Extrapancreatic Action of Truncated Glucagon-Like Peptide-I in Otsuka Long-Evans Tokushima Fatty Rats, an Animal Model for Non–Insulin-Dependent Diabetes Mellitus

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To clarify the mechanism(s) of the antidiabetic effects of truncated glucagon-like peptide-1 (GLP-1) in diabetics, we examined its insulinotropic and extrapancreatic effects in a newly established strain of spontaneously non–insulin-dependent diabetic (NIDDM) rats, Otsuka Long-Evans Tokushima Fatty (OLETF) rats, that received a continuous infusion of truncated GLP-1 620 pmol/d/kg (G group, n = 12) or of vehicle (V group, n = 12) for 4 weeks by Alzet pump. Nonfasting plasma glucose levels were significantly lower ($P < .05$) in the G group than in the V group (7.0 ± 0.67 *v* 9.1 ± 1.7 mmol/L), and fasting plasma immunoreactive insulin (IRI) levels were lower in the former than in the latter (0.63 ± 0.31 *v* 0.78 ± 0.25 nmol/L). At day 15 of infusion, the G group showed an attenuated plasma glucose response to an oral glucose load, but had plasma IRI levels comparable to those in the V group. A long-term infusion of truncated GLP-1 increased the glucose infusion rate (GIR) significantly ($P < .05$) during a euglycemic-hyperinsulinemic clamp test (59.0 ± 14.8 μmol/kg/min for group G *v* 38.9 ± 12.2 for group V), but hepatic glucose output (HGO) did not differ significantly for either group. Uptake of 2-deoxy-D-glucose (2DG) by peripheral muscles in the G group was as much as 2.4-fold higher than in the V group (5.52 ± 2.04 *v* 2.29 ± 0.97 μmol/100 g muscle weight/min). We conclude from these data that truncated GLP-1, in addition to its well-known incretin effect, is capable of augmenting insulin action in peripheral tissues of diabetics, which can contribute, in part, to improve glucose intolerance in OLETF rats.

GLUCAGON-LIKE PEPTIDE-1 (7-36) amide (truncated GLP-1) is a potent insulinotropic hormone that is released from epithelial cells in the gastrointestinal tract in response to food intake. Truncated GLP-1 is known to reduce plasma glucagon and somatostatin levels.[1,2] The insulinotropic and glucagonostatic effects of this peptide do not fully account for the increased glucose utilization induced by an infusion of this agent. These facts suggest that truncated GLP-1 may improve insulin sensitivity by modulating the insulin or glucose effect or its direct effect on peripheral tissues. Indeed, it has been recently reported that truncated GLP-1 is capable of affecting skeletal muscle[3] and adipose tissue[4,5] in vitro, and that type I diabetic subjects treated with truncated GLP-1 showed higher rates of glucose disposal than untreated subjects.[6] Truncated GLP-1 per se may therefore promote glucose uptake in peripheral tissues, in addition to augmenting insulin release. However, the mechanisms by which it reduces blood glucose levels in diabetic subjects who have both impaired insulin secretion and insulin resistance remain unclear. A rat model for non–insulin-dependent diabetes mellitus (NIDDM), Otsuka Long-Evans Tokushima Fatty (OLETF), showed insulin resistance with hyperinsulinemia, which is common to NIDDM patients. A reasonable assumption for this is that the insulinotropic action of truncated GLP-1 is obscured by the prevailing insulin resistance under these conditions, which would result in no apparent decrease in blood glucose levels. Furthermore, studies of the biological effects of GLP-1 thus far reported have involved only short-term administration of this agent, where its hypoglycemic effect was exerted mainly via an insulinotropic action.

To clarify this issue, we report herein an evaluation of the effects of long-term continuous infusion of truncated GLP-1 on glucose tolerance, insulin responsiveness, and glucose disposal rate (Gd) in OLETF rats.

MATERIALS AND METHODS

Animals

A spontaneously diabetic rat with polyuria, polydipsia, and slight obesity was discovered in an outbred colony of Long-Evans rats that had been purchased from Charles River, Canada in 1983 and subsequently maintained at the Tokushima Research Institute of Otsuka Pharmaceutical (Tokushima, Japan). After 20 generations of selective breeding, the diabetic strain, OLETF, was established in 1990.[7] As reported by Kawano et al,[7] the cumulative incidence of diabetes in 23-week-old male and female OLETF rats is 86.0% and 0%, respectively. A nondiabetic strain, Long-Evans Tokushima Otsuka (LETO), was used as a nondiabetic control. Twelve male OLETF and 12 male LETO rats were obtained from Tokushima Research Institute (Otsuka Pharmaceutical) and maintained in our animal facilities (Institute for Animal Experimentation, University of Tokushima) under specific pathogen-free conditions at controlled temperature (21° ± 2°C), humidity (55% ± 5%), and lighting (7:00 AM to 7:00 PM).

Experimental Design

OLETF and LETO rats at 20 weeks of age were randomly assigned to two groups of 12 rats each. One group (G group) was administered a

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continuous subcutaneous infusion of truncated GLP-1 (7-36) amide (620 pmol/kg/d) using a miniosmotic ALZET pump (model 2002; ALZA, Palo Alto, CA) for 4 weeks, while the other group (V group) received an infusion of the identical solution that contained no truncated GLP-1 (7-36) and served as the control group. Blood sampling for determination of nonfasting plasma GLP-1 was performed just before and at day 12 of continuous infusion.

Oral Glucose Tolerance Test

At day 12 of the infusion, a plastic catheter was inserted into the femoral vein in half the rats for collection of blood for determination of nonfasting blood glucose and immunoreactive insulin (IRI) levels. The catheter was kept patent by infusion with heparinized saline. After an overnight fast at day 15 of infusion, 2 g glucose/kg was administered orally through a metal tube while the animals were awake, and venous blood was drawn from the catheter at 0, 30, 60, and 120 minutes for measurement of blood glucose and plasma IRI and immunoreactive glucagon (IRG) levels. Rats were judged to be diabetic if their peak blood glucose was at least 16.7 mmol/L and the 120 minutes blood glucose was at least 11.1 mmol/L, according to the criteria used by Kawano et al.[7] They were judged to have impaired glucose tolerance if either one of the two exceeded the values just listed.

Measurement of In Vivo Glucose Disposal by a Hyperinsulinemic-Euglycemic Clamp Test

At day 22 to 24 of infusion, insulin-mediated whole-body glucose uptake was measured in anesthetized rats using a euglycemic clamp technique[8] in the rats in which an oral glucose tolerance test (OGTT) had been performed. After an overnight fast, six rats from each group were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and catheters were inserted into the femoral vein. The rats received an infusion of insulin (Novo Nordisk, Bagsvaerd, Denmark) at a rate of 60 pmol/kg/min over a 1-hour period. An infusion of 100 g/L glucose solution was started at time zero, and the rate was adjusted to clamp plasma glucose at approximately 6.1 mmol/L. Blood samples for determination of glucose were obtained at 2- to 5-minute intervals throughout the study. Data on total-body glucose uptake represent the mean values for glucose infusion rate (GIR) during the last 20 minutes.

Measurement of Hepatic Glucose Output

Hepatic glucose output (HGO) was measured in the same group of rats during the hyperinsulinemic-euglycemic clamp test. At time zero, 2.5 μCi (10 μCi/mL) D-[U-^{14}C]glucose (Amersham, Amersham, UK) was infused as a bolus, followed by continuous infusion at a rate of 10 μCi/h. Blood samples for determination of D-[U-^{14}C]glucose–specific activity were obtained at 55 and 60 minutes. Blood was deproteinized in $Ba(OH)_2/ZnSO_4$ as described by Somogyi[9] and centrifuged (2 minutes at 16,000 × *g*). D-[U-^{14}C]glucose in the supernatant was determined by liquid-scintillation counting (LSC-700; Aloka, Tokyo, Japan). The glucose disappearance rate (Gd) and HGO were determined by Steel's method.[10] Gd and HGO were calculated as follows:

$$Gd = \frac{\text{blood glucose (mg/dL)} \times \text{flow rate (μL/min)} \times \text{total count/plasma count}}{\text{body weight (kg)}} \times 0.2 \times 10^{-4}$$

$$\text{and } Gd - GIR = HGO.$$

The average for HGO at 55 and 60 minutes was calculated.

Determination of Insulin-Stimulated Glucose Utilization Index in Muscle Tissue

The glucose utilization index of skeletal muscle was measured in the same group of animals during the euglycemic-hyperinsulinemic clamp test using the 2-deoxy-D-[1-^{3}H]glucose ([^{3}H]2DG) technique as described by Ferre et al[11] and James et al.[12] In this procedure, [^{3}H]2DG (25 μCi, 1 mCi/mL; Amersham) was injected in 250 μL 0.9% NaCl as a bolus through the femoral vein. A blood sample (50 μL) for determination of the plasma tracer concentration was obtained at 60 minutes after bolus administration. At the end of the clamp test, rats were rapidly anesthetized with pentobarbital (60 mg/kg), and the skeletal muscle tissue was removed and frozen in liquid N_2. Blood was deproteinized in $Ba(OH)_2/ZnSO_4$ as described earlier, and the supernatant was used for determination of [^{3}H]2DG by liquid scintillation counting. The tissue sample was weighed and placed into 1 mol/L NaOH (2.5 mL/1 g tissue) and heated at 60°C for 45 minutes to totally digest the tissue, after which 1 mol/L HCl (2.5 mL/1 g tissue) was added. One milliliter of 6% $HClO_4$ was added to a 200-μL aliquot of the neutralized solution, and 1 mL $Ba(OH)_2/ZnSO_4$ was added to another 200-μL aliquot of the solution. After centrifugation, the supernatants (800 μL) of these solutions were used for determination of radioactivity after adding 10 mL scintillation solution (Atomlight; Biotechnology Systems, Boston, MA) by liquid scintillation counting. Because 2-deoxyglucose and 2-deoxyglucose 6-phosphate are both soluble in 6% $HClO_4$ and only 2-deoxyglucose is soluble in the Somogyi reagent [$Ba(OH)_2/ZnSO_4$], the content of [^{3}H]2DG 6-phosphate ([^{3}H]2DGP) in muscle tissue was obtained by subtracting the radioactivity (dpm) in the $Ba(OH)_2/ZnSO_4$ supernatant from that in the $HClO_4$ supernatant. Tissue glucose uptake (defined as the glucose metabolic index, Rg′) was calculated using the following equation described by Kraegen et al[13]:

$$Rg' \text{ (mol/100 g/min)} = \frac{Cp \times Cm^* \text{ (60)}}{\int_0^{60} Cp^*(t)\, dt}$$

where Cp is the steady-state plasma glucose concentration over a 60-minute period of observation (mmol/L), Cm* is tissue accumulation of [^{3}H]2DGP per unit mass at 60 minutes (dpm/mg wet weight), Cp*(t) is plasma [^{3}H]2DG concentration (dpm/mL), and *t* equals 0 when the tracer is administered as a bolus.

Assays

Blood glucose levels were determined by the glucose oxidase method (Fuji Dri-Chem 2000; Fuji Medical System, Tokyo, Japan). Insulin level was measured with a commercial kit (Daiichi Radioisotope, Tokyo, Japan) using rat insulin as a standard (Novo Nordisk, Bagsvaerd, Denmark). Radioimmunoassay of glucagon immunoreactivity (IRG) was performed with antiserum OAL 123 (Otsuka Pharmaceutical, Tokushima, Japan), which is directed toward the C-terminal of glucagon and recognizes glucagon but not glicentin or oxyntmodulin.[14] GLP-1 was determined radioimmunologically using specific antiserum against GLP-1 raised in rabbit and commercially available anti–rabbit immunoglobulin goat serum. The antibody showed 100% cross-reactivity with GLP-1(7-36) amide, GLP-1(7-37), and GLP-1(1-37) and 77% cross-reactivity with GLP-1(1-36) amide, but did not cross-react with glucagon, GIP, VIP, and other proglucagon products.[15] The sensitivity of the assay was 12 fmol/mL, and interassay and intraassay coefficients of variation were 4.5% and 5.0%, respectively.

Statistical Analysis

Data are expressed as the mean ± SD unless otherwise indicated. Significance was determined by ANOVA, followed by the multiple *t* test for individual comparison of means.

RESULTS

Plasma GLP-1 Levels Before and During Continuous Infusion of Truncated GLP-1

Plasma GLP-1 of OLETF rats was significantly higher than that of LETO rats ($P < .05$). Plasma GLP-1 during continuous infusion of truncated GLP-1 increased approximately twofold over preinfusion levels in both strains (112.3 ± 20.4 *v* 59.4 ± 8.2 pmol/L in OLETF rats and 90.8 ± 17.6 *v* 42.8 ± 5.9 pmol/L in LETO rats).

Fasting and Nonfasting Blood Glucose and Plasma IRI Levels

Figure 1 shows nonfasting and fasting blood glucose and plasma IRI levels at days 12 and 15 of continuous infusion of truncated GLP-1. In OLETF rats, the G group showed significantly ($P < .05$) lower nonfasting blood glucose levels compared with the V group (7.03 ± 0.70 *v* 9.12 ± 1.73 mmol/L), whereas there was no significant difference in fasting blood glucose levels between the groups (6.08 ± 0.50 *v* 6.92 ± 0.63 mmol/L). Nonfasting plasma IRI levels in the G group were slightly but not significantly higher than in the V group (2.86 ± 0.91 *v* 2.25 ± 0.72 nmol/L), whereas fasting plasma IRI levels in the G group were slightly lower than in the V group (0.63 ± 0.31 *v* 0.78 ± 0.25 nmol/L). No differences were observed for plasma glucose and IRI levels in either the nonfasting or fasting state, nor were differences observed between the G group and V group of LETO rats (Fig 1).

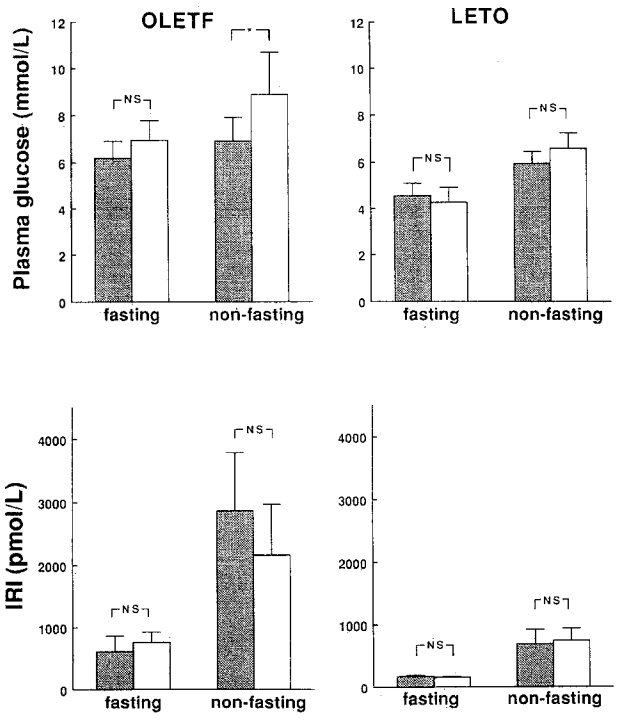

Fig 1. Fasting and nonfasting plasma glucose and IRI levels in OLETF and LETO rats with a continuous infusion of truncated GLP-1 (■) and vehicle (□). *$P < .05$; NS, no significant difference. Blood samples were obtained at days 12 and 15 of infusion.

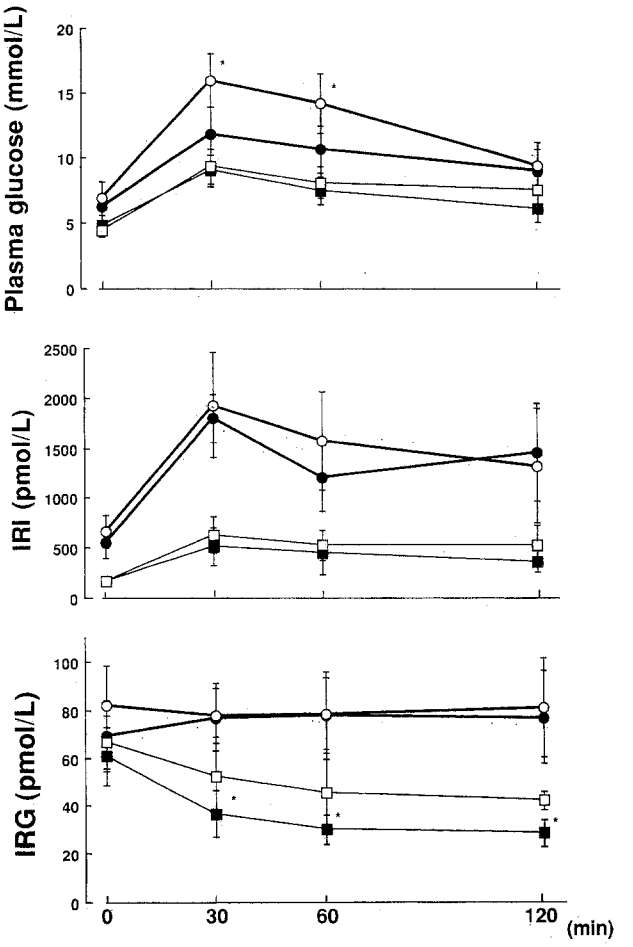

Fig 2. Plasma glucose, IRI, and IRG responses to an oral glucose load in OLETF with GLP-1 (●) or vehicle (○) and in LETO with GLP-1 (■) or vehicle (□). An OGTT was performed at day 15 of infusion, and blood samples were obtained from the catheter inserted into the femoral vein 3 days before OGTT. *$P < .05$, G *v* V group in the same strain.

Glucose Tolerance and Plasma IRI and IRG Responses to Oral Glucose

Plasma glucose, IRI, and IRG responses to an oral glucose load are shown in Fig 2. In OLETF rats, plasma glucose levels at 30 and 60 minutes for the G group were significantly lower than for the V group. However, in LETO rats, plasma glucose levels in the G group were similar to those in the V group at all time points. Based on our criteria for diabetes mellitus, all OLETF rats of the G group showed normal glucose tolerance, whereas 70% of OLETF rats of the V group showed impaired glucose tolerance to an oral glucose load, and there is no significant difference in plasma glucose level between the G group of OLETF rats and the G or V group of LETO rats. Plasma IRI responses in both G groups were similar to those for the respective V groups in OLETF and LETO rats. In OLETF rats, plasma IRG levels were not decreased in either the G group or the V group after glucose loading. However, in LETO rats, they were significantly decreased during the OGTT in the both groups, and plasma IRG levels in the G group were significantly lower at 30, 60, and 120 minutes than the corresponding values in the V group.

In Vivo Glucose Disposal, HGO, and 2-DG Uptake in Skeletal Muscle

The GIR, HGO, and 2DG uptake in skeletal muscle in OLETF and LETO rats are shown in Fig 3. Insulin-stimulated glucose disposal in vivo was significantly lower in OLETF than in LETO rats, suggesting that the former was less sensitive to insulin than the latter. A continuous infusion of trucated GLP-1 increased the GIR significantly in OLETF rats, but it had no effect in LETO rats. No significant differences were observed for HGO among all four groups. 2DG uptake of femoral muscle in the G group was significantly higher than in the respective V group for both OLETF and LETO rats, but for OLETF rats of the G group, this value was still significantly lower compared with the V group in LETO rats (6.4 ± 1.3 *v* 2.4 ± 1.0 mol/100 g muscle weight/min in OLETF and 25.5 ± 8.0 *v* 16.9 ± 2.9 mol/100 g muscle weight/min in LETO, respectively).

DISCUSSION

In long-term studies, truncated GLP-1 caused a decrease in nonfasting but not in fasting plasma glucose levels in OLETF rats, but did not decrease either the nonfasting or fasting plasma glucose level in LETO rats. The insulinotropic action of truncated GLP-1 has been reported to be glucose-dependent.[16] In our previous study, truncated GLP-1 as high as 1.0 nmol/L showed no stimulating effect on insulin secretion from the pancreas perfused with 3.3 mmol/L D-glucose, whereas it significantly increased insulin secretion stimulated by 11.1 mmol/L D-glucose at concentrations of truncated GLP-1 as low as 100 pmol/L.[17] In the present study, the plasma glucose levels may be too low to allow for truncated GLP-1 to enhance insulin secretion in both strains, except for the nonfasting state in OLETF rats, in which the mean plasma glucose level was 8.8 ± 2.2 mmol/L. Even in these conditions, plasma IRI was slightly (but not significantly) increased by exogenous truncated GLP-1 because of its low concentration. These facts suggest that the reduced increase in plasma glucose observed in the nonfasting state in the G group of OLETF rats was induced not only by an increased insulin level, but also by its extrapancreatic effect. This hypothesis is compatible with the results during the OGTT: plasma glucose levels in the G group of OLETF rats were significantly lower than in the V group, and plasma IRI levels in the former were similar to those in the latter. This suggests that truncated GLP-1 decreases plasma glucose by reducing circulating glucagon concentration. However, this is not the case for the attenuated increase in plasma glucose after an oral glucose load in the G group of OLETF rats, since no significant decrease in plasma IRG levels was observed during the OGTT. The normal A-cell response to changes in glucose concentration is known to be impaired in diabetic individuals, ie, the glucagon response to insulin-induced hypoglycemia and the suppression of glucagon secretion by hyperglycemia.[18] These impairments of A-cell function were also observed in OLETF rats at ages greater than 16 weeks.[19] This impaired response of the pancreatic A cell to hyperglycemia in OLETF rats was not ameliorated by a continuous infusion of truncated GLP-1, although it showed a clear glucagonostatic action in LETO rats. These findings suggest that A-cell dysfunction in OLETF rats is due to its intrinsic defect and not to GLP-1 derangement. A significant decrease in plasma IRG concentration during the OGTT in the G group of LETO rats resulted in no significant decrease in plasma glucose levels. Judging from these data, it is unlikely that the effect of truncated GLP-1 in decreasing plasma glucose levels in the nonfasting state and during OGTT is exclusively the result of its effect on islet hormone secretion. Indeed, the GIR, the index of insulin sensitivity, was increased by a long-term infusion of truncated GLP-1 in OLETF but not in LETO rats. The question remains as to what mechanisms are involved when long-term administration of truncated GLP-1 significantly increases the GIR in OLETF rats. No differences in HGO were observed between the G and V groups or between OLETF and LETO rats. This is consistent with recent findings that indicate an absence of receptors for GLP-1 on hepatocytes and liver membranes[20] and no direct influence of the agent on hepatocyte function such as cAMP formation, glycogen phosphorylase A activity, and glucose release in cultured or isolated rat hepatocytes,[21-23] although it is in contradiction with some reports.[24,25] Continuous infusion of truncated GLP-1 increased 2DG uptake into muscle in both OLETF and LETO rats, but the

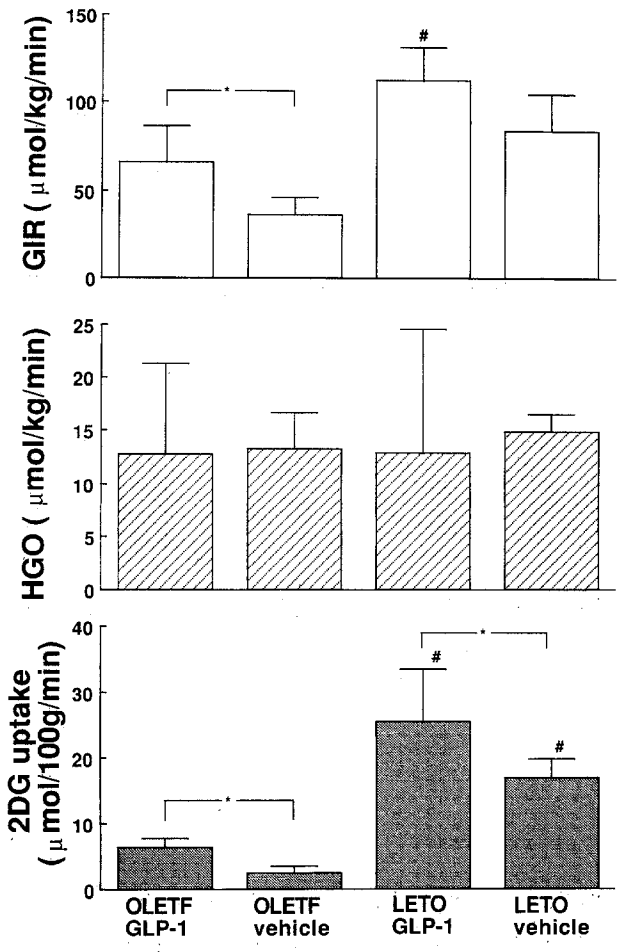

Fig 3. GIR, HGO, and 2DG uptake in skeletal muscle during hyperinsulinemic-euglycemic clamp (HEC) in 4 groups. HEC was performed at day 24 of infusion. *$P < .05$, G *v* V group in the same strain; #$P < .01$, OLETF *v* LETO with similar treatment.

rate of increase was much higher in the former than in the later (2.7-fold *v* 1.5-fold). The increased sensitivity of muscle to insulin is, in part, responsible for the increased GIR in rats treated with truncated GLP-1. Truncated GLP-1 may have some metabolic effects in adipose tissue, since receptors for the agent were found on solubilized membranes of rat epididymal adipose tissue.[26] Indeed, in explanted rat adipose tissue, GLP-1 probably stimulates fatty acid synthesis.[27] Furthermore, glucose uptake and lipogenesis in adipocytes were increased by the addition of insulin and truncated GLP-1 to culture media compared with addition of insulin alone.[4] Accordingly, truncated GLP-1 exerts its metabolic effects in adipose tissue to a greater extent in OLETF than in LETO rats, since adipose tissue is more abundant in the former. This could be attributed to the higher rate of increase in GIR in OLETF rats versus LETO rats, caused by long-term infusion of truncated GLP-1.

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