Kallikrein Gene Delivery Improves Serum Glucose and Lipid Profiles and Cardiac Function in Streptozotocin-Induced Diabetic Rats

David Montanari, Hang Yin, Eric Dobrzynski, Jun Agata, Hideaki Yoshida, Julie Chao, and Lee Chao

We investigated the role of the kallikrein-kinin system in cardiac function and glucose utilization in the streptozotocin (STZ)-induced diabetic rat model using a gene transfer approach. Adenovirus harrowing the human tissue kallikrein gene was administered to rats by intravenous injection at 1 week after STZ treatment. Human kallikrein transgene expression was detected in the serum and urine of STZ-induced diabetic rats after gene transfer. Kallikrein gene delivery significantly reduced blood glucose levels and cardiac glycogen accumulation in STZ-induced diabetic rats. Kallikrein gene transfer also significantly attenuated elevated plasma triglyceride and cholesterol levels, food and water intake, and loss of body weight gain, epididymal fat pad, and gastrocnemius muscle weight in STZ-induced diabetic rats. However, these effects were blocked by icatibant, a kinin B2 receptor antagonist. Cardiac function was significantly improved after kallikrein gene transfer as evidenced by increased cardiac output and \( \pm \Delta P/\Delta t \) (maximum speed of contraction/relaxation), along with elevated cardiac sarco(endo)plasmic reticulum \( (\text{Ca}^{2+} + \text{Mg}^{2+}) \)-ATPase (SERCA)-2a, phosphorylated phospholamban, NOx and cAMP, and GLUT4 translocation into plasma membranes of cardiac and skeletal muscle. Kallikrein gene delivery also increased Akt and glycogen synthase kinase (GSK)-3\( \beta \) phosphorylation, resulting in decreased GSK-3\( \beta \) activity in the heart. These results indicate that kallikrein through kinin formation protects against diabetic cardiomyopathy by improving cardiac function and promoting glucose utilization and lipid metabolism. Diabetes 54:1573–1580, 2005

From the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina.

Address correspondence and reprint requests to Lee Chao, PhD, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425-2211. E-mail: chao@musc.edu.

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RESEARCH DESIGN AND METHODS

Animal treatment. STZ (Sigma, St. Louis, MO) was dissolved in 0.05 mol/l citrate buffer, pH 4.5. Sprague-Dawley rats (male, 8 weeks old; Harlan Sprague-Dawley, Indianapolis, IN) were injected intravenously with 65 mg/kg STZ imme-
diately after dissolving. One week after STZ injection, animals were injected via the tail vein with 1.2 &times; 10^6 plaque-forming units of either adenovirus harboring the human tissue kallikrein tissue gene (Ad-CMV-TK) or control empty virus (Ad.Null). Each group consists of six to seven animals. For physiological parameter measurements, the bradykinin B2 receptor antagonist icatibant was delivered by osmotic minipump (0.05 μg/h) along with injection of Ad-CMV-TK. Cardiovascular function was measured at 14 days after gene delivery, and all rats were then processed for biochemical and morphological analyses. All procedures complied with 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, Bethesda, MD).

Preparation of replication-deficient adenoviral vectors Ad.CMV-TK and Ad.Null. Adenoviral vectors containing the human tissue kallikrein cDNA (Ad-CMV-TK) under the control of the cytomegalovirus enhancer/promoter (CMV) were used as described (10). Adenovirus without a reporter gene (Ad.Null) were constructed and prepared as previously described (16).

Measurements of blood glucose, insulin levels, and physiological parameters. Blood was collected via the tail vein after gene delivery and was then centrifuged to obtain serum. Serum samples were processed for glucose assay on a SYNCHRON LX System (Beckman Coulter) by the Department of Clinical Pathology, Medical University of South Carolina. Blood glucose levels were measured according to the clinical standards established for human insulin-receptor binding and curve of 1–200 ng/ml. Blood insulin levels were determined by radioimmunoassay with a kit according to manufacturer’s instructions (Linco Research, St. Charles, MI). Animals were weighed and placed into separate cages and each supplied with 500 ml water and 30 g rat food at 5 days after gene delivery. Remaining water volume and food weight were measured 24 h later, and the differences were used to calculate water and food consumption. The epididymal fat pad and the gastrocnemius muscles from both the left and right hindlimbs were removed, briefly blotted, and weighed.

Expression of human tissue kallikrein in STZ-induced diabetic rats. Twenty-four–hour urine collection was performed as previously described (17). Expression of recombinant human tissue kallikrein in rat serum and urine after gene delivery was monitored by a specific enzyme-linked immunosorbent assay (18).

Cardiac extract, plasma membrane, and cytosolic-fraction preparation. At the end of the experiment, all rats were pulse-inactivated intraperitoneally with pentobarbital at a dose of 50 mg/kg body wt. Rats were then perfused with normal saline (0.9% NaCl) via the heart. The whole heart and left ventricle were removed, blotted, and weighed. Cardiac extracts and plasma membrane fraction from both heart and skeletal muscles were isolated as previously described (10,19). Briefly, heart or skeletal muscle were minced and homogenized at 4°C in lysis buffer and then centrifuged at 2,000g for 10 min. Total tissue extract in the supernatant was collected and kept on ice. The pellet was resuspended in lysis buffer, rehomogenized for 10 s, and centrifuged for 10 min at 2,000g. Cytosolic fractions in the two supernatants were pooled. The plasma membrane fraction in the pellet was resuspended and loaded onto a 10–30% (wt/wt) continuous sucrose gradient and centrifuged at 100,000g for 1 h. Protein concentrations were determined by Lowry’s method.

Measurements of cardiac cAMP levels. Cardiac sections were done under double-blind conditions. Hearts were immediately excised after sacrificing the animal, and cardiac sections was done under double-blind conditions. Application of sections was done under double-blind conditions.

**Glycogen assay.** Quantitative analysis of cardiac glycogen content was determined as previously described (23). Briefly, 0.1 g cardiac tissue was dissolved in 30% KOH and then heated at 100°C for 10 min. The samples were diluted (1:10) with 30% KOH and precipitated by adding anhydrous ethanol and centrifuging at 5,700 rpm for 15 min. The pellet was resuspended in 0.5 ml 0.1 M 28.5% trichloracetic acid (TCA; 100 ml TCA in 100 ml 80% H2SO4) was added and then heated at 100°C for 10 min. The measurement was made using a Cary 3 UV-Visible Spectrophotometer (620 nm).

**Western blot analysis.** Cardiac extracts (80–100 μg) were subjected to Western blot analyses for SERCA2a, phospholamban, Akt, and GSK-3β, and β-actin and plasma membrane proteins from cardiac and skeletal muscle extracts were immunooblotted for GLUT4 as previously described (10). All blots immunoreacted with a primary antibody overnight at 4°C with dilutions as follows: SERCA2a 1:2,000 (Affinity BioReagents, Golden, CO), phosphor-phospholamban, phospholamban 1:2,000 (Upstate Biotechnology, Lake Placid, NY), GLUT4 1:1,000 (Santa Cruz, Santa Cruz, CA), β-actin 1:2,000 (Sigma), Akt 1:1,000 (Cell Signaling, Beverly, MA), and GSK-3β 1:1,000 (Cell Signaling, Beverly, MA). Chemiluminescence (Western Lightning; Perkin Elmer Life Sciences, Boston, MA) was used to a detect signal following the manufacturer’s instructions.

**GSK-3β activity assay.** GSK-3β activity was measured using phospho-glycogen synthase peptide-2 (Upstate Biotechnology) according to a previously published method (24). Briefly, 10 μl cardiac extract (10 μg) was mixed with 10 μl GSK-3β substrate peptide and 10 μl reaction buffer per assay, followed by 10 μl diluted [γ-32P]ATP (4,000 cpm) per sample. After incubating for 30 min at 37°C, 25 μl was spotted on the center of P81 paper. Assay papers were washed with 0.75% phosphoric acid and then with acetone. The assay papers were dried, and the radioactivity was then counted in a scintillation counter.

**Statistical analysis.** Data are expressed as means ± SE. Comparisons among groups were made by ANOVA followed by Fisher’s protected least-significant difference or by an unpaired Student’s t test. Differences were considered significant at P < 0.05.

**RESULTS.**

Expression of human tissue kallikrein in rats after gene delivery. Using a specific enzyme-linked immunosorbent assay, recombinant human tissue kallikrein levels were measured in the sera and urine of rats after STZ treatment. Immunoreactive human tissue kallikrein levels in rat serum reached a maximum level of 1,000 ± 160 ng/ml (n = 11) at 3 days after gene delivery and reduced to 580 ± 120 ng/ml (n = 11) at 14 days after gene transfer. Also, immunoreactive human tissue kallikrein was detected in the urine of rats receiving Ad.CMV-TK (5.7 ± 0.82 μg • 100 g—1 body wt • 24 h—1, n = 8). Linear displacement curves for immunoreactive kallikrein in rat sera and urine were parallel with the standard curve of human kallikrein, indicating their immunological identity (data not shown). Human tissue kallikrein was not detected in sera or urine of control rats injected with Ad.Null. These results indicate that the anti-human tissue kallikrein antisem did not cross-react with the members of the rat kallikrein gene family.

Improvement in physiological parameters after kallikrein gene delivery. STZ-induced diabetic rats injected with control virus showed stable signs of diabetes, including hyperglycemia, hypoinsulinemia, and increased food and water intake with no increase in body weight. Kallikrein gene delivery improves these physiological parameters. Most importantly, kallikrein reduced elevated blood glucose levels induced by STZ treatment (Table 1). Kallikrein has no effect on blood insulin levels but significantly improved body weight gain, food and water intake, epididymal fat pad, and gastrocnemius muscle weight in STZ-treated rats (data not shown). The improvement of kallikrein on epididymal fat pad weight and muscle weight was abrogated by icatibant, indicating a kinin B2 receptor-mediated event.
Kallikrein gene delivery decreases circulating triglyceride and cholesterol levels. Figure 1 shows the effect of kallikrein gene delivery on serum triglyceride and cholesterol levels 14 days after gene transfer. STZ-induced diabetes resulted in markedly increased triglyceride levels in the circulation, and animals receiving the kallikrein gene had significantly lower triglyceride levels than the Ad.Null group (147.1 ± 91.1 vs. 701.2 ± 247.1 mg/dl, n = 6, P < 0.01), but levels remained significantly elevated compared with control animals (40.7 ± 16.2 mg/dl, n = 5, P < 0.05). Results were similar for cholesterol levels. Diabetic rats after kallikrein gene delivery had significantly lower cholesterol levels than the Ad.Null group (96.7 ± 24.1 vs. 224.4% compared with the Ad.Null group). These results indicate that kallikrein gene transfer reduces hyperlipidemia in STZ-induced diabetes through kinin formation.

Reduced cardiac glycogen content in rats receiving kallikrein gene delivery. Left ventricle tissue was harvested and subjected to PAS staining for morphological and histochemical staining with PAS, suggesting increased cardiac glycogen content after STZ treatment. Quantification of cardiac glycogen levels was determined using a chemical method. Consistent with morphological and histochemical staining with PAS, kallikrein gene delivery significantly reduced cardiac glycogen levels compared with the Ad.Null group (3.8 ± 0.5 vs. 7.1 ± 1.04 mg/g tissue, n = 6, P < 0.05), reducing cardiac glycogen levels similar to those of control animals (2.2 ± 0.23 mg/g tissue, n = 5) (Fig. 2B). These results indicate that kallikrein gene transfer reduces glycogen accumulation in the heart induced by STZ treatment.

Kallikrein gene delivery improves cardiac function. Table 1 shows that kallikrein gene delivery significantly improved cardiac output in animals compared with the Ad.Null group, with cardiac output reaching levels similar to those of the control animals. In diabetic hearts, maximum speed of relaxation (−ΔP/Δt) was reduced by 42.9% and maximum speed of contraction (+ΔP/Δt) was reduced by 39.1%. Kallikrein gene transfer increased myocardial contractility as −ΔP/Δt increased 19.3% and +ΔP/Δt increased 24.4% compared with the Ad.Null group. These results indicate that kallikrein gene transfer can protect diabetic hearts from severe contractile dysfunction. There were no significant differences in systemic blood pressure among control animals and the animals injected with adenovirus containing the human tissue kallikrein gene or with empty virus at 3 weeks after STZ treatment (data not shown).

Kallikrein gene delivery increases phospholamban phosphorylation and SERCA2a levels. Contractile dysfunction in the diabetic state is related to an impaired sarcoplasmic reticulum function, leading to disturbed intracellular calcium handling. To further elucidate the
potential mechanism of kallikrein in the improvement of cardiac contractility, we investigated the effect of kalli-
krlein gene transfer on the sarcoplasmic reticulum calcium pump (SERCA2a) after STZ treatment. Western blot anal-
ysis showed that STZ treatment reduced SERCA2a levels
compared with the control rats, whereas kallikrein gene
transfer significantly increased SERCA2a levels (Fig. 3).
β-Actin levels remained the same among the three groups.
Increased SERCA2a is due to phosphorylation of phospho-
lamban, leading to increased Ca\(^{2+}\) transport. Similar to
SERCA2a, kallikrein gene transfer significantly increased
phosphorylated phospholamban in the left ventricle ex-
tracts compared with the Ad.Null group, whereas no change
was observed in total phospholamban levels (Fig. 3). These
results indicate that kallikrein improves cardiac contractil-
ity in diabetic cardiomyopathy by increased phosphoryla-
tion leading to increased SERCA2a levels, thus improving the calcium sequestration of the sarco-
meric reticulum. Increased phospholamban phosphoryla-
tion was accompanied by increased cAMP and NO levels
after kallikrein gene transfer (Table 1).
**Kallikrein gene delivery increases Akt and GSK-3β phos-
phorylation and reduces GSK-3β activity.** Western blot analysis showed that STZ treatment reduced Akt
phosphorylation compared with the control, whereas kal-
likrein gene transfer increased phospho-Akt (Fig. 4A).
Total Akt levels were not altered among the three groups. Similarly, kallikrein gene delivery significantly increased
the phosphorylated form of GSK-3β compared with the
Ad.Null and control groups. Total GSK-3β levels remained
unaltered. GSK-3β activity assay confirmed the results of
Western blot in that phosphorylation of GSK-3β leads to
inactivation of GSK-3β. Kallikrein gene delivery signifi-
cantly decreased GSK-3β activity compared with the
Ad.Null group (1,768.6 ± 379.6 vs. 3,450.8 ± 702.2 cpm,
n = 5, \(P < 0.05\)) and control animals (2,967.0 ± 301.7 cpm,
n = 4, \(P < 0.05\)) (Fig. 4B). These results indicate that
kallikrein gene transfer resulted in activation of Akt and
thus GSK-3β and that inactivation of GSK-3β by phosphor-
ylation led to decreased GSK activity, resulting in reduced
glycogen accumulation.

**Kallikrein gene delivery increases GLUT4 translo-
cation into plasma membranes.** Western blot analysis
shows that STZ treatment results in reduced GLUT4 levels
in the plasma membranes in both skeletal muscle and
cardiac extracts (Fig. 5A and B, upper panels). Kallikrein
gene transfer increased GLUT4 levels in the plasma mem-
branes in skeletal muscle and cardiac extracts compared
with the STZ-treated Ad.Null group (Fig. 5A and B). Con-
trarily, increased GLUT4 levels were observed in the cytoso-
latic fraction of skeletal muscle and cardiac extracts after
STZ treatment, and kallikrein attenuated GLUT4 levels in
the cytosolic fraction compared with the STZ-treated Ad.Null group. Total GLUT4 levels remained unaltered after STZ treatment with or without kallikrein gene transfer. Quantitative analysis shows that kallikrein gene transfer significantly increased GLUT4 levels in the plasma membrane but reduced GLUT4 in the cytosolic fractions (Fig. 5A and B, lower panels). These results indicated that kallikrein promoted GLUT4 translocation from cytosol to plasma membrane in the heart and skeletal muscles of diabetic rats.

**DISCUSSION**

This is the first study to investigate the potential role of the tissue kallikrein-kinin system in diabetic cardiomyopathy using a viral vector to overexpress tissue kallikrein. Our results show that adenoviral-mediated kallikrein gene delivery decreases blood glucose levels and glycogen accumulation in the heart, increases GLUT4 translocation into the plasma membrane, and improves cardiac function in the STZ diabetic rat model. Examination of signaling events important in cardiac function indicates that the beneficial role of kallikrein/kinin may be mediated by increased phosphorylation of phospholamban and SERCA2a levels. With diabetic patients having such a long history of impaired cardiac function and heart failure related to the chronic mismanagement of glucose levels, these data demonstrate a new role for kallikrein in this debilitating disease.

Increased accumulation of cardiac glycogen in STZ-treated animals is typical in both insulin-dependent and insulin-independent models of diabetes (25). Increased storage of glycogen in the myocardium results when there is a shift in energy substrate utilization, typically from a carbohydrate metabolism to a lipid metabolism (26). This switch in energy source produces an excessive accumulation of glycogen within the myocardium, which may have accelerated glycogen synthesis or an overall impairment in glycogenolysis, or a combination of the two. Our results showed that kallikrein gene transfer markedly reduced STZ-induced glycogen accumulation by nearly 50%, as identified by both PAS staining and quantitative glycogen assay. To study the mechanism of kallikrein in glycogen regulation, we examined the intracellular signal proteins Akt and GSK-3β. The phosphorylated form of Akt increases GSK-3β phosphorylation, leading to decreased GSK-3β activity and thus decreasing the rate of glycogen synthesis (27). Our results showed that kallikrein gene delivery significantly increased both phospho-Akt and phospho-GSK-3β levels and decreased GSK-3β activity in the STZ-induced diabetic rat. This study demonstrates a potential role for the kallikrein-kinin system through activation of Akt and GSK-3β in overcoming the impairment in glycogenolysis and improving the use of myocardial glycogen.

Our results show that kallikrein gene delivery promotes a significant reduction in blood glucose levels independent of insulin levels. To examine the potential mechanisms of this significant drop in blood glucose, we examined the effect of kallikrein gene transfer on the glucose transporter GLUT4 translocation. Kinin has previously been shown to increase GLUT4 translocation in cardiac and skeletal muscles as well as in adipocytes (13–15). Western blot analysis confirmed that kallikrein gene delivery significantly increases GLUT4 translocation into plasma membranes in both skeletal and cardiac muscle in STZ-induced diabetic rats. Increased GLUT4 translocation after kallikrein gene delivery resulted in improved glucose utilization in response to an increased glucose load resulting from the STZ treatment.

In addition to hyperglycemia, diabetic patients also commonly suffer from dyslipidemia, which can lead to increased atherogenesis and incidence of heart disease (28). To determine if kallikrein gene delivery affects lipid metabolism, we examined serum triglyceride and cholesterol levels. STZ treatment resulted in markedly elevated serum triglyceride and cholesterol levels compared with nondia-
betic control animals, but both were reduced to those of control animals after kallikrein gene delivery. It is of interest to note that use of the kinin B2 receptor antagonist icatibant abrogated the reductions in triglyceride and cholesterol levels in animals receiving kallikrein gene transfer, indicating that kinin receptors have an essential role in mediating the lipid-lowering effect in STZ-induced diabetic rats. Insulin action has a significant role in lipid biosynthesis and regulation by inhibiting VLDL production in the liver and the clearance and breakdown of LDLs in circulation (29,30). Because no change occurred in insulin levels in the diabetic rats after kallikrein gene delivery, it is possible that kallikrein/kinin is promoting an insulin-like effect, similar to GLUT4 translocation, on the management of serum lipid profiles. Currently, the mechanism of action for kallikrein in lipid production and management is unknown and must be further explored.

Along with decreased glucose and lipid levels, water and food intake was also significantly decreased in animals receiving kallikrein gene delivery. It has been previously observed that animals treated with STZ without insulin have increased food and water intake (31). The potential effect of kallikrein gene delivery on reduction of water and food consumption could be attributed to better glucose management through increased GLUT4 translocation into plasma membrane, thus resulting in reduction of blood glucose levels. These results indicate that kallikrein gene expression in the heart can reduce the impact of diabetes on the development of cardiomyopathy by reducing glycogen accumulation and hyperlipidemia through increased glucose utilization.

Diabetic cardiomyopathy is a well-characterized pathological condition that develops throughout the life of the diabetic patient. The underlying dysfunction of the diabetic heart can be linked to two important proteins: phospholamban and SERCA2a. A previous report has
noted that a decrease in protein content of phospho-phospholamban and SERCA2a results in a significant reduction in heart function (32). Increased phospho-phospholamban and SERCA2a levels were observed after kallikrein gene delivery, indicating a beneficial role of kallikrein in cardiac function. Kallikrein, through kinin formation, triggers activation of second messengers, such as cAMP and NO/cGMP. Increased cAMP contributes to the phosphorylation of phospholamban by binding to and activating protein kinase A. Phosphorylation of phospholamban results in a significant reduction of cardiac cAMP levels and can improve cardiac output and $\Delta P/\Delta t$ through the regulation of phospholamban and SERCA2a.

The present study demonstrates that adenovirus-mediated delivery of human tissue kallikrein leads to significant improvements in cardiac function, glucose utilization, and lipid metabolism in the STZ model of diabetes. Kallikrein/kinin, through second messengers cAMP and NO/cGMP, protects diabetic hearts from severe contractile dysfunction through activation of the Akt-GSK-3β signaling pathway and increased GLUT4 translocation.

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