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

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Hepatic insulin gene therapy prevents deterioration of vascular function and improves adipocytokine profile in STZ-diabetic rats. Am J Physiol Endocrinol Metab 290: E114–E122, 2006. First published August 23, 2005; doi:10.1152/ajpendo.00134.2005.—Hepatic insulin gene therapy (HIGT) ameliorates hyperglycemia in diabetic rodents, suggesting that similar approaches may eventually provide a means to improve treatment of diabetes mellitus. However, whether the metabolic and hormonal changes produced by HIGT benefit vascular function remains unclear. The impact of HIGT on endothelium-dependent vasodilation, 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. Rats made diabetic with streptozotocin (STZ) were treated with subcutaneous insulin pellets dosed to sustain body weights and hyperglycemia or with HIGT; nondiabetic rats served as controls. Hyperglycemic rats demonstrated impaired endothelium-dependent vasodilation, reduced levels of NO-Hb, and diminished insulin, leptin, and adiponectin concentrations compared with controls. In contrast, HIGT treatment significantly reduced blood sugars and sustained both endothelium-mediated vasodilation and NO-Hb at control levels. HOMA-IR calculations and ITT indicated enhanced insulin sensitivity among HIGT-treated rats. HIGT partially restored suppressed leptin levels in hyperglycemic rats and increased adiponectin concentrations to supranormal levels, consistent with indicators of insulin sensitivity. Our findings indicate that the metabolic milieu produced by HIGT is sufficient to preserve vascular function in diabetic rodents. These data suggest that improved glycemia, induction of a beneficial adipocytokine profile, and enhanced insulin sensitivity combine to preserve endothelium-dependent vascular function in HIGT-treated diabetic rats. Consequently, HIGT may represent a novel and efficacious approach to reduce diabetes-associated vascular dysfunction.

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), homeostasis model assessment of insulin sensitivity (HOMA-IR) calculations, and insulin tolerance testing (ITT), as well as hormone and adipocytokine concentrations, were determined in HIGT-treated diabetic rats ~40 days after induction of hyperglycemia. Our findings indicate that, although failing to fully normalize metabolism, HIGT preserved endothelium-dependent vasorelaxation, induced a potentially beneficial adipocytokine profile, and improved insulin sensitivity. To our knowledge, these studies are the first to demonstrate that HIGT represents a novel and potentially efficacious treatment to reduce vascular dysfunction caused by diabetes.

MATERIALS AND METHODS

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 [(GIRE)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 Adsorption Technologies, Sunnyvale, CA) (40). The resulting adenovirus had a titer of 2 x 10^11 plaque-forming units/ml.

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Biotec..., Morrisville, NC), were obtained 3–5 times each week. Animals routinely
values, determined by hand-held glucose meter on tail blood (One-
200 mg/dl, was induced by intravenous injection of freshly prepared
Emory University Institutional Animal Care and Use Committee.
by and conformed to the stipulations of the Atlanta Veterans Affairs/
Wilmington, MA) were used for all studies were housed singularly in
sucrose buffer containing 10 mM Tris and 2 mM MgCl2, aliquoted,
by double CsCl density gradient centrifugation, dialyzed in a 4%
insulin injections in HIGT rats were tapered as blood
euglycemia in two models of type 1 diabetes in rats (48, 62).
This adenoviral dosage was previously determined to induce near
were separated by electrophoresis and blotted onto polyvinylidene
concentrations were determined using a bicinchoninic acid system
(4°C). Proteins were visualized using a peroxidase-coupled
anti-mouse IgG in the presence of LumiGlo reagent (Kirkegaard &
Santa Cruz, CA). Proteins were visualized using a peroxidase-coupled
at 4°C with antibodies (1:1,000) to endothelial NO synthase (eNOS;
were isometrically mounted onto a Harvard Apparatus differential
capacitor force transducer (Holliston, MA). Raising tension on each
cooked aorta 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 t-phenylephrine. Relaxation responses to acetylcholine (1
mM to 10 μM) and the NO donor sodium nitroprusside (SNP, 0.1 mM to 1 μM) were determined in aortas precontracted with 300 mM
phenylephrine, a concentration that yields ~80% of maximal contrac-
tion. Data were obtained and analyzed using a Powerlab system
(A/D Instruments, Cranfield 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 pyrophos-
phate; 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 biocinchoninic acid system
(Pierce, Rockford, IL). Equal amounts of protein (50 μg/lane) were
loaded into each well of a 4–12% bis-tris PAGE minigel. 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
(Bio-Rad, 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 equal 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 × 103.

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 postest
unless otherwise specified. All analyses were performed using Graph-
Pad Prism v. 3 (GraphPad Software, San Diego, CA) unless otherwise
specified. Variability was determined using an F-test resident 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)
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, r² = 0.93) applied to HIGT body weights was similar to that of nondiabetic controls (control slope = 4.52 g/day, r² = 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, r² = 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>
<tr>
<th>Lipid</th>
<th>Control</th>
<th>Hyperglycemic</th>
<th>HIGT</th>
</tr>
</thead>
<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>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>89.5 ± 12.7</td>
<td>171.6 ± 47.4</td>
<td>91.3 ± 12.2</td>
</tr>
<tr>
<td>NEFA, pg/ml</td>
<td>0.31 ± 0.05</td>
<td>0.25 ± 0.04</td>
<td>0.28 ± 0.02</td>
</tr>
<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.
with control and HIGT animals. As shown in Fig. 3 for NO-Hb was reduced in hyperglycemic animals compared and demonstrate that the amplitude of the signature spectrum individual animals within each group are presented in Fig. 3 an index of bioavailable NO (8). Representative spectra from eNOS expression.
similar at individual time points across groups (Fig. 2 (Fig. 2 (Fig. 2). Relaxation responses to SNP (10^{-10} to 10^{-6} 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 high glycemic 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 glucose 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 adipocytokines 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 resolved 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
muscular TG content (73). Adiponectin also increases NO
sensitivity by stimulating NEFA oxidation and reducing intra-
uptake in both muscle and fat (63, 70), and improves insulin
dexpression of hepatic glucose output (6, 11), stimulates glucose
and leptin levels. Adiponectin enhances insulin-mediated sup-
disposal will be needed to resolve this issue.

In conclusion, HIGT treatment of STZ-diabetic rats normal-
izes average random blood sugars. Despite abnormally large
glucose fluctuations, HIGT preserved endothelium-me-
diated vascular relaxation similar to that in nondiabetic con-
tral 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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