Basal Insulin Gene Expression Significantly Improves Conventional Insulin Therapy in Type 1 Diabetic Rats

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Although a conventional insulin regimen for type 1 diabetes with twice-daily insulin injections is effective in preventing postprandial blood glucose excursions, this treatment is limited by its inadequate control of fasting hyperglycemia. Alternatively, sustained basal hepatic insulin gene expression has been shown to result in fasting normoglycemia in type 1 diabetic rats, although the treated animals still exhibited moderate postprandial hyperglycemia. To test the hypothesis that basal hepatic insulin production can be used as an auxiliary treatment to conventional insulin therapy for achieving better glycemic control, streptozotocin-induced diabetic rats were treated with twice-daily insulin injections, basal hepatic insulin production, or both in combination. Diabetic rats treated by conventional insulin therapy still suffered from fasting hyperglycemia, but when complemented with basal hepatic insulin production, near-normoglycemia under both fed and fasting conditions was achieved without fasting hypoglycemia. In addition, the combination-treated animals showed significantly enhanced glucose tolerance and markedly improved profiles in lipid metabolism. Furthermore, the combination treatment reduced the elevated fructosamine, glycated hemoglobin, and advanced glycation end products concentrations to normal. These results provide a proof of concept for basal hepatic insulin production as an adjuvant treatment to conventional insulin therapy in type 1 diabetes. Diabetes 51:130–138, 2002

In type 1 diabetes, insulin deficiency accompanied by elevated glucagon secretion results in impaired peripheral glucose utilization and augmented hepatic glucose production, the combined effect of which contributes significantly to elevated blood glucose levels. As a result, type 1 diabetic patients are chronically hyperglycemic and must receive at least twice-daily injections of a mixture of short- and delayed-action insulin before breakfast and the evening meal. It is thought that delayed-action insulin provides a relatively constant background level of insulin to serve the basal requirement, on which short-action insulin is superimposed to meet the postprandial demand of insulin after meals. However, this conventional insulin therapy is inadequate for blood glucose control between meals and during the night because treated patients still experience fasting hyperglycemia and elevated HbA1c, which are causative for the development of long-term diabetic complications, including nephropathy, neuropathy, retinopathy, and cardiovascular disease. Whereas much tighter glycemic control can be achieved through frequent glucose monitoring and multiple daily insulin injections, such intensive insulin therapy entails undue body weight gain and is associated with markedly increased episodes of hypoglycemia (1). Therefore, the focus of alternative therapy development has been to achieve better glycemic control by replacing or reducing the frequency of insulin injection.

Among alternative strategies designed to improve glycemic control in type 1 diabetes, hepatic insulin gene expression is being exploited to provide an endogenous source of insulin. The liver was chosen as a surrogate organ for insulin production because, as a major target organ of insulin action, it plays a critical role in glucose homeostasis. In normal individuals, the liver initiates glycogenesis in response to glucose uptake, whereas during fasting or prolonged starvation, the liver undergoes glycogenolysis and gluconeogenesis (2). This intricate interplay between glycogenesis and glycogenolysis/gluconeogenesis ultimately dictates net glucose utilization versus glucose production in the liver (2–4). In addition, the liver is also an essential organ for ketogenesis. In type 1 diabetes, the elevated ketogenesis along with unrestrained lipolysis is a major causative factor for diabetic ketoacidosis (5,6). Both ketogenesis and lipolysis are highly sensitive to insulin inhibition, and basal levels of plasma insulin are effective in achieving 50% of maximal inhibition of hepatic ketogenesis (7–9). Thus, even basal levels of hepatic insulin production by gene transfer would significantly inhibit ketogenesis and restrain lipolysis, producing a profound impact on glucose and fat metabolisms.

Using defective viruses as gene delivery vehicles, we and others have validated the concept of hepatic insulin gene expression for improving glycemic control in type 1 diabetic animals (10–16). Hepatically produced insulin was shown to reverse the severe insulin-deficient phenotype, reduce ketoacidosis, and prevent death in diabetic animals. Recently, we have achieved a dose-dependent expression of hepatic insulin by adenovirus-mediated gene transfer and defined a maximal tolerable level of hepatic insulin production, which is sufficient for significant reduc-
tion of nonfasting hyperglycemia without fasting hypoglycemia in diabetic animals (15). In this article, we show that basal hepatic insulin gene expression, when applied as an adjuvant treatment to conventional insulin therapy, significantly improved glycemic control without fasting hypoglycemia in STZ-induced diabetic rats.

**RESEARCH DESIGN AND METHODS**

**Recombinant adenoviral vectors.** The recombinant adenoviral vector ADV-EF1-erINS encodes the engineered rat preproinsulin-1 (erINS) cDNA under the control of the constitutive elongation factor EF1-α promoter (15). Such a genetically modified rat preproinsulin contains the consensus furin recognition sequence between the Bc and Ca junctions to allow processing of proinsulin to mature insulin by the ubiquitous protease furin (11,17). The control adenoviral vector ADV-RSV-lacZ expresses the bacterial lacZ gene driven by the RSV promoter, as previously reported (15).

**STZ-induced diabetic nude rats.** Athymic NIH nude rats (C3H/HeN-nu) at 5 weeks of age with an average body weight of 110 g were purchased from the National Cancer Institute and kept in isolator cages in a barrier animal facility with a 12-h light/dark cycle. Animals were fed ad libitum with a regular diet, except during fasting. Animals were rendered diabetic by intravenous injection of streptozotocin (STZ) at the dose of 80 mg/kg body wt, as previously reported (15). All STZ-treated rats developed ketotic hyperglycemia (blood glucose levels >550 mg/dl and urinary ketone levels >80 mg/ml) 3 days after STZ injection, and their plasma insulin concentrations were reduced to nondetectable levels (<0.08 ng/ml) when assayed by rat insulin enzyme-linked immunosorbent assay (ELISA) (ALPCO, Windham, NH). Blood glucose and urinary ketone levels were determined with a Glucometer Elite (Bayer, Elkhart, IN) and Ketostix Strips (Bayer), respectively.

**Insulin gene transfer and insulin injection.** The ADV-EF1-erINS vector at the dose of 3 × 10^{12} particles/kg body wt was injected intravenously to individual diabetic rats via the tail vein. This dose of insulin vector has been shown to result in hepatic insulin production at a defined basal level (~0.5 ng/ml) and reduce blood glucose levels from >550 to >300 mg/dl in STZ-induced diabetic rats (15). For insulin administration, Novolin (Novo Nordisk, Bagsvaerd, Denmark) 70/30 (70% NPH and 30% regular human insulin) was intraperitoneally injected to diabetic rats twice daily (8:00 a.m. and 6:00 p.m.).

**Blood chemistries.** Plasma concentrations of triglyceride, cholesterol, β-hydroxybutyrate, and fructosamine were measured using Sigma Diagnostics kits for each assay (Sigma, St. Louis, MO). Serum nonesterified fatty acid (NEFA) concentrations were measured using the Wako NEFA kit (Wako Chemicals, Richmond, VA). To determine glycated hemoglobin (HbA1), aliquots (50 μl) of blood collected in EDTA-coated microtubes were hydrolyzed with hemolyzing reagents (Sigma). Negatively charged glycated hemoglobin variants were fractionated from nonglycated forms by cation-exchange column chromatography using a Glycated Hemoglobin Kit (Sigma). HbA1 levels were determined and expressed as the percentage of total blood hemoglobin. Serum advanced glycation end products (AGEs) were determined by ELISA, as described previously (18).

**Intraperitoneal glucose tolerance test.** Animals were fasted for 5 h and injected intraperitoneally with 50% dextrose solution (Abbott Laboratories, Chicago) at the dose of 5 g/kg body wt. Blood glucose was measured before and at 30-min intervals after glucose infusion.

**Renal functional analysis.** Urine samples were collected from individual animals for determination of urine protein concentrations by the Pierce protein assay reagents (Pierce, Rockford, IL), of urine creatinine by Releotron creatinine reagents in the Releotron system (Boehringer Mannheim Diagnostics, Indianapolis, IN), and of urine albumin using the rat albumin ELISA kit (ALPCO, Windham, NH).

**Histopathology.** Animals were killed at the end of the study. Liver and kidney tissues were collected and fixed in 10% buffered formalin and embedded in paraffin (Fisher Scientific, Fair Lawn, NJ) for periodic acid-Schiff (PAS) reaction and hematoxylin and eosin staining or placed in an O.C.T. compound (Sakura, Torrance, CA) and snap-frozen in liquid nitrogen for fat staining with Oil Red-O, as previously described (15).

**Statistical analysis.** Statistical analyses of data were performed by analysis of variance (ANOVA) using StatView software (Abacus Concepts, Berkeley, CA). Pairwise comparisons were performed to study the significance between different treatments. Data were expressed as the mean ± SE. P values <0.05 were regarded as statistically significant.

**RESULTS**

**Prevention of ketoacidosis and improvement in survival rates.** To study the effect on glycemic control of the combination therapy, STZ-induced diabetic rats were randomly assigned to multiple groups, which were respectively treated with insulin injection, insulin vector, insulin injection plus insulin vector, or control lacZ vector. In addition, one group of mock-treated diabetic animals and one group of normal nude rats were used as diabetic and nondiabetic controls, respectively. For insulin gene transfer, the insulin vector was administered to individual diabetic animals at a moderate dose that was previously shown to produce basal levels of plasma insulin in STZ-induced diabetic rats, leading to significant reduction of nonfasting blood glucose levels without fasting hypoglycemia (15). For insulin treatment, we followed the protocol of conventional insulin therapy, which consists of twice-daily injections of Novolin.

To determine the maximal effective insulin dose, Novolin at doses ranging from 4 to 20 units/kg body wt was intraperitoneally administered to diabetic rats in a dose-escalating experiment (Fig. 1A). Diabetic rats treated at insulin doses <12 units/kg body wt still exhibited severe hyperglycemia, whereas those treated with 20 units/kg insulin developed hypoglycemia (3/10 animals). Insulin injection at a moderate dose of 16 units/kg resulted in a significant reduction of nonfasting hyperglycemia (from 554 ± 32 to 340 ± 70 mg/dl) without fasting hypoglycemia. In the vector-treated animals, their nonfasting blood glucose levels were reduced to ~300 mg/dl by hepatically produced insulin. Twice-daily injections of 8 units/kg insulin were sufficient for achieving near-normoglycemia (124 ± 38) without fasting hypoglycemia. Higher insulin doses (12 units/kg) resulted in hypoglycemia (3/8 animals). These data have defined the maximal effective dose of insulin at 16 units/kg for insulin treatment alone and 8 units/kg for the combination treatment. Further increases in the insulin dose in both groups by 4 units/kg (from 16 to 20 units/kg vs. 8 to 12 units/kg) resulted in hypoglycemia at similar frequencies (3/10 vs. 3/8 animals) in insulin treatment and combination treatment groups, respectively. Thus, the comparisons between the two treatment groups were based on the respective optimal treatment conditions.

Insulin injection and insulin vector treatment alone or in combination rapidly reversed the phenotype of ketonuria as the urinary ketone levels in those treated animals were decreased to an undetectable background value (<5 mg/dl) 2 days after treatment. In contrast, mock-treated or lacZ control vector–treated diabetic animals continued to manifest significantly elevated urinary ketone levels varying from medium to large grades (40–160 mg/dl). Determination of blood ketone levels showed that the plasma ketone levels were also markedly elevated in mock-treated or lacZ vector–treated diabetic animals (Fig. 1B). Insulin gene transfer and insulin injection alone or in combination reduced the elevated plasma ketone levels to background levels.

Blood ketone levels in treated and control diabetic animals closely correlated with their survival rates. As shown in Fig. 1C, mock-treated and lacZ vector–treated diabetic animals died progressively during the course of
this study, whereas no deaths occurred in diabetic rats treated with insulin injection, insulin vector, or both. This confirmed the results from previous studies (10,11,15) showing that basal levels of plasma insulin provided by either insulin gene transfer or insulin injection are sufficient for prevention of lethal ketoacidosis associated with type 1 diabetes.

Furthermore, the combination treatment normalized body weight in STZ-induced diabetic rats. As shown in Fig. 1D, there was an initial loss of body weight immediately after STZ treatment, but progressive body weight gains were achieved in diabetic animals after twice-daily insulin injections or after insulin gene transfer. Insulin injection complimented by insulin vector treatment appeared to produce an additive effect on body weight gain because the growth curve of the treated animals paralleled that of nondiabetic controls.

FIG. 1. A: Insulin dose responses in diabetic rats treated with (●, n = 8) and without (■, n = 10) insulin vector. B: Serum ketone levels. C: Survival curves. D: Body weight changes. ○, Nondiabetic group (normal, n = 6); ■, mock-treated diabetic group (diabetic, n = 12); △, lacZ control vector–treated group (lacZ vector, n = 8); ▲, insulin vector–treated group (INS vector, n = 8); ▼, insulin injection group (insulin, n = 8); ●, combination treatment group (combined, n = 8). *P < 0.001 vs. diabetic control. In C, the survival curves of animals treated with insulin injection, insulin vector, or both behaved like those of nondiabetic controls. In D, data of diabetic controls were not shown because animals died on different days.

animals were measured for an experimental period of 10 weeks (Fig. 2A). Insulin gene transfer or twice-daily insulin injections alone resulted in significant blood glucose reduction compared with persistently elevated blood glucose levels in mock-treated and lacZ vector–treated diabetic animals. Basal hepatic insulin production in combination with twice-daily insulin injections further reduced the elevated blood glucose levels of treated diabetic animals to near normal. Significantly, the insulin dose used in the combination treatment group is half the dose of insulin applied to diabetic animals treated with insulin injection alone. Thus, the combination therapy completely reversed ketotic hyperglycemia, and it did so at a reduced insulin dose.

As expected, basal hepatic insulin production was achieved in insulin vector–treated diabetic rats (plasma insulin concentrations, 0.54 ± 0.07 vs. 0.06 ± 0.02 ng/ml in mock-treated diabetic controls) (Fig. 2B). Basal insulin production plus insulin injection resulted in normal plasma insulin levels, which contributed to near-normalization of hyperglycemia in the combination therapy–
treated animals. However, in insulin-treated diabetic rats, a relatively higher mean plasma insulin level was detected, but with a high degree of variation between animals, which were attributable to the observed intersubject variability in blood glucose control by insulin treatment alone (Fig. 2A).

To assess the safety of the combination treatment for blood glucose control, treated animals in different groups were fasted for 16 h. As shown in Fig. 3, the fasting blood glucose levels in the combination treatment group were reduced to normal (60–100 mg/dl). Importantly, no fasting hypoglycemia (blood glucose levels <50 mg/dl) was detected in the combination treatment group. In contrast, the mock-treated and control vector–treated diabetic animals still exhibited hyperglycemia.

**Improvement in glucose tolerance.** To study the efficacy of blood glucose disposal, an intraperitoneal glucose tolerance test (IPGTT) was performed. As shown in Fig. 4, insulin injection or insulin gene treatment alone significantly improved the IPGTT in treated animals, and their elevated blood glucose levels after the glucose challenge returned to the prechallenge levels within 2.5 h. However, the combination treatment further improved IPGTT to near normal as blood glucose levels in the combination-treated animals decreased to a normal range within 1.5 h and their profile approximated that of nondiabetic controls.

**Reduction of blood fructosamine and glycated hemoglobin.** To study the quality of glycemic control by the combination treatment over time, serum fructosamine and blood HbA1c concentrations in different treatment groups of animals were determined. As shown in Fig. 5, both serum fructosamine and blood HbA1c levels were elevated.
in mock-treated or lacZ vector–treated diabetic animals. Insulin injection and insulin gene treatment alone significantly reduced their serum fructosamine and blood HbA1c concentrations. The combination treatment further reduced serum fructosamine and blood HbA1c concentrations to near-normal levels. Furthermore, the extent of reduction for both parameters correlated with the degree of nonfasting blood glucose reduction in different treatment groups of animals (Fig. 2A).

This significantly improved glycemic control was reflected in the reduction of serum AGE levels in the combination treatment group of animals. It has been previously shown that chronic hyperglycemia in uncontrolled diabetes is associated with an enhanced accumulation of AGE, which has been implicated as a predisposing factor for microvascular complications (19–21). We showed that serum AGE concentrations were increased in mock-treated and lacZ vector–treated diabetic rats (Fig. 5C). Both insulin injection and insulin gene transfer significantly lowered serum AGE concentrations, but the combination treatment further reduced serum AGEs to background levels.

**Protective effects on renal function.** To study the potential beneficial effects of the combination treatment on renal function, urine protein, urine creatinine, and urine albumin concentrations were determined. As shown in Fig. 6, renal function was severely impaired as a result of insulin deficiency, as reflected in markedly increased urine protein/creatinine and urine albumin/creatinine ratios in diabetic control rats. Insulin gene transfer or insulin injection treatment alone significantly lowered the urine protein/creatinine and urine albumin/creatinine ratios, but the combination treatment completely reversed the phenotypes of proteinuria and albuminuria. Despite the presence of proteinuria and albuminuria, histopathological examination under light microscopy did not reveal significant morphological lesions in the kidney of diabetic animals with or without further treatment.

**Effects on lipid metabolism.** To examine the effects of improved glycemic control on lipid metabolism, serum triglyceride and NEFA levels were determined in different treatment groups. As shown in Fig. 7, serum triglyceride and NEFA concentrations were markedly elevated in STZ-treated nude rats. These observed abnormalities in lipid metabolism were partially corrected by insulin injection or hepatic insulin gene expression. However, the combination therapy reduced the elevated plasma triglyc-

![Fig. 4. Blood glucose profiles in a glucose tolerance test.](image)

*Fig. 4. Blood glucose profiles in a glucose tolerance test. ■, Diabetic controls; ▲, insulin vector–treated; ◊, insulin injection treated; ●, combination-treated; ○, nondiabetic controls.*

![Fig. 5. A: Serum fructosamine concentrations. B: Blood HbA1c levels. C: Serum AGE concentrations in different treatment groups of animals.](image)

*Fig. 5. A: Serum fructosamine concentrations. B: Blood HbA1c levels. C: Serum AGE concentrations in different treatment groups of animals. *P < 0.05 vs. diabetic controls; †P < 0.01 vs. diabetic controls and P < 0.05 vs. insulin treatment and insulin (INS) vector treatment.*
eride and NEFA concentrations to normal. In contrast, serum total cholesterol concentrations remained unchanged in diabetic rats with and without further treatment.

**Effects on glycogen and fat contents in the liver.** To assess the beneficial effects of the combination treatment on glycogen and fat metabolisms in the liver, liver sections from treated diabetic rats were stained for PAS and stained with Oil Red-O stain. Consistent with previous observations (11,15), STZ treatment caused nearly complete depletion of liver glycogen, accompanied by increased fat distribution in the liver (Fig. 8). However, these metabolic defects in excessive glycogen breakdown and fat deposition in the liver were corrected by insulin injection and hepatic insulin production alone and in combination. Finally, histopathological examination did not reveal any discernible morphological abnormality in the livers of diabetic rats that have been treated by insulin vector or insulin vector plus twice-daily insulin injections.

**DISCUSSION**

We have assessed the efficiency and outcome of glycemic control in STZ-induced diabetic rats treated with an insulin vector in the presence and absence of twice-daily insulin injections.
injections. As expected, insulin treatment and insulin gene transfer each reduced the severity of ketotic hyperglycemia. However, the combination treatment completely reversed ketotic hyperglycemia. A great concern remains as to whether an improvement in glycemic control by the combination therapy is associated with an increased risk of hypoglycemia. To address this concern, we periodically subjected treated diabetic animals to an overnight fast. Treated diabetic animals were able to maintain their fasting blood glucose levels within a normal range (60–100 mg/dl) and no fasting hypoglycemia was detected in the combination treatment group of animals. Taken together, these data provided the proof of principle that hepatic insulin gene transfer, when applied in conjunction with conventional insulin therapy, can achieve tight glycemic control without fasting hypoglycemia in type 1 diabetic animals.

These significantly improved blood glucose profiles under both fed and fasting conditions conferred profound physiological benefits in this model of type 1 diabetes. This is partially reflected in significantly improved glucose profiles as well as markedly reduced NEFA and triglyceride concentrations. In addition, persistently improved glycemic control in diabetic rats treated by the combination therapy resulted in significant reductions in both serum fructosamine and blood HbA1c levels, the two commonly used clinical markers for the quality of glycemic control (22). It is known that prolonged exposure to hyperglycemia as a result of poor glycemic control can lead to enhanced formation and accumulation of AGEs in circulation, which has been linked with hyperglycemia-induced tissue damage and renal failure in diabetes (19–21). Previous experiments in diabetic animals have demonstrated that a reduction in serum AGE levels helps delay or prevent the development of diabetic complications (23–26). In this study, we showed that a significant reduction in serum AGE levels by the combination therapy greatly improved renal function in diabetic rats, as reflected in the phenotypic correction of proteinuria and albuminuria.

This increased efficacy in glycemic control by the combination treatment versus insulin injection alone is intriguing. In normal individuals, insulin is released from the pancreas to the portal circulation in response to a rise in blood glucose levels. As a result, the portal insulin levels are relatively higher than peripheral insulin concentrations (27). Obviously, such a gradient cannot be achieved by peripheral insulin infusion in type 1 diabetes, which has been implicated as a causative factor for impaired sup-

FIG. 8. Liver histology. The liver tissues isolated from dead animals were stained for glycogen (A and B) and fat (C and D). A and C: Mock-treated diabetic rats. B and D: Combination therapy–treated animals. Bar equals 50 μm.
pression of hepatic gluconeogenesis. As a result, continuous hepatic glucose production after subcutaneous insulin injection exacerbates the degree of hyperglycemia (28,29). In contrast, hepatic insulin production has the potential to restore to at least partially the portal/peripheral insulin concentration gradient because insulin is synthesized locally in the liver.

In summary, we have demonstrated the efficacy as well as the feasibility of combining hepatic insulin production and conventional insulin therapy to significantly improve blood glucose profiles without causing fasting hypoglycemia in STZ-induced diabetic animal models. Unlike intensive insulin therapy, by which tight glycemic control is achieved at an increased frequency of insulin injection with concomitant risk of hypoglycemia, the significantly improved glycemic control by combination therapy is accomplished without the need for multiple insulin injections and without excessive body weight gain.

However, a major limitation in this study is the adenovirus-mediated gene delivery system, which has prevented us from performing combination treatment in genetic models of type 1 diabetes to address experimentally its effects on the prevention of long-term diabetic complications. Because of viral gene expression, the E1-deleted adenoviral vector is immunogenic, and the vector-transduced cells do not persist in immune-competent hosts (30,31). Recently, a gutless adenoviral vector was developed by removing all viral protein sequences (32), and gutless vector–mediated transgene expression was shown to be both persistent and nontoxic in rodents and nonhuman primates (33,34). It would be of interest to use this vector system in future studies for assessing long-term therapeutic benefits and potential side effects of the combination therapy in genetic rodent models of type 1 diabetes.

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