gentle palpation of the nonischemic region of the heart. The pericardium was widely opened, and the atria, great vessels, and right ventricle were dissected. The left ventricle was cut into 4 slices transversely from base to apex. The slices were incubated at 37°C with 4% triphenyltetrazolium chloride for 30 minutes. Each slice was photographed, and information was downloaded into the computer. The infarct area (unstained), area at risk (brick-red stained), and noninfarct area (blue stained) were measured with use of NIH Image program. The following parameters were averaged for 4 slices from each heart: (1) infarct size expressed as a percentage of the area at risk and (2) area at risk expressed as a percentage of the total area of the slice.

Detection of Apoptosis With In Situ Nuclear DNA Fragmentation
DNA fragmentation was determined through a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay in deparaffinized 4-μm-thick sections from tissue blocks.\textsuperscript{20} This procedure was performed by using an in situ cell death detection kit according to the manufacturer’s instructions (Boehringer Mannheim). The sections were then counterstained with 0.05% light green. Cardiomyocytes in the left ventricle were analyzed in ≥10 separate fields for each tissue section under a light microscope at a magnification of $\times 400$. More than 300 cardiomyocytes in the ischemic area per rat were counted, and cell numbers from 6 rats per group were averaged. TUNEL-positive cardiomyocytes in the ischemic myocardium were scored by an individual who was unaware of the experimental design and were carefully distinguished from TUNEL-positive noncardiomyocytes, such as leukocytes, macrophages, or endothelial cells. The ratio of TUNEL-positive cardiomyocytes to the total number of cardiomyocytes was calculated.

Tissue Preparation
At the end of the experiment, blood samples of 6 to 8 rats from each group were collected through direct cardiac puncture and chilled at 4°C. These samples were centrifuged at 1000g for 20 minutes, and sera were collected and frozen at $-20^\circ$C. At the same time, rats were perfused with normal saline from the heart, and the heart was rapidly excised and rinsed in cold normal saline. The left ventricle was separated from the right ventricle and atria and then stored at −80°C until further analysis.

Reverse-Transcription Polymerase Chain Reaction
Southern Blot Analysis of Human Tissue Kallikrein mRNA
Total RNA was extracted from fresh rat tissues with the use of guanidine isothiocyanate.\textsuperscript{21} Reverse-transcription polymerase chain reaction Southern blot analysis with specific oligonucleotide probes for human tissue kallikrein (5′-primer, 5′-AACACAGCCCATGGTTG-3′; 3′-primer, 5′-CTTCACTAAAGACAGA-3′; internal probe, 5′-GACCTCAATCTTGCC-3′) was performed as previously described.\textsuperscript{22}

ELISA for Human Tissue Kallikrein
Human tissue kallikrein levels in rat serum were determined with the use of an ELISA specific for human tissue kallikrein.\textsuperscript{23} Human tissue kallikrein standard ranges from 0.4 to 25 ng/mL. Because the antibody recognizes only the active kallikrein, the immunoreactive kallikrein levels determined with ELISA represent active kallikrein.

Radioimmunoassays for Kinin, cGMP, and cAMP
The heart was homogenized in 10 vol of 0.1 N HCl at 4°C. The homogenates were centrifuged at 15 000g for 30 minutes, and aliquots of the supernatants were used for the assay. Protein concentrations were measured according to the method of Lowry et
Cardiac kinin, cGMP, and cAMP levels were measured with the use of radioimmunoassays.\textsuperscript{25–28}

**Statistical Analysis**

Data are expressed as mean±SEM and were compared between experimental groups with the use of 1-way ANOVA and Fisher’s PLSD. HR and MAP were compared among groups with the use of repeated measures ANOVA. Binomially distributed data (VT and VF incidence) were compared with the use of the $\chi^2$ test and Fisher’s exact probability test. Differences were considered statistically significant at a value of $P<0.05$.

**Results**

**Hemodynamic Parameters After Gene Delivery**

Table 1 summarizes the hemodynamic variables throughout the experiment. The results show that at the basal level, these parameters are comparable in all groups. Two-way repeated measures ANOVA indicated no significant changes in HR but did indicate a significant time-related reduction in MAP during the experiment. No group-related effects were detected for either MAP or HR. These results suggest that the effects of Ad.CMV-cHK, Ad.CMV-LacZ, icatibant, and a combination of Ad.CMV-cHK and icatibant did not alter the time course of these hemodynamic variables in this model under the experimental conditions.

**Effects of Kallikrein Gene Delivery on Myocardial Infarct Size**

Figure 1 shows the effect of kallikrein gene delivery on infarct size after coronary artery occlusion/reperfusion. The ratio of infarct size to area at risk was significantly lower in rats receiving Ad.CMV-cHK than in control rats receiving either saline or Ad.CMV-LacZ (44.5±2.0% versus 72.3±3.5% or 69.2±2.7%, n=10 or 8, $P<0.01$, respectively; Figure 1A). Icatibant alone did not alter the infarct size after myocardial ischemia/reperfusion compared with control rats injected with either saline or Ad.CMV-LacZ groups (68.2±2.4% versus 72.3±3.5% or 69.2±2.7%). However, icatibant abolished the beneficial effect of kallikrein gene delivery on myocardial ischemia/reperfusion (72.8±2.4% versus 44.5±2.0%, n=8 and 10, $P<0.01$; Figure 1A). The ratio of the area at risk to the left ventricle was similar among groups. Values are expressed as mean±SEM (n=10 or 8). *$P<0.01$ vs all other groups.
Effects of Kallikrein Gene Delivery on Left Ventricular Arrhythmias

Figure 2 shows the effect of kallikrein gene delivery on the incidence of ventricular arrhythmias induced by myocardial ischemia/reperfusion. Ventricular premature beats were observed in all rats after coronary artery occlusion. Kallikrein gene delivery significantly attenuated the incidence of VF from 64.3% to 16.7% (n = 14 and 24, P < 0.01, Figure 2A) and reduced the incidence of VT from 85.7% to 41.7% (n = 14 and 24, P < 0.01, Figure 2B) compared with the group injected with control adenovirus. Neither Ad.CMV-LacZ nor icatibant affected these incidence rates. However, icatibant reversed the protective effect of kallikrein gene delivery on ventricular arrhythmias (Figure 2, A and B).

Expression of Human Tissue Kallikrein After Gene Delivery

At 7 days after the intravenous injection of adenovirus containing the human tissue kallikrein gene into the jugular vein of rats, human tissue kallikrein mRNA was identified through reverse-transcription polymerase chain reaction (RT-PCR) analysis. Human tissue kallikrein mRNA was detected in the heart, liver, lung, kidney, and adrenal gland (Figure 3, top left). The expression of human tissue kallikrein mRNA was not detected in control rats receiving Ad.CMV-LacZ (Figure 3, top right). Similar levels of β-actin mRNA were detected in tissue of both experimental and control groups, indicating the integrity of RNA in these samples (Figure 3, bottom). With the use of ELISA in a time-dependent manner, recombinant human tissue kallikrein was also detected in rat serum from 1 to 7 days after kallikrein gene delivery, with the highest level of 274.7 ± 10.9 ng/mL (n = 3) occurring at 3 days after gene transfer. The results are consistent with our previous studies showing the time course of human tissue kallikrein levels after gene delivery.17,18 Because of the high efficiency of adenovirus infection, the highest level of human tissue kallikrein in rat serum was found at 3 to 4 days after gene transfer and was detectable within 5 to 6 weeks.

Kallikrein Gene Delivery Increased Cardiac Kinin and cGMP Levels

Figure 4 shows cardiac kinin and cGMP levels after myocardial ischemia/reperfusion injury in rats receiving gene transfer. Adenovirus-mediated kallikrein gene delivery significantly increased cardiac kinin levels compared with control rats receiving Ad.CMV-LacZ (29.6 ± 12.7 versus 6.1 ± 2.1 pg/mg protein, n = 5, P < 0.05), whereas icatibant and Ad.CMV-chK administration did not change kinin levels (31.9 ± 7.5 pg/mg protein, n = 5). Cardiac cGMP levels also increased in the kallikrein group compared with the control group (1.30 ± 0.06 versus 0.86 ± 0.09 pmol/mg protein, n = 7, P < 0.01), and icatibant treatment significantly reduced cGMP levels to those of control rats receiving Ad.CMV-LacZ. No significance difference was detected in cardiac cAMP levels between experimental and control rats (0.56 ± 0.06 and 0.47 ± 0.02 nmol/mg protein, respectively; n = 5).

Kallikrein Gene Delivery Attenuated Apoptotic Myocardiocytes

Figure 5 shows representative apoptotic figures in myocardiocytes after reperfusion injury. TUNEL-positive staining of cardiomyocytes was significantly reduced in the group of rats
receiving Ad.CMV-cHK compared with the group receiving Ad.CMV-LacZ, whereas icatibant abolished the protective effect of kallikrein gene delivery on programmed cell death in myocardiocytes. Figure 5B shows that kallikrein gene delivery significantly reduced the ratio of TUNEL-positive cardiomyocytes to total cardiomyocytes compared with the control group (24.8±3.0% versus 40.8±2.6%, n=6, P<0.01). Icatibant abolished the beneficial effect of kallikrein on programmed cell death in myocardiocytes (34.6±3.6% versus 24.8±3.0%, n=6, P<0.05).

**Discussion**

This study demonstrates that adenovirus-mediated kallikrein gene delivery protects cardiac function as demonstrated by the reduction in myocardial infarct size and the incidence of ventricular arrhythmias and apoptotic cardiomyocytes after acute coronary artery occlusion/reperfusion in vivo. These cardioprotective effects of kallikrein were blocked by icatibant, a specific bradykinin B2 receptor antagonist, indicating a kinin-mediated event. Human kallikrein mRNA was detected in rat heart and immunoreactive human kallikrein was secreted into the circulation after the kallikrein gene transfer. Kallikrein gene delivery resulted in increased cardiac kinin and cGMP levels. These results indicate that the protective effects of kallikrein gene delivery in myocardial ischemia/reperfusion injury are mediated via kinin through an NO-cGMP–dependent signal transduction pathway.

**Figure 4.** Effect of kallikrein gene delivery on cardiac kinin and cGMP levels after myocardial occlusion/reperfusion. Both kinin (left) and cGMP (right) levels were significantly increased in Ad.CMV-cHK group compared with that of rats receiving Ad.CMV-LacZ. Values are expressed as mean±SEM (n=5). **P<0.05 vs Ad.CMV-LacZ group. *P<0.01 vs Ad.CMV-LacZ and Ad.CMV-cHK/icatibant groups.

**Figure 5.** Effect of kallikrein gene delivery on apoptotic cardiomyocytes identified with TUNEL assay. A, Representative TUNEL-positive cardiomyocytes identified with TUNEL staining. B, Ratio of TUNEL-positive cardiomyocytes to total cardiomyocytes. Ad.CMV-cHK significantly attenuated TUNEL-positive cardiomyocytes compared with Ad.CMV-LacZ, and icatibant reversed protective effects of Ad.CMV-cHK. Magnification ×400. Values are expressed as mean±SEM (n=6). *P<0.01 vs Ad.CMV-LacZ. **P<0.05 vs Ad.CMV-cHK group.
Ischemia/reperfusion injury is a multifactorial event that includes calcium overload, free radical production, metabolic abnormalities with associated acidosis, and inflammatory reactions. Many studies have demonstrated myocardial protection from ischemia/reperfusion injury. Although reports on this topic are controversial, it is generally accepted that myocardial protection during reperfusion is beneficial and necessary. A protective role of bradykinin B₁ receptor in this regard has been implicated, but the mechanisms by which kinin-mediated protection is provided in ischemic damage are not clear. There are several possibilities regarding the cardioprotective effects of kinin. First, locally and systemically administered kinins can increase coronary and capillary nutritional flow. Second, kinins may change cardiac metabolism, such as the preservation of high-energy–enriched phosphates and increase in myocardial glucose uptake and use; these 2 combined events improve cardiac function and reduce the risk of necrosis. Third, infusions of kinines reduce ischemia-induced norepinephrine overflow and reperfusion-induced arrhythmias. The protection may lead to decreases in cytosolic enzyme leakage, as well as in superoxides that induce cell damage. This in turn may break a vicious circle of cell injury and death. Therefore, these combined effects of kinines on the myocardium may provide protection against ischemia.

The inhibition of ACE has been shown to reduce infarct size and improve cardiac function after coronary artery occlusion/reperfusion injury. ACE inhibitors not only inhibit angiotensin II production but also augment kinin accumulation by decreasing kinin degradation. The finding that a specific bradykinin B₂ receptor antagonist could reverse the cardioprotective effects of ACE inhibition in myocardial ischemia was thought to be mediated, at least in part, via activation at the B₂ receptor. In this study, we observed that kallikrein gene delivery reduced not only the infarct size but also the incidence of ventricular arrhythmias (Figure 2). It is apparent that minimization of the infarct size leads to a reduced incidence of arrhythmias. Previous studies showed that low doses of bradykinin reduced ischemic arrhythmias and improved myocardial electrical stability while not having an effect on coronary blood flow. Bradykinin reduced the severity of ventricular arrhythmias induced by short-term occlusion/reperfusion, even before necrosis occurred.

These findings indicate that the activation of bradykinin receptors may cause an alteration in myocardial electrophysiological activities against ventricular arrhythmias. Taken together, the results of this study indicate that increased cardiac kinin might directly reduce the incidence of VT and VF.

The cardioprotective effects of kinines could be mediated by the activation of bradykinin B₂ receptor via the pathways through either phospholipase A₂ or phospholipase C. The stimulation of phospholipase A₂ results in increased prostacyclin formation and its metabolites, such as 6-keto-prostaglandin F₁ and prostaglandin F₂, were implicated as a consequence of elevated kallikrein activity and local kinin formation. The binding of prostacyclin to its receptor may result in the stimulation of adenylate cyclase and increased cAMP levels. Our results show that cardiac cAMP levels were not changed after kallikrein gene delivery compared with the control, indicating that cAMP may not serve as a second messenger in kinin-mediated cardioprotective effects. Alternatively, activation of bradykinin B₂ receptor may stimulate phospholipase C and thus trigger NO formation. Increased NO formation may result in the stimulation of guanylate cyclase and increased cGMP levels. It has been shown in cultured porcine aortic endothelial cells that bradykinin stimulates the release of NO, which in turn induces the production of cGMP via activation of bradykinin B₂ receptors. In this study, we detected increased cardiac cGMP levels in the group receiving kallikrein gene therapy (Figure 4). These results indicate that the NO-cGMP signal transduction pathway is more likely to serve as the second messenger after the binding of intact kinin to the bradykinin B₂ receptor in this model.

In the present study, we show that kallikrein gene delivery significantly reduced myocardocyte apoptosis after ischemia/reperfusion injury as demonstrated with the TUNEL assay and that icatibant administration reversed the kallikrein-mediated beneficial effect. Myocardial ischemia/reperfusion induces myocardial infarction and apoptosis. Most cardiomyocytes that yield to myocardial infarction during the most damaging phase of the insult undergo apoptosis within 6 hours, whereas necrosis becomes the major contributor to cell death 6 hours after the onset of the ischemic insult. Therefore, we mainly identified apoptosis in cardiomyocytes after a 30-minute coronary occlusion followed by a 2-hour reperfusion. Kinin and cGMP levels in rat heart were significantly increased after kallikrein gene delivery in this model, and icatibant treatment reduced cGMP levels to those of control rats. These findings suggest that the binding of cardiac kinines to bradykinin B₂ receptors activates second messengers such as NO and cGMP and thus leads to inhibition of apoptosis in the ischemia/reperfusion injury model. This notion is consistent with previous studies showing that exogenous NO could inhibit caspase-3–like activity and prevent tumor necrosis factor-α–induced apoptosis in endothelial cells via a cGMP–dependent pathway.

Furthermore, inhibition of caspase activity by its specific inhibitor has been shown to attenuate both apoptosis and ischemia/reperfusion injury in rat myocardium. Apoptosis that contributes to the extension of the infarct is one of the major events responsible for ventricular remodeling. Therefore, the potential benefits of antiapoptotic effects after kallikrein gene delivery may ascribe to a reduction in myocardial infarct size and thus the development of congestive heart failure. Taken together, the present results indicate that the cardiac kallikrein-kinin system plays a protective role in the protection against apoptosis and myocardial infarction.

In clinical situations, ischemia/reperfusion–induced myocardial damage commonly occurs during thrombolytic therapy for myocardial infarction, restoration of blood flow after cardioplegic arrest in cardiovascular surgery, and heart transplantation. The present study showed that the delivery of the kallikrein gene was effective in reducing myocardial ischemia/reperfusion injury in rats. The successful application of kallikrein gene therapy may have potential value in the treatment of individuals with coronary artery diseases.
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References
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