Tissue Kallikrein Elicits Cardioprotection by Direct Kinin B2 Receptor Activation Independent of Kinin Formation

Julie Chao, Hang Yin, Lin Gao, Makoto Hagiwara, Bo Shen, Zhi-Rong Yang, Lee Chao

Abstract—Tissue kallikrein exerts various biological functions through kinin formation with subsequent kinin B2 receptor activation. Recent studies showed that tissue kallikrein directly activates kinin B2 receptor in cultured cells expressing human kinin B2 receptor. In the present study, we investigated the role of tissue kallikrein in protection against cardiac injury through direct kinin B2 receptor activation using kininogen-deficient Brown Norway Katholiek rats after acute myocardial infarction. Tissue kallikrein was injected locally into the myocardium of Brown Norway Katholiek rats after coronary artery ligation with and without coinjection of icatibant (a kinin B2 receptor antagonist) and Nω-nitro-L-arginine methylester (an NO synthase inhibitor). One day after myocardial infarction, tissue kallikrein treatment significantly improved cardiac contractility and reduced myocardial infarct size and left ventricle end diastolic pressure in Brown Norway Katholiek rats. Kallikrein attenuated ischemia-induced apoptosis and monocyte/macrophage accumulation in the ischemic myocardium in conjunction with increased NO levels and reduced myeloperoxidase activity. Icatibant and Nω-nitro-L-arginine methylester abolished kallikrein’s effects, indicating a kinin B2 receptor NO-mediated event. Moreover, inactive kallikrein had no beneficial effects in cardiac function, myocardial infarction, apoptosis, or inflammatory cell infiltration after myocardial infarction. In primary cardiomyocytes derived from Brown Norway Katholiek rats under serum-free conditions, active, but not inactive, kallikrein reduced hypoxia/reoxygenation-induced apoptosis and caspase-3 activity, and the effects were mediated by kinin B2 receptor/nitric oxide formation. This is the first study to demonstrate that tissue kallikrein directly activates kinin B2 receptor in the absence of kininogen to reduce infarct size, apoptosis, and inflammation and improve cardiac performance of infarcted hearts. (Hypertension. 2008;52:715-720.)

Key Words: apoptosis ■ cardiac function ■ infarct size ■ kinin B2 receptor ■ tissue kallikrein

Tissue kallikrein is a serine proteinase that specifically processes low-molecular-weight kininogen to produce the potent vasoactive kinin peptides bradykinin and Lys-bradykinin (kallidin),1 which bind to and activate the kinin B2 receptor.2 Kinins have been shown to protect against cardiac injury through kinin B2 receptor activation.3-5 Yang and coworkers5 demonstrated that the cardioprotective effect of preconditioning was abolished in kinin B2 receptor knockout mice and in kininogen-deficient rats. The cardioprotective response to inhibition of angiotensin-converting enzyme (ACE) and angiotensin II type 1 receptor was diminished in B2 receptor-deficient mice.6 Similarly, kinins appear to play an important role in the cardioprotective effect of ACE inhibition in kininogen-deficient rats.7 However, recent studies from Erdös’ group demonstrated that tissue kallikrein directly activates the kinin B2 receptor in cultured Chinese hamster ovary cells.8,9 This novel finding is consistent with our earlier report that tissue kallikrein directly induced rat uterine contraction independent of detectable kinin formation.10 In contrast, contraction of isolated jugular vein by tissue kallikrein appears to be dependent on blood vessel-derived kininogen and B2 receptor activation.11 Taken together, these results indicate that tissue kallikrein’s actions are mediated by kinin B2 receptor activation with or without kinin formation. However, whether tissue kallikrein can directly act on the kinin B2 receptor in the absence of kinin formation in triggering cardioprotective effects in vivo has not been demonstrated.

Brown Norway Katholiek (BNK) rats, genetically deficient in kininogen secretion, retain kinin B2 receptor expression. Therefore, the kininogen-deficient BNK rat is an ideal model to investigate whether tissue kallikrein has a direct effect on kinin B2 receptor in triggering biological functions. In this study, we determined whether tissue kallikrein has a cardioprotective role by direct activation of the kinin B2 receptor in BNK rats after myocardial infarction (MI). Our results showed that tissue kallikrein through kinin B2 receptor activation and NO formation improved cardiac performance and reduced ischemia-induced infarction, cardiomyocyte apoptosis, and intramyocardial inflammation in BNK rats. This is the first study to identify a direct biological function of tissue kallikrein through kinin B2 receptor activation independent of kinin formation in vivo.

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715
Animals and Treatments

Brown Norway Katholiek rats were kindly provided by Dr. Edward Shesely of Hypertension and Vascular Research Division, Henry Ford Hospital (Detroit, Mich) and were initially obtained from the Department of Pharmacology, Kitasato University School of Medicine. Male BNK rats weighing 200 to 250 g were subjected to ligation of the left coronary artery as previously described.12 This study complied with the Guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences). Rat tissue kallikrein was purified and characterized as previously described.13 Animals were randomly divided into 6 groups (n=10 in each group). In 2 control groups, rats were subjected to either sham surgery or left anterior descending coronary artery ligation followed by saline injection. In the third group, tissue kallikrein (25 μg in 150 μL saline) was injected at 7 different sites into the border area of the infarcted left ventricle immediately after coronary artery ligation. The fourth group received tissue kallikrein together with coinjection of the kinin B2 receptor antagonist (icatibant, 15 mg/kg; obtained from Hoechst Marion Roussel). The fifth group received tissue kallikrein followed by intravenous injection of Nω-nitro-L-arginine-methyl-ester (L-NAME) (35 mg/kg). The sixth group received inactive tissue kallikrein (25 μg in 150 μL saline) followed by saline injection.

Methods

Inactivation of Tissue Kallikrein by Aprotinin

Tissue kallikrein was inactivated by prior incubation with 5-fold molar excess of aprotinin at 37°C for 1 hour. Inactivation of tissue kallikrein was determined by enzymatic assay with S2266, a chromogenic substrate.15 Aprotinin-treated tissue kallikrein exhibited less than 5% of active kallikrein activity.

Myocardial Infarct Size Determination

The middle part of the heart (2 mm) was sectioned transversely and incubated with 1.5% 2,3,5-triphenyltetrazolium chloride (Sigma) for 5 minutes at 37°C. The ratio of infarcted area to the area at risk was then calculated. The infarcted area was distinguished by 2,3,5-triphenyltetrazolium chloride staining using computer-assisted planimetry (National Institutes of Health Image 1.57).

Histological and Immunohistological Analysis

For histological analyses, the left ventricle was fixed with 4% paraformaldehyde, dehydrated, embedded, and cut into 4-μm sections. Primary antibody against ED-1 (Chemicon, 1:200) was used for immunostaining of monocytes/macrophages. The number of ED-1-positive cells was counted in a double-blind fashion from 8 to 10 different fields of each section (n=10) at 400× magnification. Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling).16 The ratio of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling-positive cardiomyocytes to the total number of cardiomyocytes was calculated.

Hypoxia/Reoxygenation of Primary Cultured Cardiomyocytes

Cardiomyocytes were isolated from the hearts of 2- to 3-day-old BNK rats as previously described.17 Cardiomyocytes were grown in DMEM supplemented with 10% fetal bovine serum. Cardiomyocyte origin was confirmed immunocytochemically using antibody to sarcomeric α-actinin (Sigma). Subcultured cells were maintained in serum-free DMEM for 24 hours and then incubated with serum-free DMEM supplemented with active tissue kallikrein (0.2 μmol/L) or aprotinin-inactivated tissue kallikrein (0.2 μmol/L) under the condition of hypoxia for 12 hours (95% N2 and 5% CO2) followed by 24-hour reoxygenation (95% O2 and 5% CO2). Apoptotic cardiomyocytes were identified by Hoechst 33342 staining. Hoechst-positive apoptotic cells were determined by counting cardiomyocytes in 6 randomly chosen fields. Caspase-3 activity in cardiomyocyte lysates was determined using a fluorometric caspase-3 assay kit (Oncogene) according to the manufacturer’s instructions.

Nitrare/Nitrite and Myeloperoxidase Assays

Nitrate/nitrite levels, an indicator of NO production, were measured by a fluorometric assay as previously described.18 Myeloperoxidase activity in cardiac extracts was measured as previously described.19

Table. Hemodynamic Parameters 1 Day After MI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>Control</th>
<th>TK</th>
<th>TK/Icatibant</th>
<th>TK/L-NAME</th>
<th>Inactive TK</th>
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</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>105.8±3.6</td>
<td>108.3±3.7</td>
<td>109.2±2.5</td>
<td>108.3±4.1</td>
<td>104.2±3.5</td>
<td>106±4.1</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>2.2±0.5</td>
<td>11.9±1.1</td>
<td>5.6±0.5*</td>
<td>12.1±0.9</td>
<td>11.8±0.5</td>
<td>12.9±1.3</td>
</tr>
<tr>
<td>dp/dt max, mm Hg/s</td>
<td>3559±89</td>
<td>2503±120</td>
<td>3054±77*</td>
<td>2535±100</td>
<td>2529±61</td>
<td>2326±99</td>
</tr>
<tr>
<td>dp/dt min, mm Hg/s</td>
<td>3022±81</td>
<td>2004±112</td>
<td>2549±84*</td>
<td>2019±107</td>
<td>1931.9±55</td>
<td>1800±89</td>
</tr>
</tbody>
</table>

*P<0.01 vs other MI groups.

TK indicates tissue kallikrein; MAP, mean arterial pressure; LVEDP, left ventricular end diastolic pressure; dp/dt max, maximum first derivative of pressure; dp/dt min, minimum first derivative of pressure.
Statistical Analysis
Data were compared among experimental groups using analysis of variance followed by Fisher’s partial least squares difference. Data are expressed as mean±SEM. Differences were considered statistically significant at a value of $P<0.05$.

Results
Kallikrein Improves Cardiac Function and Reduces Infarct Size in Kininogen-Deficient Rats
Tissue kallikrein injection significantly improved cardiac function in BNK rats 1 day after MI (Table). MI induced a significant increase of left ventricular end diastolic pressure compared with the sham group, whereas kallikrein significantly reduced left ventricular end diastolic pressure. Cardiac contractility (dP/dt max and dP/dt min) was markedly reduced after MI, but was significantly increased by kallikrein. Both icatibant and L-NAME blocked kallikrein’s cardioprotective effects. Unlike active kallikrein, aprotinin-inactivated kallikrein did not improve cardiac contractility or reduce left ventricular end diastolic pressure in BNK rats after MI. Mean arterial pressure was not altered among all groups.

Intramyocardial injection of tissue kallikrein, but not inactive kallikrein, significantly reduced infarct size in the left ventricle 1 day after MI compared with the MI control group, as determined by 2,3,5-triphenyltetrazolium chloride staining and quantitative analysis (Figure 1A and 1B). Coadministration of icatibant and L-NAME abrogated kallikrein’s effect. However, icatibant, L-NAME, or aprotinin alone had no effect on myocardial infarct size as compared with the MI control (data not shown).

Kallikrein Reduces MI-Induced Cardiomyocyte Apoptosis and Intramyocardial Inflammation
Apoptotic cardiomyocytes were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling in the infarcted myocardium 1 day after MI, and kallikrein treatment reduced the number of apoptotic cells (Figure 2A). Icatibant and L-NAME abrogated kallikrein’s effect, and inactive kallikrein had no effect. Quantitative analysis showed that active, but not inactive, kallikrein significantly reduced the ratio of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling-positive cardiomyocytes to total number of cardiomyocytes as compared with the control group. However, kallikrein’s protective effect was blocked by icatibant and L-NAME (Figure 2B). Furthermore, inflammatory cell accumulation in the infarcted tissue was significantly reduced in the kallikrein-treated group compared with the MI control group (Figure 3A and 3B).

Figure 2. Active tissue kallikrein inhibits cardiomyocyte apoptosis induced by MI in the infarcted heart of kininogen-deficient BNK rats, and the effect is blocked by icatibant and L-NAME; treatment with inactive tissue kallikrein had no effect. A, Representative apoptotic cells stained by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling. B, Quantitative results of apoptotic cells. *$P<0.05$ vs other groups, $n=6$ to 10.

Figure 3. Tissue kallikrein administration reduces monocyte/macrophage infiltration in heart tissue of kininogen-deficient BNK rats after MI, and the effect is blocked by icatibant and L-NAME; treatment with inactive tissue kallikrein had no effect. A, Representative monocyte/macrophage infiltration in infarcted heart tissue as determined by ED-1 immunohistochemical staining. B, Quantitative analysis of monocyte/macrophage number in infarcted heart after MI. *$P<0.01$ vs other groups, $n=6$ to 10.
region of the heart was identified by ED-1 immunostaining (Figure 3A). ED-1 positive cells were counted for quantification of monocyte/macrophage number (Figure 3B). Increased inflammatory cell infiltration was detected in the infarcted area of the heart after acute MI, but kallikrein injection significantly decreased monocytes/macrophages compared with the control. Icatibant and L-NAME blocked kallikrein’s effect, but aprotinin-inactivated kallikrein had no protective effect against the inflammatory response.

**Kallikrein Increases NO Levels and Reduces Myeloperoxidase Activity**

Kallikrein treatment resulted in a significant increase in cardiac nitrate/nitrite production compared with the MI control group, and this effect was abrogated by icatibant and L-NAME (Figure 4A). Moreover, kallikrein prevented the increase in cardiac myeloperoxidase levels induced by MI damage, and the effect of kallikrein was blocked by icatibant and L-NAME (Figure 4B). Again, inactive kallikrein had no effect in increasing NO formation or inhibiting oxidative stress.

**Active Kallikrein Reduces Hypoxia/Reoxygenation-Induced Apoptosis and Caspase-3 Activity in Primary Cultured Cardiomyocytes**

Representative Hoechst-positive staining and quantitative analysis showed that active tissue kallikrein, but not aprotinin-inactivated tissue kallikrein, significantly reduced hypoxia/reoxygenation-induced apoptosis in cultured primary cardiomyocytes derived from BNK rats (Figure 5A and 5B). Similarly, active kallikrein, but not inactive kallikrein, significantly reduced hypoxia/reoxygenation-induced caspase-3 activity (Figure 5C). Icatibant and L-NAME abrogated kallikrein’s effects on both apoptosis and caspase-3 activity.

**Discussion**

This study establishes that tissue kallikrein elicits cardioprotection independent of kinin formation. Using kininogen-deficient BNK rats, which are unable to produce kinin peptides, we clearly demonstrated that tissue kallikrein, but not aprotinin-inactivated tissue kallikrein, significantly reduced hypoxia/reoxygenation-induced apoptosis in cultured primary cardiomyocytes. Our results showed a direct function independent of its kinin-releasing activity in protection against cardiovascular disease. It is important to note that the involvement of local cardiac low-molecular-weight kininogens in BNK rats can be dismissed due to the finding that this rat strain possesses a point mutation in the kininogen gene that causes low-molecular-weight kininogen to accumulate inside the cell and thus prevent its secretion for cleavage by kallikrein. Moreover, rat T-kininogen should have no effect on our observations because it cannot be cleaved by tissue kallikrein.

It has been shown that only active tissue kallikrein, but not active site-inhibited kallikrein, can stimulate the kinin B2 receptor in cultured cells, suggesting that cleavage of a peptide bond in the receptor is necessary for its activation by kallikrein. To determine whether tissue kallikrein has a direct proteolytic action on the kinin B2 receptor, we evaluated the effect of purified active and inactive forms of tissue kallikrein on hypoxia/reoxygenation-induced programmed cell death in cultured cardiomyocytes. Our results showed that active kallikrein, but not inactive kallikrein, inhibited hypoxia-induced apoptosis and caspase-3 activity in cultured cardiomyocytes derived from Sprague-Dawley (data not available).
shown) and kininogen-deficient rats. These results indeed indicate that cleavage of a peptide bond in the kinin B2 receptor is necessary for direct activation of kinin B2 receptor by tissue kallikrein.

Kinin is capable of generating NO by causing an increase in the phosphorylation of endothelial NO synthase. Similarly, tissue kallikrein gene transfer has been shown to promote muscular neovascularization by endothelial NO synthase upregulation and Akt activation. Moreover, endothelial NO synthase gene delivery protected against cardiac remodeling through reduction of oxidative stress after MI. NO is a potent antioxidant and is capable of inhibiting neutrophil superoxide anion production through a direct action on the membrane components of NADPH oxidase and the assembly of NADH/NADPH oxidase subunits. Our present finding showed that icatibant and L-NAME abolished kallikrein's effects in promoting nitrate/nitrite (an indicator of NO) levels as well as suppressing superoxide production in infarcted hearts. These combined results indicate that kallikrein/kinin is capable of improving cardiac function through increased NO formation.

A recent study showed that the kinin B1 and B2 receptors may serve a protective role in cardiac dysfunction. However, we previously demonstrated that myocardial hypertrophy induced by aortic occlusion is mediated by B2 receptor, but not by B1 receptor, using kinin receptor knockout mice. In addition, intact bradykinin, but not des-Arg9-bradykinin (a kinin B1 receptor agonist), prevented cardiomyocyte apoptosis and ventricular remodeling after acute ischemia/reperfusion, supporting a role of kinin B2 receptor, but not B1 receptor, in cardiac protection. The potential role of tissue kallikrein in protection against other organ damage such as the kidney, blood vessels, and brain through direct kinin B2 receptor activation awaits further investigation.

Perspectives
This is the first study to demonstrate that tissue kallikrein improves cardiac function by inhibiting apoptosis and inflammation through direct activation of the kinin B2 receptor without kinin formation in the infarcted myocardium. Comparison of active and inactive tissue kallikrein indicates that cleavage of a peptide bond is required for B2 receptor activation.
stimulation by kallikrein in vitro and in vivo. This is an innovative finding because it is well characterized that tissue kallikrein exerts biological functions through generation of kinin peptides from kinogen substrate. Because ACE inhibition can potentiate kallikrein’s effect on kinin B2 receptor activation, tissue kallikrein, in addition to kinin formation, could provide advanced therapeutic benefits of ACE inhibition in protection against cardiovascular and renal diseases.

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Disclosures
None.

References
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Independent of Kinin Formation

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