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

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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 harboring 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 (Ca^{2+} + Mg^{2+})-ATPase (SERCA)-2a, phosphorylated phospholamban, NOx and cAMP levels, and GLUT4 translocation into plasma membranes of cardiac and skeletal muscle. Kallikrein gene delivery also increased Akt and glycogen synthase kinase (GSK)-3β phosphorylation, resulting in decreased GSK-3β 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**

Tissue kallikrein specifically processes low–molecular weight kininogen to produce potent vasoactive kinin peptides (1). Intact kinin binds to the bradykinin B2 receptor and transduces signals through nitric oxide (NO)-cGMP and prostacyclin-cAMP pathways, thereby modulating a broad spectrum of cellular functions (2). The B2 receptor can be blocked by the specific B2 receptor antagonist icatibant (also known as HOE140) (3). Previous reports have shown that the kallikrein-kinin system (KKS) components are locally expressed in the heart (4), and streptozotocin (STZ)-induced diabetes results in a decrease of active cardiac tissue kallikrein levels (5,6), resulting in increased thickness of the left ventricle wall and cardiac hypertrophy (7). The STZ animal model develops characteristic symptoms of diabetes such as hyperglycemia, hyperlipidemia, and increased water and food intake without body weight gain. In addition, STZ diabetes also induces key symptoms including increased glycogen storage in the myocardium, depressed ventricular performance, and cardiac hypertrophy (8). Our recent studies using gene transfer approaches have demonstrated that the KKS improves cardiac function in animal models of myocardial ischemia, chronic heart failure, and cardiac hypertrophy (9–11). In addition, transgenic rats overexpressing the human tissue kallikrein gene resulted in reduction of isoproterenol-induced cardiac hypertrophy and fibrosis, and these protective effects were abolished by icatibant (12). These findings indicate a potential protective role of the KKS in diabetic cardiomyopathy.

STZ-induced diabetes results in hyperglycemia and hyperlipidemia, and without insulin treatment, animals have poor control over glucose and circulating lipid levels. Previous studies have shown that the KKS is involved in glucose management by stimulating GLUT4 translocation (13), improving insulin stimulation of GLUT4 (14), and preventing dephosphorylation of insulin receptor substrate-1 (15). Whether the KKS plays a role in improving glucose utilization and lipid metabolism in STZ-induced diabetes has not been explored.

In this study, we used a gene transfer approach to determine the role and potential mechanisms of the KKS in diabetic cardiomyopathy, as well as glucose and lipid metabolism. We showed that kallikrein gene transfer improves myocardial contractility, reduced glycogen accumulation, and hyperlipidemia through increased phospholamban phosphorylation and sarco(endo)plasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase (SERCA)-2a levels, GLUT4 translocation, and activation of the Akt–glycogen synthase kinase (GSK) signaling pathway in STZ-induced diabetic rats.

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-

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$+\Delta P/\Delta t$, maximum speed of contraction; $-\Delta P/\Delta t$, maximum speed of relaxation; GSK, glycogen synthase kinase; KKS, kallikrein-kinin system; PAS, periodic acid Schiff; SERCA, sarco(endo)plasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase; STZ, streptozotocin.

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diately after dissolving. One week after STZ injection, animals were injected via the tail vein with 1.2×10^{10} 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 (0.65 μg/h) along with injection of Ad.CMV-TK. Cardiac function was evaluated at 14 days after gene delivery, and tissues 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) and control 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 with a linear standard curve of 1–1,200 mg/dl. 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 anesthetized 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 2,000*g* for 10 min. Total tissue extracts 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 2,000*g*. 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 190,000*g* for 1 h. Protein concentrations were determined by Lowry's method.

Measurements of NO content and cAMP levels. Nitrate and nitrite (NOx) levels in cardiac extracts were measured by a fluorimetric assay as previously described (20). Radioimmunoassay of cardiac cAMP levels was conducted according to previously described procedures (21).

Measurements for serum triglyceride and cholesterol levels. Circulating triglycerides and cholesterol levels in rats were measured using a diagnostic kit following the manufacturer's protocol (INFINITY Triglycerides Reagent TR22421, INFINITY Cholesterol Reagent TR13421, Triglyceride Standard TR22923, Cholesterol Standard TR13923; Thermo Electron, Woburn, MA). Briefly, serum samples were diluted 1:100 in either triglyceride or cholesterol reagent and incubated for 15 min, and 0.2 ml of the reaction mixture was used to measure at 500 nm on a Molecular Devices E^{max} plate reader.

Cardiac function. Cardiac function was performed as previously described (22). Briefly, animals were anesthetized with pentobarbital sodium (50 mg/kg body wt). The femoral and carotid arteries were cannulated. Heart rate and arterial blood pressure were recorded. Fluorescent microspheres (FluoSpheres; Molecular Probes, Eugene, OR) were injected directly into the left ventricle, whereas arterial blood was collected for a total of 90 s from the femoral artery. The collected blood and one kidney were subjected to digestion to release the microspheres, which were then quantitated in a spectrofluorometer, with excitation at 570 nm and emission at 598 nm.

Morphological analysis. Heart tissue sections embedded in paraffin were cut at 4 μm and stained with the periodic acid Schiff (PAS) reagent and then analyzed microscopically and morphometrically. Adobe Photoshop 5.5 (Adobe) was used for imaging and preparation of photomicrographs. Evaluation 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 H_2O, and 1 ml 0.2% anthrone reagent (0.2 g in 100 ml 98% H_2SO_4) 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 immunoblotted 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), phospho-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-^{32}P]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 $\mu g \cdot 100\ g^{-1}$ body wt $\cdot\ 24\ h^{-1}$, $n = 8$). Linear displacement curves for immunoreactive kallikrein in rat sera and urine were parallel with the standard curve of human tissue 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 antiserum 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.

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TABLE 1
Effects of tissue kallikrein gene delivery on physiological parameters

	Control	STZ-Ad.Null	STZ-Ad.CMV-TK
Blood glucose (mg/dl)	101.3 ± 8.3	575.8 ± 28.3*	450.8 ± 29.9*†
Cardiac output (ml/min)	100 ± 8.0	75 ± 5.0*	102 ± 6.0*†
$+\Delta P/\Delta t$ (mmHg/s)	7,512.3 ± 392.4	4,518.8 ± 810.9*	6,054.2 ± 487.6*†
$-\Delta P/\Delta t$ (mmHg/s)	6,816.4 ± 490.1	3,919.6 ± 837.1*	4,856.3 ± 667.1*†
NO (pmol/mg protein)	152.1 ± 31.6	214.2 ± 12.0*	371.5 ± 51.8*†
cAMP (nmol/mg protein)	2.3 ± 1.7	2.2 ± 1.5	6.3 ± 1.3*†

Data are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. STZ-Ad.Null.

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 ± 16.5 vs. 137.4 ± 10.3 mg/dl, $n = 6$, $P < 0.05$), and levels remained elevated compared with control animals (82.4 ± 18.1 mg/dl, $n = 5$, $P < 0.05$). Co-administration of icatibant, a kinin B2 receptor antagonist with the kallikrein gene, significantly abrogated the effect of kallikrein on increased cholesterol levels and partially blocked the effect of kallikrein on triglyceride levels. These results indicate that kallikrein significantly reduced 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 evaluation and glycogen accumulation. PAS stained carbohydrates a bright red/purple and was clearly visible within the cardiomyocytes. Significant differences were apparent between PAS-stained tissues from animals receiving kallikrein gene delivery and the Ad.Null group after STZ injection (Fig. 2*A*). The cardiac tissue of the Ad.Null group stained a more intense red than the kallikrein group, 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 ± 0.85 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. 2*B*). 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 ($-\Delta P/\Delta t$) was reduced by 42.9% and maximum speed of contraction ($+\Delta P/\Delta t$) was reduced by 39.1%. Kallikrein gene transfer increased myocardial contractility as $-\Delta P/\Delta t$ increased 19.3% and $+\Delta P/\Delta t$ 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

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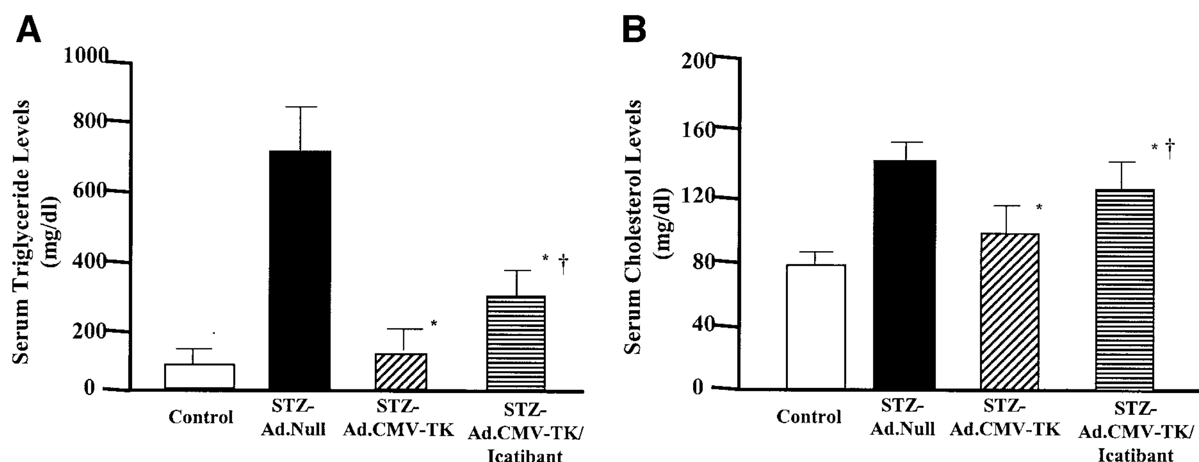

FIG. 1. Effect of kallikrein gene delivery on serum triglyceride and cholesterol levels in STZ-induced diabetic rats. *A*: Triglyceride levels. *B*: Cholesterol levels. Results are expressed as means ± SE (n = 5 or 6). *P < 0.01 vs. STZ-Ad.Null group; †P < 0.01 vs. Ad.CMV-TK.

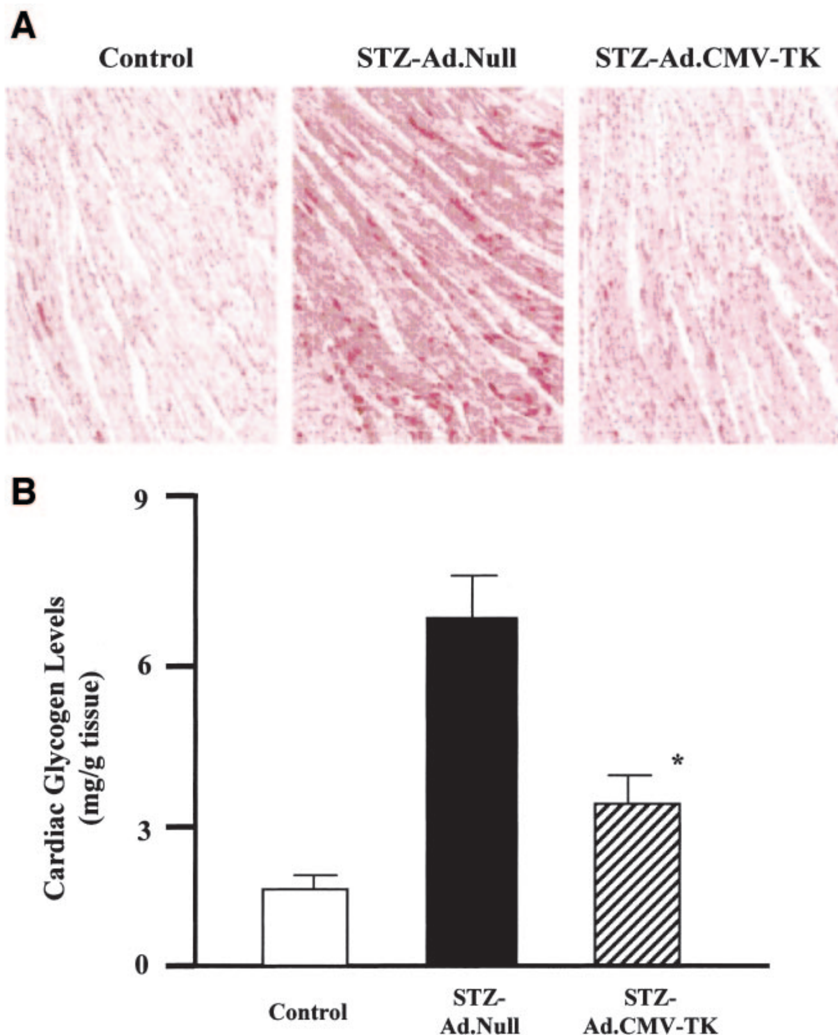

FIG. 2. Effect of kallikrein gene delivery on cardiac glycogen levels. *A*: Glycogen staining in cross-section of left ventricle identified by PAS staining (100× magnification). *B*: Cardiac glycogen levels determined by the chemical assay. Results are expressed as means ± SE (n = 6). **P* < 0.01 vs. STZ-Ad.Null.

potential mechanism of kallikrein in the improvement of cardiac contractility, we investigated the effect of kallikrein gene transfer on the sarcoplasmic reticulum calcium pump (SERCA2a) after STZ treatment. Western blot analysis 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 phospholamban, leading to increased Ca^{2+} transport. Similar to SERCA2a, kallikrein gene transfer significantly increased phosphorylated phospholamban in the left ventricle extracts compared with the Ad.Null group, whereas no change was observed in total phospholamban levels (Fig. 3). These results indicate that kallikrein improves cardiac contractility in diabetic cardiomyopathy by increased phospholamban phosphorylation leading to increased SERCA2a levels, thus improving the calcium sequestration of the sarcomeric reticulum. Increased phospholamban phosphorylation was accompanied by increased cAMP and NO levels after kallikrein gene transfer (Table 1).

Kallikrein gene delivery increases Akt and GSK-3β phosphorylation and reduces GSK-3β activity. Western blot analysis showed that STZ treatment reduced Akt phosphorylation compared with the control, whereas kallikrein gene transfer increased phospho-Akt (Fig. 4*A*). 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 significantly 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. 4*B*). These results indicate that kallikrein gene transfer resulted in activation of Akt and thus GSK-3β and that inactivation of GSK-3β by phosphorylation led to decreased GSK activity, resulting in reduced glycogen accumulation.

Kallikrein gene delivery increases GLUT4 translocation 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. 5*A* and *B*, upper panels). Kallikrein gene transfer increased GLUT4 levels in the plasma membranes in skeletal muscle and cardiac extracts compared with the STZ-treated Ad.Null group (Fig. 5*A* and *B*). Contrarily, increased GLUT4 levels were observed in the cytosolic fraction of skeletal muscle and cardiac extracts after STZ treatment, and kallikrein attenuated GLUT4 levels in

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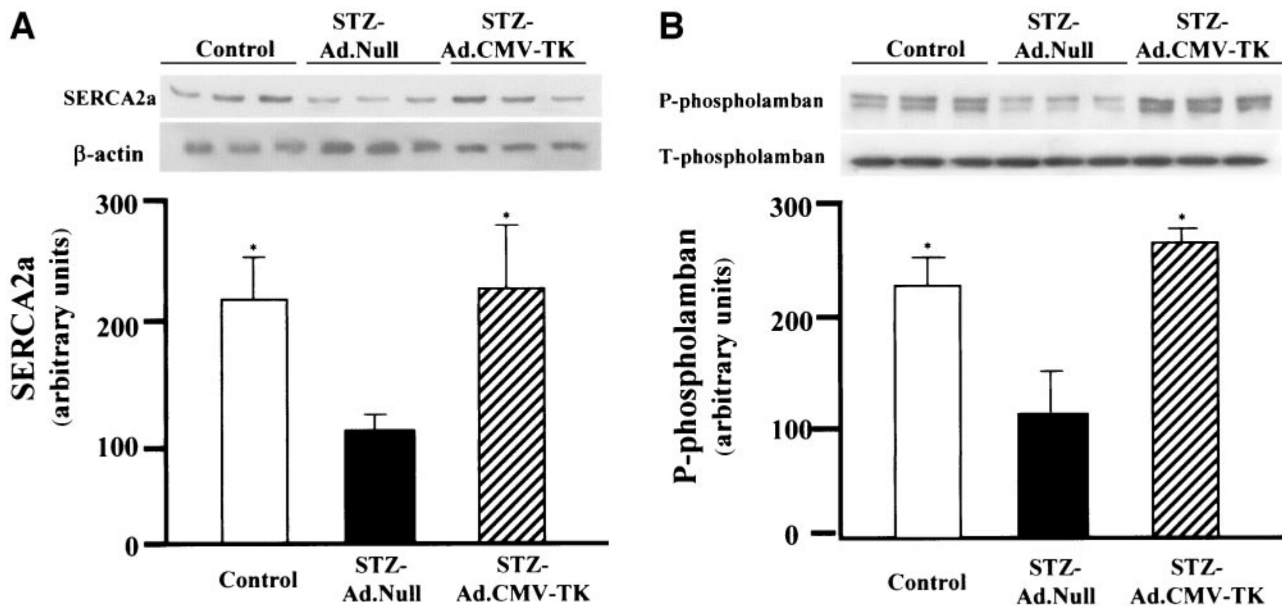

FIG. 3. Effect of kallikrein gene delivery on phospholamban and SERCA2a phosphorylation. *Upper panels*: Western blot analysis. *Lower panels*: Quantitative analysis. *A*: Kallikrein gene delivery increased SERCA2a phosphorylation but had no effect on β-actin levels. *B*: Kallikrein gene delivery increases phosphorylated phospholamban levels. Results are expressed as means ± SE ($n = 6$). *$P < 0.01$ vs. STZ-Ad.Null.

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. 5*A* 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-

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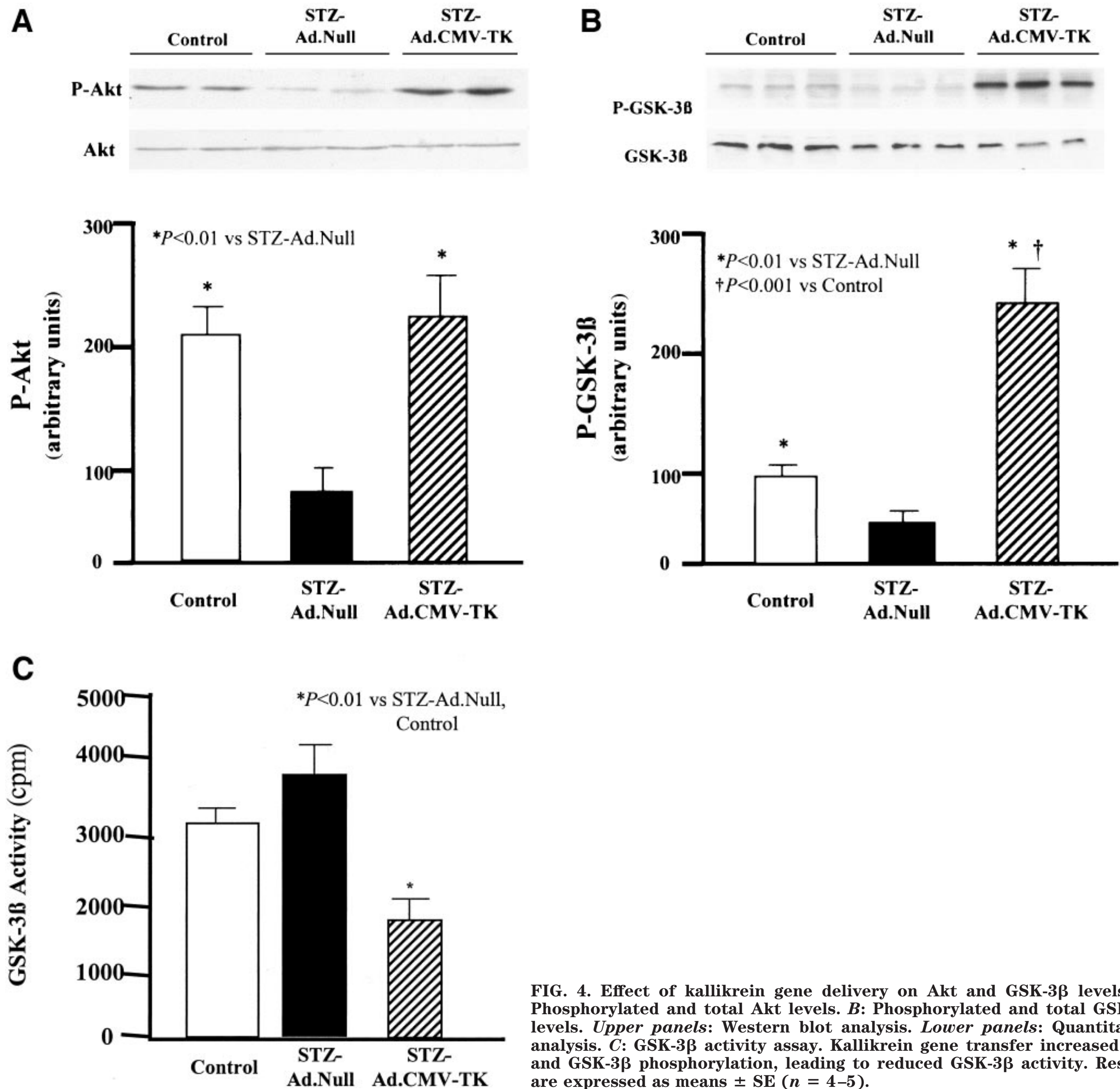

FIG. 4. Effect of kallikrein gene delivery on Akt and GSK-3β levels. *A*: Phosphorylated and total Akt levels. *B*: Phosphorylated and total GSK-3β levels. *Upper panels*: Western blot analysis. *Lower panels*: Quantitative analysis. *C*: GSK-3β activity assay. Kallikrein gene transfer increased Akt and GSK-3β phosphorylation, leading to reduced GSK-3β activity. Results are expressed as means ± SE (n = 4–5).

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 pathophysiological 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

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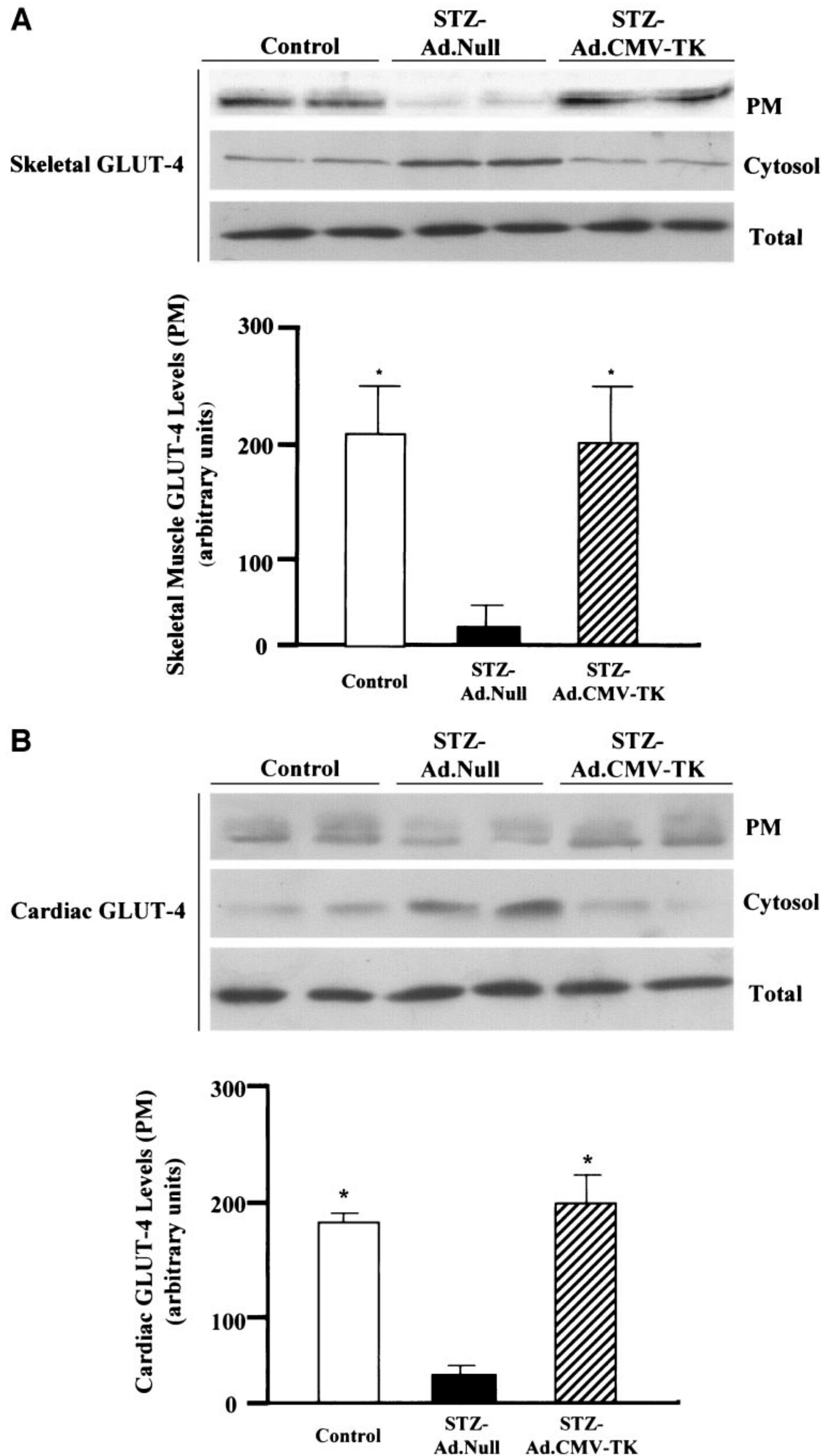

FIG. 5. Effect of kallikrein gene delivery on GLUT4 translocation into plasma membrane. *Upper panels*: Western blot analysis. *Lower panels*: Quantitative analysis. *A*: Skeletal muscle. *B*: Cardiac tissues. PM, plasma membrane. Results are expressed as means ± SE (n = 4–5). *P < 0.01 vs. STZ-Ad.Null.

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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 leads to the dissociation of the phospholamban pentameric structure and results in the release of free SERCA2a, thus increasing affinity for Ca^{2+}. Therefore, a significant influx of Ca^{2+} transients into cardiomyocytes, via SERCA2a, leads to an increased relaxation process, which is normally depressed in this animal model (33). The expression of human tissue kallikrein mRNA in the heart after intravenous injection of Ad.CMV-TK was detected by RT-PCR followed by Southern blot analysis, whereas human kallikrein mRNA was not detected in rats injected with Ad.Null, as published in several of our previous studies (17,26). In this study, our results showed that expression of recombinant kallikrein in the diabetic heart increases cardiac cAMP levels and can improve cardiac output and $\pm\Delta P/\Delta t$ 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 though increased phospho-phospholamban and SERCA2a levels, inhibits glycogen accumulation, and improves glucose utilization and lipid metabolism through activation of the Akt-GSK-3β signaling pathway and increased GLUT4 translocation.

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