Hepatic insulin gene therapy prevents deterioration of vascular function and improves adipocytokine profile in STZ-diabetic rats

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MORE THAN 18 MILLION PEOPLE IN THE US, ~6.3% of the population, suffer from diabetes mellitus (47). The number of Americans with diabetes is predicted to increase as our population ages and becomes more sedentary and obese. In addition, there has been a global increase in the incidence of childhood diabetes (1). Consequently, long-term diabetic complications, including vascular disease, will increasingly affect people at younger ages. Vascular complications account for up to 80% of diabetes-related deaths (5, 18). Perhaps the earliest indicator of vascular disease is abnormal endothelial function (30, 53).

Multiple aspects of the diabetic state combine to negatively impact endothelial health, including hyperglycemia, lipid abnormalities, and hypertension (13, 26, 57), and both thought leaders and clinical studies suggest that a multifaceted clinical approach directed toward risk factor modification is beneficial (17, 41). Yet the need for polypharmacy and the inability of most patients to attain good glycemic control using available therapies limits compliance with such recommendations (23, 46, 54, 66).

Gene therapy approaches may eventually provide a means to improve and simplify treatment of diabetes mellitus. Hepatic insulin gene therapy (HIGT) that couples insulin production to metabolic requirements can ameliorate hyperglycemia in multiple rodent models of diabetes (35, 48, 62). However, blood sugars, hormones, and lipids do not completely normalize in HIGT-treated animals (48, 62). Consequently, whether the milieu produced by HIGT is beneficial, innocuous, or even damaging with respect to vascular function remains unclear.

To evaluate the impact of HIGT on the vasculature, we examined functional and metabolic indicators of vascular health in streptozotocin (STZ)-treated diabetic rats. Endothelium-dependent vasodilation of aortic rings, nitrosyl-hemoglobin content (NO-Hb), and insulin sensitivity were studied using aortic ring preparations, electron spin resonance spectroscopy (ESR), homeostasis assessment of insulin resistance (HOMA-IR) calculations, and insulin tolerance testing (ITT). Data were correlated with selected hormone and adipocytokine concentrations.

Production of adenoviral vector. The (GIRE)3BP1–2xfur transgene was constructed by combining a glucose- and insulin-responsive, liver-specific promoter consisting of an inverted, head-to-tail, trimer of the compound rat liver pyruvate kinase (L-PK) glucose-responsive element [(GlRE)3] inserted at bp 111 of the basal rat insulin-like growth factor-binding protein-1 (IGFBP-1) promoter (bp −111 to +96) with a human proinsulin gene modified to permit posttranslational processing in non-β-cells (2xfur, gift of Genentech, South San Francisco, CA) (62). The (GIRE)3BP1–2xfur sequence was used to produce infectious adenovirus Ad/(GIRE)3BP1–2xfur, using the Adeno-Quest kit per the manufacturer’s instructions (Quantum Clontech, San Francisco, CA). Adenovirus preparations were determined to be free of contaminating endotoxin (10 pg/mL).

MATERIALS AND METHODS
Biotecnomies, Montreal, QC, Canada), as previously described (61). Synthetic capacity of the transgene was verified by human insulin-specific enzyme-linked immunosorben assay (ELISA) (Merckodia, Uppsala, Sweden) of medium conditioned by primary cultured hepatocytes infected with crude lysates of expanded viral plaques. After threefold plaque purification, viral preparations were prepared by double CsCl density gradient centrifugation, dialyzed in a 4% sucrose buffer containing 10 mM Tris and 2 mM MgCl₂, aliquoted, and stored at −70°C prior to use. Viral concentrations were determined by an adaptation of the tissue culture infectious-dose method (TCID₅₀) (43).

**Animals.** Male Sprague-Dawley rats (150–175 g, Charles River, Wilmington, MA) used for all studies were housed singularly in Plexiglas shoe-box hanging cages, with alternating 12:12-h light-dark cycles, and free access to chow and water. All studies were approved by and conformed to the stipulations of the Atlanta Veterans Affairs/Emory University Institutional Animal Care and Use Committee. Diabetes, determined by two successive daily blood glucose values of >200 mg/dl, was induced by intravenous injection of freshly prepared STZ (Ferro Pfanstiehl Laboratories, Waukegan, IL) dissolved in 0.2N HCl (pH 4.0). Body weights and random blood glucose values, determined by hand-held glucose meter on tail blood (One-Touch test strips, a kind gift of Theodore Clark, LifeScan, Johnson & Johnson) were obtained 3–5 times each week. Animals routinely developed hyperglycemia within 2 days and were treated with daily subcutaneous protamine zinc insulin (PZI, U-40 PZI bovine insulin; Johnson) were obtained 3–5 times each week. Animals were euthanized by clotting whole blood in a microisolator (One-Touch test strips, a kind gift of Theodore Clark, LifeScan, Johnson & Johnson) were obtained 3–5 times each week. Animals routinely developed hyperglycemia within 2 days and were treated with daily subcutaneous protamine zinc insulin (PZI, U-40 PZI bovine insulin; BlueRidge Pharmaceuticals, Greensborough, NC) to induce near weight loss and severe ketosis as assessed by ketonuria (±5 on urine test strips; Ketostix, Boehringer Mannheim). Three to four days after receiving STZ (100 mg/kg), hyperglycemic animals received sustained-release subcutaneous nuchal implants of bovine insulin (one-to-two-thirds pellet of Linplant; Linshin Canada, Scarborough, ON, Canada) sufficient to maintain body weight while sustaining hyperglycemia. At similar times, following induction of deep anesthesia with 5% isoflurane-O₂, diabetic rats in the HIGT group received jugular venous injections of Ad/(GlRE)₃BP1-2xfur (2 × 10⁷ pfu/kg). This adenoviral dosage was previously determined to induce near euglycemia in two models of type 1 diabetes in rats (48, 62). Subcutaneous insulin injections in HIGT rats were tapered as blood sugars normalized and then discontinued.

**HOMA-IR calculation and ITT.** HOMA-IR was calculated as blood glucose (mM) × insulin concentration (mU/l)/R, utilizing serum obtained at the time the animals were euthanized (36) (27). R = 211.7 was determined empirically as the divisor producing an average HOMA-IR of 1 in controls, analogous to the linearizing assumptions applied in the development of HOMA in humans and assuming that steady state had been achieved in all groups at the time of blood sampling (42). Insulin tolerance testing (ITT) was performed on animals fasted for 15 h by administering 0.75 U/kg recombinant human insulin (Novolin; Novo Nordisk, Princeton, NJ) via intraperitoneal injection, and determining glucose on tail blood via hand-held blood glucose monitor (OneTouch, Johnson & Johnson) for the subsequent 90 min (74).

**Serum assays.** Serum was collected from all animals at the time the animals were euthanized by clotting whole blood in a microisolator tube at room temperature for 10 min (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ) and centrifuging in a tabletop microcentrifuge at 10,000 rpm for 10 min. Serum was immediately aliquoted and stored at −70°C until assayed. Total cholesterol, nonesterified free fatty acids (NEFA), and triglycerides (TG) were assayed using commercially available kits per the manufacturer’s instructions (Waco Chemicals, Richmond, VA). Human insulin was assayed using the ultra-sensitive human insulin ELISA (Mercodia, Uppsala, Sweden). Bovine insulin and rat insulin were measured using a standard dual-antibody ELISA (both Mercodia, Uppsala, Sweden). Glucagon and leptin concentrations were determined using an RIA (Linco, St. Charles, MO). Rat adiponectin was measured with a dual-antibody ELISA (Linco).

**Aorta contractility and relaxation.** Aorta contractile and relaxation properties were measured as described previously (60). Briefly, aortas were excised and maintained in PSS (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 0.025 mM EDTA, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 11 mM glucose, 25 mM NaHCO₃, pH 7.4, in 95% O₂-5% CO₂ at 37°C). Loose fat and connective tissue were removed, and 5-mm aorta rings were isometrically mounted onto a Harvard Apparatus differential capacitor force transducer (Holliston, MA). Rounding tension on each aortic ring was set to 40 mN, and this tension was maintained throughout the experiment. For experiments using denuded aortas, the endothelium was removed by rubbing the aorta between the thumb and index finger. Removing the endothelium in this manner does not affect force development in response to either potassium chloride (KCl) or L-phenylephrine. Relaxation responses to acetylcholine (1 nM to 10 μM) and the NO donor sodium nitroprusside (SNP, 0.1 mM to 1 μM) were determined in aortas precontracted with 300 nM phenylephrine, a concentration that yields ~80% of maximal contraction. Data were obtained and analyzed using a Powerlab system (ADInstruments, Colorado Springs, CO).

**Western analysis.** Immediately after the animals were euthanized, aortas were isolated and cleaned of any blood or periadventitial fat. The arteries were ground using a Pro 200 homogenizer (Pro Scientific, Oxford, CT) in lysis buffer (20 mM Tris pH 7.4, 2.5 mM EDTA, 100 mM NaCl, 10 mM NaF, 1 mM NaVO₄, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate, 1 tablet/10 ml EDTA-free Complete protease inhibitor cocktail, 1 mM β-glycerophosphate, 2.5 mM Na pyrophosphate; Roche Diagnostics Indianapolis, IN) followed by sonication (10 × 2 s burst at low power). The lysate was spun at 28,000 g for 15 min, and the supernatants were then transferred to new tubes. Protein concentrations were determined using a bichinchoninic acid system (Pierce, Rockford, IL). Equal amounts of protein (50 μg/ lane) were loaded into each well of a 4–12% bis-tris PAGE. Proteins were separated by electrophoresis and blotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated overnight at 4°C with antibodies (1:1,000 to endothelial NO synthase (eNOS); BD Biosciences, San Jose, CA) or actin (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized using a peroxidase-coupled anti-mouse IgG in the presence of LumiGlo reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD) with a Chemidoc XRS/HQ (Biorad, Hercules, CA). Densitometric analysis was accomplished using Bio-Rad Quantity One (version 4.5.0) software.

**Measurement of NO-Hb.** At the time the animals were euthanized, blood (1 ml) was collected via cardiac puncture using a heparinized 1-ml syringe. After centrifugation (2,000 g for 10 min at 4°C), red blood cells were resuspended in a volume of gaseous nitrogen-saturated phosphate-buffered saline equivalent to the aspirated plasma volume and snap-frozen in liquid nitrogen. Samples were subjected to electron spin resonance (ESR) spectroscopy, essentially as described (34), using an EMX ESR spectrometer (Bruker, Karlsruhe, Germany) with a superhigh Q microwave cavity. The ESR settings for detection of NO-Hb were: field sweep, 300 G; microwave frequency, 9.78 GHz; microwave power, 10 mW; modulation amplitude, 3 G; conversion time, 2,624 ms; time constant, 5,248 ms; receiver gain, 1 × 10⁵.

**Statistical analysis.** One-way or two-way ANOVA was used to determine significance assuming an a priori value of P < 0.05. Intergroup comparisons were made using the Newman–Keuls posttest unless otherwise specified. All analyses were performed using GraphPad Prism v. 3 (GraphPad Software, San Diego, CA) unless otherwise specified. Variability was determined using an F-test statistic in Microsoft Excel 2002.

**RESULTS**

HIGT controls hyperglycemia and normalizes the growth curve of STZ-diabetic rats. Diabetic animals were treated with either a single intravenous injection of adenovirus carrying a metabolically responsive insulin transgene (n = 14)
HIGT AND ENDOTHELIAL DYSFUNCTION

Fig. 1. Hepatic insulin gene therapy (HIGT) produces near-normal glycemia and maintains normal growth in streptozotocin (STZ)-diabetic rats. A: random blood glucose of control, HIGT, and hyperglycemic (Hypergly) rats. Values are means ± SE; n = 3–14 for each data point. B: average random blood glucose from day 7 through the time the animals were euthanized for control, HIGT, and hyperglycemic rats. Line, mean; Box, 95% confidence interval; error bars, range. C: body weight of control, HIGT, and hyperglycemic rats. Line, mean; Box, 95% confidence interval; error bars, range.

[Ad/[GIRE]3BP1-2xfur, 2 × 10^{10} pfu/kg] or subcutaneous implantation of continuous-release insulin pellets (n = 14), dosed to maintain body weight in the face of persistent hyperglycemia (blood glucose >200 mg/dl). Nondiabetic rats of similar weight served as controls (n = 10). Observation periods were similar for the control group, and for the hyperglycemic and HIGT groups following the onset of hyperglycemia (41 vs. 38.9 vs. 36.9 days, for control, hyperglycemic, and HIGT, respectively, P > 0.05).

Due to limited dosing of exogenous insulin, random blood glucose remained elevated among the hyperglycemic rats (>200 mg/dl; Fig. 1A). In contrast, blood glucose values in the HIGT-treated animals declined within 4 days of viral administration and remained less than in the hyperglycemic rats for the duration of the study. Average random blood glucose in HIGT-treated animals from 5 days postviral administration until the end of the study was similar to that in controls and less than in hyperglycemic animals (P > 0.05 HIGT vs. controls, P < 0.001 HIGT vs. hyperglycemic; Fig. 1B). However, blood glucose values for HIGT rats failed to completely normalize. The normal range of blood glucose was arbitrarily defined as 52–105 mg/dl, a range derived from the mean ± 2SD of control values (mean = 78.34, range = 51–111 mg/dl, SD = 13.11 control; Fig. 1B). HIGT blood glucose averaged within the normal range (mean = 101.03, range = 21–401 mg/dl, SD = 67.16 HIGT). However, variability was greater in HIGT animals than in control animals (F-test, P < 0.001).

Body weight of all rats increased throughout the study period. HIGT rats grew at a rate similar to controls. The slope of a linear regression (HIGT slope = 5.18 g/day, r^2 = 0.93) applied to HIGT body weights was similar to that of nondiabetic controls (control slope = 4.52 g/day, r^2 = 0.99, P > 0.05). Body weights of the hyperglycemic animals increased less rapidly and were less uniform. A linear regression derived from body weights of hyperglycemic animals varied significantly from those of HIGT and controls (hyperglycemic slope = 1.71 g/day, r^2 = 0.44, P < 0.05 vs. HIGT and control; Fig. 1C) (75).

Serum levels of NEFA, TG, and total cholesterol. To evaluate the potential role of serum lipid derangements on vascular function in control, hyperglycemic, and HIGT animals, serum NEFA, TG, and total cholesterol were measured. NEFA concentrations were similar across groups (Table 1). Although average levels of TG tended to be elevated among hyperglycemic animals, this difference was not significant (Table 1). In contrast, total cholesterol values were minimally, but significantly, diminished in hyperglycemic animals compared with both control and HIGT rats (Table 1). Total cholesterol, TG, and NEFA values among HIGT were not different from controls (P > 0.05).

Serum insulin and glucagon levels. To verify the efficacy of STZ treatment, serum concentrations of rat insulin were measured in all groups. Consistent with successful β-cell ablation, levels of rat insulin were diminished in both STZ-treated groups compared with levels in control animals (Table 1). The greater values for rat insulin in the hyperglycemic compared with the HIGT animals is attributed to cross-reactivity of bovine insulin from the subcutaneous insulin implants in the rat insulin assay (Thulé PM, personal observation and Ref. 55), and to the greater stimulation of endogenous insulin secretion from remnant β-cells in the hyperglycemic rats. To assess functional insulin concentrations, levels of rat insulin in controls were compared with bovine insulin levels in hyperglycemic rats, and human insulin levels in HIGT animals. The subcutaneous pellets in hyperglycemic rats produced bovine

Table 1. Lipids, insulin, and glucagon

<table>
<thead>
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<th>Control</th>
<th>Hyperglycemic</th>
<th>HIGT</th>
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<tbody>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>71.4±3.4</td>
<td>56.9±3.5†</td>
<td>69.0±4.5</td>
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<tr>
<td>Triglycerides, mg/dl</td>
<td>89.5±12.7</td>
<td>171.6±47.4</td>
<td>91.3±12.2</td>
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<tr>
<td>NEFA, meq/l</td>
<td>0.31±0.05</td>
<td>0.25±0.04</td>
<td>0.28±0.02</td>
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<tr>
<td>Insulin, pmol</td>
<td>490.7±123.5†</td>
<td>147.6±45.2‡</td>
<td>46.0±21.0*‡</td>
</tr>
<tr>
<td>Rat insulin, pmol</td>
<td>490.7±123.5†</td>
<td>252.0±84.4†</td>
<td>23.0±2.4*</td>
</tr>
<tr>
<td>Glucagon, pg/ml</td>
<td>86.0±6.3</td>
<td>201.6±30.5*</td>
<td>163.5±23.4*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. HIGT, hepatic insulin gene therapy. Total cholesterol, triglycerides, nonesterified fatty acid (NEFA), insulin, rat insulin, and glucagon were measured in sera obtained at time animals were euthanized; n = 10–14 for each value; †rat, ‡bovine, †human insulin assay. *P < 0.05 vs. control; †P < 0.05 vs. control and HIGT.
insulin concentrations that were lower than levels of rat insulin in controls (Table 1). Human insulin concentrations in HIGT were also less than rat insulin among controls and less than bovine insulin in hyperglycemic rats (Table 1).

Insulin inhibits glucagon secretion from islet α-cells (37). Consistent with models describing intra-islet blood flow carrying endogenous insulin from the central β-cell mass outward toward peripherally oriented α-cells, the lowest glucagon values were observed in control rats (Table 1 and Ref. 44). In both hyperglycemic and HIGT animals, glucagon levels were elevated (P < 0.05 vs. controls), consistent with a peripheral insulin effect insufficient to inhibit α-cell secretion.

HIGT preserves endothelium-dependent vascular relaxation. To determine whether the metabolic milieu induced by HIGT treatment is sufficient to inhibit the development of diabetes-associated vascular dysfunction, endothelium-dependent relaxation was evaluated in freshly prepared aortic rings from control, hyperglycemic, and HIGT animals. Phenylephrine-induced ring contraction was not different in aortic preparations from each of the three groups (data not shown). Similarly, aortic rings from all groups responded to low and intermediate concentrations of acetylcholine (10⁻⁹ to 10⁻⁶ M) with similar magnitudes of relaxation (Fig. 2A). In contrast, rings from hyperglycemic animals exhibited impaired responsiveness to the highest acetylcholine concentrations (10⁻³⁻⁵ to 10⁻³⁻⁵ M), whereas there was no difference between aortic rings from control animals and those from HIGT-treated rats (Fig. 2A). Relaxation responses to SNP (10⁻¹⁰ to 10⁻⁹ M), an indicator of endothelium-independent vasorelaxation, were similar at individual time points across groups (Fig. 2B).

HIGT prevents diabetes-induced alterations in NO-Hb and eNOS expression. The whole blood content of NO-Hb, a natural reaction product of vascular NO production, was measured as an index of bioavailable NO (8). Representative spectra from individual animals within each group are presented in Fig. 3A and demonstrate that the amplitude of the signature spectrum for NO-Hb was reduced in hyperglycemic animals compared with control and HIGT animals. As shown in Fig. 3B, HIGT prevented the decline in NO-Hb observed in hyperglycemic animals. To determine whether hyperglycemia-related alterations in NO-Hb were associated with alterations in eNOS expression, eNOS protein levels were measured in aortic tissue lysates. Protein levels of eNOS were similar in HIGT and controls (Fig. 3C). Aortic tissue from hyperglycemic animals tended to demonstrate increased eNOS expression, although this effect did not achieve statistical significance (Fig. 3C).

HIGT improves insulin sensitivity. In humans, endothelial function is reported to decline with increasing insulin resistance (26, 58). To determine the impact of HIGT on insulin sensitivity, a value of HOMA-IR was calculated, and intraperitoneal ITTs were performed on control, hyperglycemic, and HIGT animals. Calculated HOMA-IR values were marginally but insignificantly greater in hyperglycemic rats compared with controls, whereas values in HIGT animals were reduced (Fig. 4A). Following intraperitoneal administration of 0.75 U/kg recombinant human insulin, blood sugar variation was similar in control and hyperglycemic groups (Fig. 4B). However, the same insulin dose suppressed blood sugars in HIGT rats, indicating a greater sensitivity to exogenous hormone (Fig. 4B).

HIGT increases adiponectin and leptin levels. Although both adiponectin and leptin impact carbohydrate metabolism and insulin sensitivity, the impact of HIGT on these adipokines has not been previously reported. Adiponectin levels were suppressed in the hyperglycemic group compared with controls, and HIGT increased adiponectin levels (Fig. 5A). Serum leptin levels were suppressed in hyperglycemic animals, and HIGT partially restored leptin toward control levels (Fig. 5B).

DISCUSSION

To evaluate HIGT effects on diabetes-induced vascular dysfunction, we compared HIGT rats, nondiabetic rats, and rats with partially treated diabetes mellitus. STZ administration followed by treatment with continuous-release insulin pellets produced a moderate diabetic state characterized by hyperglycemia and retarded weight gain. As observed by others (39), this degree of diabetes is accompanied by TG and NEFA levels similar to those of controls. Although STZ-induced diabetes was associated with a roughly twofold increase in serum TG
(Table 1), this degree of dyslipidemia did not achieve statistical significance, suggesting that diabetes-associated derangements in total cholesterol, TG, or NEFA are not likely mediators of vascular dysfunction in this model (40, 64). Insulin levels in controls (Table 1) were elevated compared with literature reports of similarly sized rats, suggesting that chow ad libitum and inactivity combined to diminish insulin sensitivity in control animals (4, 39). Despite similar insulin levels in both hyperglycemic and HIGT groups (Table 1), blood sugars among HIGT rats were significantly lower (Fig. 1). In rodents, intravenous administration of replication-defective adenovirus tends to selectively transduce liver (20, 24, 29), from which the (GIRE)3BP1 promoter is known to drive transgenic insulin expression in vivo (62). Although hepatic insulin production in HIGT animals is likely to affect glycemia predominantly by inhibiting glucose output from the liver, both a normal growth curve and NEFA concentrations, consistent with restrained fatty acid release from peripheral tissues, indicate that some peripheral actions of insulin in HIGT were sustained as well. As previously reported, glucagon in HIGT and hyperglycemic animals was increased (48). Taken together, these findings indicate the presence of peripheral insulin action sufficient to restrain NEFA efflux but insufficient to inhibit glucagon secretion (37).

To further investigate the ability of HIGT-derived insulin to modulate important end points in diabetic complications, we analyzed vascular endothelial function. Consistent with the short duration of the study and similar serum lipid measurements across groups, endothelium-independent vasorelaxation assessed with SNP (Fig. 2B) was comparable at individual time points for control, hyperglycemic, and HIGT groups (25, 28, 32). However, metabolic derangements among hyperglycemic animals were sufficient to impair endothelium-dependent vasorelaxation (Fig. 2A and Refs. 25 and 32). Because hyperglycemia plays a central role in the pathology of diabetic vascular dysfunction, HIGT-induced improvements in glycemic control could contribute significantly to preserving endothelium-dependent relaxation in HIGT rats (14, 19, 21, 57). Consistent with this hypothesis, HIGT restored diabetes-induced impairments in acetylcholine-induced, endothelium-dependent vasodilation (Fig. 2A). However, it must be noted that HIGT failed to fully normalize glycemia and resulted in a normal mean glucose level but greater fluctuations in random blood sugars than observed in controls (Fig. 1B). Recent data in humans suggest that even sporadic, temporary elevations in blood sugar contribute to poor vascular outcomes (15, 22). Nonetheless, altered endothelium-dependent vasorelaxation is among the earliest manifestations of atherosclerosis (26, 53). Thus our studies demonstrate for the first time that HIGT is capable of restoring glycemic control in an animal model of diabetes sufficiently to reduce associated vascular dysfunction.

Fig. 3. Effects of gene therapy on erythrocyte nitrosyl-hemoglobin (NO-Hb) formation and vascular endothelial NO synthase (eNOS) expression. Red blood cells collected at the time the animals were euthanized from control (n = 9), HIGT (n = 11), and hyperglycemic (n = 10) rats were subjected to electron spin resonance (ESR) analysis to determine NO-Hb content. A: representative spectrum from each treatment group. Dashed lines indicate amplitude of peak used in calculations. B: amplitude of the initial peak of each ESR spectrum was quantified and used to calculate group statistics. Data are expressed as arbitrary unit means ± SE; *P < 0.05 vs. control and HIGT. C: tissue lysates from aortic segments collected at the time the animals were euthanized were resuspended on SDS-PAGE, transferred to a PVDF membrane, and probed with primary antibody (1:1,000). Representative blot is shown above a scanning densitometric analysis of multiple (n = 3/group) samples expressed as arbitrary unit means ± SE; P = 0.12
The mechanisms by which HIGT ameliorates vascular dysfunction in this animal model of diabetes continue to be defined. Because acetylcholine-induced vascular relaxation is mediated by endothelial NO generation, we further examined the impact of HIGT on NO-Hb, a marker of bioavailable NO (8). Compared with controls, NO-Hb levels were reduced in hypoglycemic animals, a derangement prevented by HIGT. Reductions in bioavailable NO can arise from either diminished eNOS activity and reduced NO production or from increased destruction of NO by free radicals such as superoxide (25, 34). Hyperglycemia-induced free radicals can deplete the NOS cofactor tetrahydrobiopterin (2, 55), leading to NOS production of superoxide rather than NO (59, 69). In the current study, hyperglycemia was associated with reduced NO-Hb levels and normal to increased aortic eNOS expression. Taken together, these findings suggest that STZ-induced diabetes is associated with enhanced eNOS expression but that eNOS function is “uncoupled,” resulting in superoxide rather than NO production, as previously described in STZ-diabetic rats (25). These diabetes-associated derangements were attenuated by HIGT.

Although HIGT appears to restore diabetes-induced reductions in endothelial NO production, other factors may contribute to HIGT effects on the vasculature. For example, insulin resistance is associated with deterioration of endothelial function independently of hyperglycemia (26, 58), raising the possibility that HIGT-induced improvement in insulin sensitivity, in addition to glycemic control, may have contributed to preservation of vascular function. HIGT reversed the insulin resistance induced by insulin-deficient diabetes in hyperglycemic rats, as determined by both HOMA-IR calculations and ITT. HOMA-IR calculations were originally derived from human data and assume steady state at sampling and minimal metabolic differences between groups (42). Further linearizing assumptions permit a simple algebraic approximation to relate insulin resistance to the product of glucose and insulin (42). The constant (22.5) that normalizes HOMA-IR to 1 for non-diabetic individuals was derived from multivariate equations describing the relationship between insulin and glucose and was confirmed empirically (42). Because neither multivariate calculations nor empiric confirmation have been performed for rodents (67), the HOMA-IR calculated here cannot be extrapolated to other studies. However, if similar assumptions of...
atheroma formation in apoE production from endothelial cells in vitro (10, 50) and reduces muscular TG content (73). Adiponectin also increases NO uptake in both muscle and fat (63, 70), and improves insulin function, and insulin sensitivity, HIGT increased adiponectin disposal will be needed to resolve this issue.

In conclusion, HIGT treatment of STZ-diabetic rats normalizes average random blood sugars. Despite abnormally large blood glucose fluctuations, HIGT preserved endothelium-mediated vascular relaxation similar to that in nondiabetic controls. HIGT also sustained NO-Hb levels with apparently normal eNOS expression. Strikingly, parameters of insulin sensitivity were improved in HIGT rats compared with both hyperglycemic and control animals. In addition, adiponectin and leptin levels were favorably altered and may play a role in both the mechanisms of HIGT-mediated glycemic control and preservation of vascular health. Additional studies are required to determine whether such alterations are durable over time.

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