Sustained Expression of Exendin-4 Does Not Perturb Glucose Homeostasis, β-Cell Mass, or Food Intake in Metallothioninein-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 t1/2 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),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, mammalian GLP-1, a mammalian exendin-4 gene has not yet been identified (7, 12).

The finding that exendin-4 represents a potent GLP-1 analogue has prompted studies of exendin-4 activity in normal and diabetic rodents. Exendin-4 potentiates glucose-stimulated insulin secretion and lowers blood glucose in both rats and mice (11, 13–16). Exendin-4 also inhibits food and water intake, raising the possibility that chronic exendin-4 treatment may decrease satiety and promote weight loss in vivo (17, 18). Furthermore, recent studies demonstrate that exendin-4 administration leads to induction of pancreatic endocrine cell differentiation, islet proliferation, and expansion of β-cell mass (11, 13–16).

Although the biological activities of exendin-4 and GLP-1 have been examined in numerous short term studies, limited information is available regarding the physiological actions of these peptides in experimental paradigms characterized by prolonged exposure to increased levels of GLP-1R agonists. To assess the feasibility and physiological effects of chronic expression of lizard exendin-4 in vivo, we have generated transgenic mice in which lizard exendin-4 expression is under the control of the mouse metallothionein I promoter. We now report the characterization and metabolic consequences of sustained exendin-4 expression in mice in vivo.
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**MATERIALS AND METHODS**

**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 electroeluted from a 1% (w/v) agarose gel and further purified on an Elutip-D column (Schleicher & Schuell). Transgenic mice were generated by Charsalis (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 mM 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% Drotin 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 BioRadisil column (21) for on-column elution for exendin-like immunoreactivity. Another fractionation was carried out using a rabbit anti-exendin-4 antiserum (Cocalico Biologicals Inc., Reamstown, PA), synthetic exendin-4 (California Peptide Research Inc., Napa, CA) as standard, and 125II-exendin-4, prepared by the chloramine T method (20, 21).

**Glucose Tolerance Tests and Measurement of Plasma Insulin Levels**—Ox and intraperitoneal glucose tolerance tests were carried out following overnight fast (16–18 h). Glucose (1.5 mg/ml body weight) was administered orally through a gavage tube or via injection into the peritoneal cavity. Blood was drawn from a tail vein at 0, 10, 20, 30, 60, and 120 min after glucose administration, and blood glucose levels were measured by the glucose oxidase method using a One Touch Basic Glucometer (Lifescan Ltd., Burnaby, British Columbia, Canada). To measure plasma insulin, a blood sample was removed from the tail vein during the 10–20 min time period following oral or intraperitoneal glucose administration. Plasma was assayed for insulin content using a rat insulin enzyme-linked immunosorbent assay kit (Crystal Chem Inc., Chicago, IL) with mouse insulin as a standard.

**Measurement of Food and Water Intake**—For feeding studies, mice were fasted for 18 h and then placed into individual cages containing preweighed rodent chow, with free access to water. The indicated time points were recorded. The food and water intake (mg) was calculated. Food intake was monitored for a total of 24 h. For drinking studies, mice were water deprived for 13 h and then placed into individual cages containing preweighed water bottles, with free access to food. At 0.5, 1, 2, and 24 h, the water bottles were weighed, and water intake (ml) was determined.

**Estimation of β-Cell Mass**—The entire pancreas was removed, fixed overnight in either 10% buffered formalin or 4% paraformaldehyde, and embedded in paraffin. Sections were obtained and stained with hematoxylin and eosin using standard protocols. Immunostaining for insulin and glucagon was carried out as described previously (22–24).

**Histology and Immunohistochemistry**—The pancreas was removed, fixed overnight in either 10% buffered formalin or 4% paraformaldehyde, and embedded in paraffin. Paraffin sections were cut and stained with hematoxylin and eosin using standard protocols. Immunostaining for insulin and glucagon was carried out as described previously (22–24).

**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 proexendin-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 transgene 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.** 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. 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 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. As GLP-1 receptor signaling is essential for control of blood glucose and glucose-stimulated insulin secretion (1), we examined these parameters in control and control-exendin 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 excision and glucose-stimulated insulin was comparable in +/- and MT-Ex

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2 D. J. Drucker and P. L. Brubaker, unpublished observations.
3 P. L. Brubaker, manuscript in preparation.
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FIG. 2. Detection of exendin-4-like immunoreactivity (exendin IR) in the plasma of transgenic mice. A, radioimmunoassay for detection of exendin-like immunoreactivity in plasma from control litters (nontransgenic) and transgenic male (MT-Ex M) and female (MT-Ex F) mice. Mice were given either standard drinking water (−Zn) or water supplemented with 25 mM ZnSO4 (+Zn) to up-regulate transgene expression. Zinc supplementation was for a period of 72 h. Values are expressed as means ± S.E. **p < .01, transgenic versus control (nontransgenic). B, HPLC elution profile of exendin-like immunoreactivity extracted from the plasma of a 4-month-old zinc-treated MT-exendin male mouse. The elution position of synthetic exendin-4 is indicated by the arrow.

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transgenic mice following either oral (Fig. 3A) or intraperitoneal (Fig. 3C) glucose challenge. In contrast, induction of transgene expression with zinc treatment resulted in a significant reduction in glycemic excursion following oral (Fig. 3B) and intraperitoneal (Fig. 3D) glucose loading. The reduced glycemic excursion was associated with a significant increase in plasma levels of glucose-stimulated insulin after oral but not intraperitoneal glucose challenge (0.38 ± 0.04 versus 0.21 ± 0.02 ng/ml, for insulin in Mt-Ex versus control mouse, respectively; Fig. 3B).

The physiological importance of GLP-1 receptor signaling for central nervous system control of food intake and body weight remains unclear (29). Administration of intracerebroventricular GLP-1 or exendin-4 inhibits short term feeding, whereas repeated administration of the GLP-1 receptor antagonist exendin (9–39) increases food intake and promotes weight gain in rats (30, 31). In contrast, mice with complete disruption of GLP-1R signaling do not exhibit defects in feeding control or body weight homeostasis (32, 33). Basal levels of exendin precursor in murine tissues with the correct processing and secretion of the lizard preproexendin-4 are expressed as means ± S.E. **p < .01, transgenic versus control (nontransgenic). B, HPLC elution profile of exendin-like immunoreactivity extracted from the plasma of a 4-month-old zinc-treated MT-exendin male mouse. The elution position of synthetic exendin-4 is indicated by the arrow.

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The observation that GLP-1 exhibits a very short plasma half-life due to its rapid degradation by dipeptidyl peptidase IV (2, 3) has prompted a search for DP IV-resistant GLP-1 analogues that exhibit longer durations of action and enhanced potency in vivo. Several GLP-1 analogues have now been reported that exhibit improved potency in both normal and diabetic rodents (5, 35). Furthermore, fatty acid derivatives of GLP-1 may also result in enhanced albumin binding and more prolonged bioactivity in vivo (36). The naturally occurring lizard exendin-4 peptide is not a substrate for DP IV and consequently exhibits a much longer half-life and greater potency in vivo (9, 11, 13).

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 prolonged 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-
mal. 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-

![Fig. 3](image-url). Oral and intraperitoneal glucose tolerance and levels of plasma insulin in control and MT-exendin transgenic female mice. Values, averaged over three independent experiments, are expressed as means ± S.E.; n = 8–12 mice/group, * p < 0.05, transgenic versus control mice. A, oral glucose tolerance in control (open circles) and MT-exendin (solid squares) mice. Plasma insulin concentrations (inset) following oral glucose in control (open bar) and MT-exendin (solid bar) mice were measured in plasma obtained at the 10–20 min time point. B, oral glucose tolerance in control (open circles) and MT-exendin (solid squares) mice following treatment with 25 mM ZnSO₄ to up-regulate transgene expression. Plasma insulin concentrations (inset) in control (open bar) and MT-exendin (hatched bar) mice were obtained at the 10–20 min time point following oral glucose. C, intraperitoneal glucose tolerance in control (open circles) and MT-exendin (solid squares) mice. Plasma insulin concentration (inset) were obtained in samples obtained at the 10–20 min time point following intraperitoneal glucose in control (open bar) and MT-exendin (hatched bar) mice.

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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-
Fig. 6. Normal islet morphology and β-cell mass in MT-exendin transgenic mice. Hematoxylin & eosin (H&E) and immunohistochemical staining for glucagon and insulin in the pancreatic islets of control (A) and MT-exendin transgenic (B) mice. Pancreata were obtained from control and transgenic animals that were given either standard drinking water (Zn+−) or water supplemented with 25 mM ZnSO4 (Zn++) for 5−7 days to up-regulate transgene expression. C, β-cell mass in control (open bars) and MT-exendin transgenic (solid bars) mice. Values are expressed as means ± S.E.; n = 3–8 mice/group. All mice were maintained on water supplemented with 25 mM ZnSO4 for 5−7 days to up-regulate transgene expression.


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