Sustained Expression of Exendin-4 Does Not Perturb Glucose Homeostasis, β-Cell Mass, or Food Intake in Metallothionein-Preproexendin Transgenic Mice*

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Activation of glucagon-like peptide (GLP)-1 receptor signaling promotes glucose lowering via multiple mechanisms, including regulation of food intake, glucose-dependent insulin secretion, and stimulation of β-cell mass. As GLP-1 exhibits a short \( t_{1/2} \) in vivo, the biological consequences of prolonged GLP-1 receptor signaling remains unclear. To address this question, we have now generated metallothionein promoter-preproexendin (MT-Ex) transgenic mice. MT-Ex mice process preproexendin correctly, as is made evident by detection of circulating plasma exendin-4 immunoreactivity using high pressure liquid chromatography and an exendin-4-specific radioimmunoassay. Despite elevated levels of exendin-4, fasting plasma glucose and glucose clearance following oral and intraperitoneal glucose tolerance tests are normal in MT-Ex mice. Induction of transgene expression significantly reduced glycemic excursion during both oral and intraperitoneal glucose tolerance tests (\( p < 0.05 \)) and increased levels of glucose-stimulated insulin following oral glucose administration (\( p < 0.05 \)). Despite evidence that exendin-4 may induce β-cell proliferation, β-cell mass and islet histology were normal in MT-Ex mice. MT-Ex mice exhibited no differences in basal food intake or body weight; however, induction of exendin-4 expression was associated with reduced short term food ingestion (\( p < 0.05 \)). In contrast, short term water intake was significantly reduced in the absence of zinc in fluid-restricted MT-Ex mice (\( p < 0.05 \)). These findings illustrate that sustained elevation of circulating exendin-4 is not invariably associated with changes in glucose homeostasis, increased β-cell mass, or reduction in food intake in mice in vivo.

Glucagon-like peptide-1 (GLP-1), a product of the progluca-

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1 The abbreviations used are: GLP, glucagon-like peptide; GLP-1R, GLP-1 receptor; MT-Ex, metallothionein promoter-preproexendin; Exendin-4-IR, exendin-4-like immunoreactivity; HPLC, high pressure liquid chromatography.
MT-Exendin Transgene Construction and Generation of Transgenic Mice—To generate the MT-exendin transgene, a 492-base pair cDNA encoding lizard preproexendin-4 (7) was cloned into the BglII site of the pEV142 expression vector (19), under the control of an inducible mouse metallothionein-I promoter. A 1.9-kilobase EcoRI fragment containing the MT-exendin transgene was electrophoresed from a 1% (w/v) agarose gel and further purified on an Elutip-d column (Schleicher & Schuell). Transgenic mice were generated by Chrystalis (DNX Transgenic Sciences, Princeton, NJ) on a C57BL/6 × SJL genetic background. Two lines of MT-exendin mice were generated that exhibited comparable phenotypes. All mice used in these studies were 16–20 weeks old. Control animals were age- and sex-matched transgene-negative mice from the same litter or family. For induction of metallothionein-I promoter activity, drinking water was supplemented with 25 mg/ml ZnSO4 for a minimum of 72 h. All procedures were conducted according to protocols and guidelines approved by the Toronto Hospital Animal Care Committee.

Plasma Extraction—Blood samples were obtained by cardiac puncture and mixed with 10% (v/v) TEG (50,000 IU/ml Trasylol, 1.2 mg/ml EDTA, and 0.1% Drotten A). Plasma was collected by centrifugation at 4 °C and mixed with 2 volumes of 1% (v/v) trifluoroacetic acid, pH 2.5. Peptides and small proteins were adsorbed from plasma extracts by passage through a C18 silica cartridge (Waters Associates, Milford, MA). Adsorbed peptides were eluted with 4 ml of 80% (v/v) isopropanol containing 0.1% (v/v) trifluoroacetic acid.

High Pressure Liquid Chromatography (HPLC) and Radioimmunoassay—HPLC was performed on a Waters system using a C18 MA). Adsorbed peptides were eluted with 4 ml of 80% (v/v) isopropanol at 4 °C and mixed with 2 volumes of 1% (v/v) trifluoroacetic acid, pH 2.5. A coherent double-lattice grid was used for point counting. Sampling and sections was also performed by systematic uniform random sampling. A grid—cells within tissue blocks was estimated according to the principle of the MT-exendin transgene was purified and used to generate transgenic mice. Electrical activity was carried out using a rabbit anti-exendin-4 antisemur (Calbiochem Biologicals Inc., Reamstown, PA), synthetic exendin-4 (California Peptide Research Inc., Napa, CA) as standard, and 125I-exendin-4, antibody binding. In wild-type nontransgenic mice, basal levels of Ex-4-IR were similar to the major exendin-immunoreactive molecular forms of circulating Ex-4-IR. The major exendin-immunoreactive (Ex-4-IR) using exendin-4 antiserum generated in our laboratory.2 The exendin-4 antiserum used for these studies does not cross-react with glucagon, glicentin, oxyntomodulin, gastric inhibitory polypeptide, vasoactive intestinal polypeptide, GLP-1, or GLP-2, nor does it require a free N terminus for binding.3 In wild-type nontransgenic mice, basal levels of Ex-4-IR were less than 27 pg/ml. In contrast, basal plasma levels of Ex-4-IR were 434 ± 59 and 330 ± 84 pg/ml in male and female transgenic mice, respectively (Fig. 2A), and induction of transgene expression with zinc treatment resulted in an ~2.5-fold increase in the circulating levels of Ex-4-IR in both male and female mice (p < 0.01, Fig. 2A).

To determine whether preproexendin was both processed appropriately and secreted into the circulation, HPLC and radioimmunoassay analyses were used to characterize the molecular forms of circulating Ex-4-IR. The major exendin-immunoreactive peptide detected in plasma extracts from MT-exendin-4 transgenic mice eluted at the same position as synthetic exendin-4 (Fig. 2B). Significant amounts of exendin-4-immunoreactivity eluting in the same position as synthetic exendin-4 were also detected in several tissues.3 As GLP-1 receptor signaling is essential for control of blood glucose levels, we used this assay to determine whether endogenous levels of exendin-4 could contribute to glucose homeostasis in transgenic mice. Fasting blood glucose levels were normal in MT-exendin mice under conditions of either basal or induced transgene expression (Fig. 3). Despite clearly detectable levels of circulating exendin-4 immunoreactivity, blood glucose excursion and glucose-stimulated insulin secretion were comparable in +/+ and MT-Exendin-4 transgenic mice.

FIG. 1. Structure of the MT-exendin transgene. The H. suspensa preproexendin-4 cDNA (7) was cloned into the pEV142 expression vector (58), downstream of an inducible mouse metallothionein-I promoter (MT-1) and upstream of 3′ flanking sequences from the human growth hormone (hGH) gene. The 1.9-kilobase EcoRI fragment containing the MT-exendin transgene was purified and used to generate transgenic mice.

grid density was calibrated such that approximately 100–200 points hitting β-cells and approximately the same number of points hitting pancreas were counted per pancreas (26). Estimates of β-cell mass were determined in a blinded manner.

Statistics—Results are expressed as mean ± S.E. Statistical significance was calculated by analysis of variance and Student’s t test using INSTAT 1.12 (Graph-Pad Software, Inc., San Diego, CA). A p value < 0.05 was considered to be statistically significant.

RESULTS

To study the generation of MT-exendin transgenic mice, we used a 1.9-kilobase fragment (Fig. 1) containing the following: (i) 770 base pairs of the mouse metallothionein I promoter (including 5′ flanking and exon 1 sequences) (27), (ii) the 492-base pair lizard preproexendin-4 cDNA (7), and (iii) 625 base pairs of the human growth hormone gene (containing the polyadenylation signal and 3′-flanking sequences) (28). Transgenic mice were identified by Southern blot analysis (data not shown). Male and female MT-exendin transgenic mice were viable and fertile and appeared to develop normally.

Northern blot analysis detected exendin gene expression in several tissues, including heart, duodenum, jejunum, colon, and adipose tissue (data not shown). Tissue and plasma extracts from MT-exendin mice were analyzed by radioimmunoassay for exendin-4-like immunoreactivity (Ex-4-IR) using exendin-4 antiserum generated in our laboratory.2 The exendin-4 antiserum used for these studies does not cross-react with glucagon, glicentin, oxyntomodulin, gastric inhibitory polypeptide, vasoactive intestinal polypeptide, GLP-1, or GLP-2, nor does it require a free N terminus for binding.3 In wild-type nontransgenic mice, basal levels of Ex-4-IR were less than 27 pg/ml. In contrast, basal plasma levels of Ex-4-IR were 434 ± 59 and 330 ± 84 pg/ml in male and female transgenic mice, respectively (Fig. 2A), and induction of transgene expression with zinc treatment resulted in an ~2.5-fold increase in the circulating levels of Ex-4-IR in both male and female mice (p < 0.01, Fig. 2A).

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2 D. J. Drucker and P. L. Brubaker, unpublished observations.
3 P. L. Brubaker, manuscript in preparation.
GLP-1 and exendin-4 have been administered daily to humans and diabetic rodents for periods of up to several weeks (11, 13, 16, 18, 37, 38); however, the long term consequences of exendin-4 administration have not been examined. Although cell-based delivery systems for GLP-1 and exendin-4 have been proposed (39), there is little information available on the viability or efficacy of this strategy in rodents in vivo. The generation of mice expressing lizard preproexendin-4 provides an opportunity to assess the safety and feasibility of continuous exendin-4 delivery in mice in vivo. Although studies of the molecular determinants of preproexendin-4 processing have not yet been reported, the finding of detectable levels of circulating exendin-4 in MT-exendin transgenic mice is consistent with the correct processing and secretion of the lizard preproexendin precursor in murine tissues in vivo. Furthermore, the levels of circulating bioactive exendin-4 detected in MT-exendin transgenic mice are clearly much higher than plasma levels of less potent GLP-1 (1) and are certainly within the range of or higher than the plasma levels of exendin-4 noted to decrease blood glucose in diabetic db/db mice (11, 40). Hence, the findings observed in our studies cannot simply be attributable to a failure to achieve sufficient levels of bioactive exendin-4 in vivo.

Exogenous GLP-1/exendin-4 treatment has been shown to reduce both fasting and postprandial blood glucose levels and enhance glucose-stimulated insulin secretion in both human and rodent studies (1, 41–46). In complementary experiments, mice with a targeted disruption of the GLP-1 receptor gene exhibit mild fasting hyperglycemia (32), and immunoneutralization or blockade of GLP-1 action increased fasting blood glucose in baboon, rodent, and human studies (47–49). These findings implicate an important role for basal GLP-1 signaling, even in the fasting state, for control of glucose homeostasis. Although basal levels of circulating exendin-4 were clearly detectable in MT-exendin mice, fasting blood glucose was nor-
Furthermore, hypoglycemia was not observed in MT-exendin mice despite further induction of transgene expression with zinc. As exendin-4 has been estimated to be up to 5000 times more potent than GLP-1 with respect to glucose lowering in vivo (11), our findings of normoglycemia in MT-Ex mice further emphasize the glucose dependence of GLP-1R signaling for glucoregulation in vivo (1, 46).

Although incretins such as gastric inhibitory polypeptide and GLP-1 have been proposed as possible treatments for patients with diabetes, short term infusion of gastric inhibitory polypeptide has been associated with diminished effectiveness in diabetic patients (50) and desensitization of the gastric inhibitory polypeptide receptor in diabetic rats in vivo (51). Both homologous and heterologous desensitization of GLP-1 receptor signaling has also been observed in islet cell lines in vitro (52–54). However, daily administration of exendin-4 to diabetic mice for 13 weeks reduced levels of blood glucose and decreased glycosylated hemoglobin and increased plasma insulin (13), demonstrating that a single daily exendin-4 injection does not produce significant desensitization in vivo. The results of our studies in MT-exendin transgenic mice extend these observations by demonstrating that despite continuous exposure to transgene-derived exendin-4 for several months, acute induction of transgene expression in older mice led to reduced glycemic excursion and significantly increased levels of glucose-stimulated insulin following oral glucose challenge. These findings suggest that ongoing continuous exposure to exendin-4 in the mouse is not associated with significant impairment of GLP-1 receptor-dependent actions, such as loss of the glucose-lowering effects of exendin-4 in vivo. Nevertheless, whether β-cell desensitization to GLP-1 receptor agonists will prove to be an issue in long term human studies cannot be inferred from our transgenic mouse studies.

The physiological importance of GLP-1 receptor signaling for control of food and water intake remains unclear (29); however, several studies have demonstrated that exogenous administration of GLP-1 or exendin-4 clearly reduces food intake. Intracerebroventricular administration of GLP-1 reduced short but not long term food and water intake (17, 30, 55, 56), whereas peripheral GLP-1 administration inhibited water intake but had no effect on feeding in rodents (17). In both normal and type 2 diabetic humans, intravenous administration of GLP-1 was found to promote satiety and reduce energy intake (56, 57).

Although chronic intracerebroventricular administration of exendin (9–39) increased feeding and weight gain in rats (31), we found no evidence for sustained dysregulation of food intake or change in body weight in MT-exendin transgenic mice. The effects of exendin-4 on food intake may be related to the mode and timing of exendin-4 delivery and the variation in the levels of systemic exendin-4. Rats treated with a single daily dose of exendin-4 exhibited no significant changes in food intake or body weight after the first few days of exendin-4 administra-
tion, whereas twice daily exendin-4 dosing led to a sustained reduction in food intake and body weight (18). In contrast, basal transgenic expression of exendin-4 in MT-Ex mice was associated with a significant reduction in short term water intake; however, only induced (but not basal) exendin-4 expression was associated with a significant reduction in short term food intake. These findings have implications for future studies designed to deliver therapeutic levels of exendin-4 that promote sustained reductions in food intake and body weight over a long term treatment period.

Several experiments implicate a role for exogenous exendin-4 in the induction of β-cell neogenesis and proliferation. Treatment of pancreatic AR42J cells with exendin-4 induced differentiation into insulin-secreting islet cells (15), and exendin-4 stimulated β-cell replication and neogenesis, enhanced ductal pdx-1 expression, and improved glucose control in rats and mice (14, 16). In contrast, we observed no differences in islet morphology or β-cell mass in normoglycemic MT-exendin-4 transgenic mice. The findings of normal islet histology in MT-exendin-4 transgenic mice may reflect the need for ad-
The study of exendin-4 transgenic mice has been pivotal in elucidating the physiological roles of GLP-1 in regulating food intake and body weight. These models have been instrumental in understanding the potential therapeutic efficacy of GLP-1 receptor agonists. The long-acting GLP-1 analogues and their transgenic counterparts have provided insights into the mechanisms underlying their anorectic and hypoglycemic effects. For instance, the sustained expression of exendin-4 in transgenic mice has been shown to lead to reductions in food intake, body weight, and blood glucose levels, thereby mimicking the effects observed in vivo. These findings have underscored the importance of GLP-1 signaling in the treatment of diabetes and other metabolic disorders.

Moreover, the metabolic outcomes observed in MT-exendin transgenic mice suggest that bioactive exendin-4 expression alone may not be sufficient for the induction of islet proliferation. The data presented in the figure, which shows that sustained activation of GLP-1 receptor signaling is essential for the development of beta-cell mass in these mice. This is supported by the observation that exendin-4 expression in the mouse pancreas is not sufficient for the induction of beta-cell proliferation or neogenesis. These results imply that additional factors, such as cellular context and environmental cues, may play critical roles in the functional implications of GLP-1 signaling.

In conclusion, the use of exendin-4 transgenic mice as a model system has provided valuable insights into the physiological consequences of prolonged GLP-1 expression. These models continue to be a valuable tool for the investigation of GLP-1 receptor signaling and its potential therapeutic applications in the treatment of diabetes and other metabolic conditions.
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