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Effect of GLP-1 Treatment on GLUT2 and GLUT4 Expression in Type 1 and Type 2 Rat Diabetic Models

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Glucagon-like peptide-1 (GLP-1) is an incretin with glucose-dependent insulinotropic and insulin-independent antidiabetic properties that exerts insulin-like effects on glucose metabolism in rat liver, skeletal muscle, and fat. This study aimed to search for the effect of a prolonged treatment, 3 ds, with GLP-1 on glucotransporter GLUT2 expression in liver, and on that of GLUT4 in skeletal muscle and fat, in rats. Normal rats and streptozotocin-induced type 1 and type 2 diabetic models were used; diabetic rats were also treated with insulin for comparison. In normal rats, GLP-1 treatment reduced in the three tissues the corresponding glucotransporter protein level, without modifying their mRNA. In the type 2 diabetic model, GLP-1, like insulin, stimulated in liver and fat only the glucotransporter translational process, while in the muscle an effect at the GLUT4 transcriptional level was also observed. In the type 1 diabetic model, GLP-1 apparently exerted in the liver only a posttranslational effect on GLUT2 expression; in muscle and fat, while insulin was shown to have an action on GLUT4 at both transcriptional and translational levels, the effect of GLP-1 was restricted to glucotransporter translation. In normal and diabetic rats, exogenous GLP-1 controlled the glucotransporter expression in extrapancreatic tissues participating in the overall glucose homeostasis—liver, muscle, and fat—where the effect of the peptide seems to be exerted only at the translational and/or posttranslational level; in muscle and fat, the presence of insulin seems to be required for GLP-1 to activate the transcriptional process. The stimulating action of GLP-1 on GLUT2 and GLUT4 expression, mRNA or protein, could be a mechanism by which, at least in part, the peptide exerts its lowering effect on blood glucose.

Key Words: Diabetes; glucotransporters; glucagon-like peptide-1.

Introduction

The facilitated transport of glucose across the plasma membrane of mammalian cells is catalyzed by a family of glucose transport proteins (GLUTs); GLUT2 is the most abundant isoform in liver and pancreatic B-cells, whereas GLUT4 is in muscle and fat tissue. GLUT2 has a high K_m value for glucose (17–20 m*M*), which ensures a high in-and-out hexose transport capacity, never saturated under normal physiologic conditions or during the hyperglycemia observed in moderate diabetes *(1)*; its expression level —protein and mRNA—is modulated by the nutritional and hormonal enviroment *(2–5)*. Results from in vitro studies, however, indicated that insulin would not be required for glucose to stimulate the glucotransporter expression in cultured rat hepatocytes *(5,6)*.

Muscle is the principal site for insulin-stimulated glucose disposal in vivo, and, although to a much lesser extent, glucose is also transported into the adipocyte *(7)*; in muscle, glucose transport is a major rate-limiting step in normal and diabetic states, the resistance to a stimulatory effect on glucose utilization being a key pathogenic feature of obesity, syndrome X, and most forms of type 2 diabetes (see ref. *8* for review). Insulin stimulates the intracellular translocation of GLUT4 from the storage vesicles to the plasma membrane, which is impaired in diabetic subjects *(9)*, raising the maximal velocity of glucose transport into the cell *(10)*. GLUT4 expression seems to be regulated in a different manner in muscle and fat *(11)*, with its protein concentration reduced in adipocytes, but not in muscle, in obese patients and those with impaired glucose tolerance or type 2 diabetes. Nevertheless, an induced overproduction of the glucotransporter in normal and diabetic mice increases glucose tolerance and insulin sensitivity *(12,13)* and also reduces hyperglycemia and enhances insulin sensitivity in the adipose tissue of streptozotocin-induced diabetic mice *(14)*. An increase in GLUT4 concentration and insulin sensitivity has been observed in the muscle of insulin-resistant and type 2 diabetic patients after exercise *(15)*.

Because a reduced insulin-stimulated glucose uptake in muscle cannot always be attributable to a decreased production of GLUT4 *(11)*, the defect leading to insulin resistance could be located in the intracellular insulin-signaling pathway, which regulates the mechanism of glucotransporter

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translocation *(16)*. Or, as already suggested, muscle GLUT4 protein may be subjected to regulation at the pretranslational level *(17)*. Insulin treatment induces a restoration of both GLUT4 mRNA and protein to control levels in streptozotocin-treated diabetic animals *(18)*, while prolonged fasting in normal rats induces a rise in the gene expression that also returns to normal after feeding is resumed *(19)*.

Since molecular defects in glucose disposal leading to diabetic states are suggested to reside within the glucose or insulin-signaling pathways *(1,20–22)*—although cause and effect are not yet well established—it is of considerable interest to elucidate possible regulators of GLUT gene expression, as well as their mode of action, for the development of new therapies to stimulate glucose storage in the liver and its uptake by adipocytes and muscle.

Glucagon-like peptide-1 (GLP-1), proposed as a possible tool for the therapy of type 2 diabetes *(23)*, is a naturally occurring glucose-dependent insulinotropic peptide, with incretin characteristics and insulin-independent antidiabetic properties *(23,24)*. GLP-1 has insulin-like effects on glucose metabolism in liver and skeletal muscle from normal *(25,26)* and diabetic *(27)* rats; in these two tissues, the peptide seems to act through specific receptors *(28–30)*, apparently different in structure or signaling pathway *(31–33)* from that in the pancreas *(34)*. In adipose tissue, where the presence of specific receptors has also been reported *(35,36)*, GLP-1 exerts a dual action, lipogenic and lipolytic, on lipid metabolism; in addition, it stimulates glycogen synthesis, glycogen synthase *a* activity, and other parameters involved in glucose metabolism *(37,38)*. Also GLP-1 has been shown to be capable of modulating glucose transporter level in cultured 3T3-L1 adipocytes *(39)*.

Here we have studied the effect of a prolonged treatment with GLP-1 on GLUT2 protein and mRNA expression in the liver, and on that of GLUT4 in skeletal muscle and adipose tissue, in normal rats and in type 1 and 2 diabetic models.

Results

Effect of GLP-1 on GLUT2 Expression in Normal and Diabetic Rats

In normal rats, 3-d treatment with GLP-1 induced a significant ($p < 0.001$) decrease (29 ± 7% normal control) in GLUT2 protein levels; in type 2 and 1 groups, both initially showing lower (51 ± 11% normal control; $p < 0.001$) and higher (146 ± 6% normal control; $p<0.001$) values, respectively, GLP-1 treatment, like that of insulin, produced a normalization of the glucotransporter protein content (Fig. 1). By Northern blot analysis, it was shown (Fig. 2) that GLUT2 mRNA expression was initially significantly higher in the type 2 group (240 ± 36% normal control; $p < 0.05$); GLP-1 treatment did not modify the glucotransporter gene expression in normal rats but reduced to normal values expression in the type 2 rats (93 ± 32% normal control; $p < 0.05$ vs type 2 control), without changing expression in the type 1 diabetic animals. Insulin treatment apparently exerted in both diabetic groups the same effects as GLP-1.

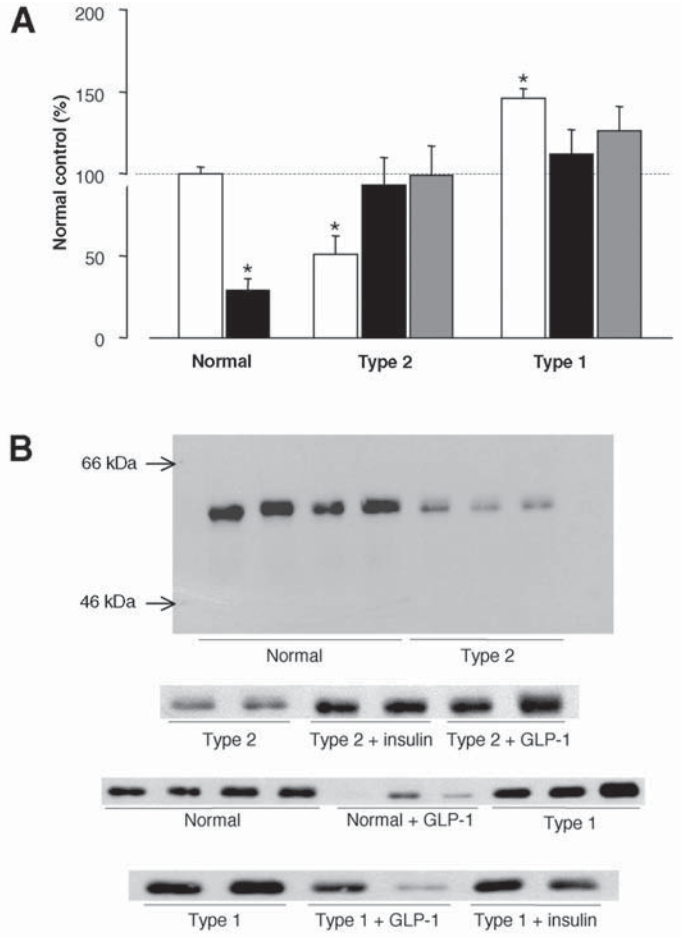

Fig. 1. Effect of a 3-d treatment with GLP-1 (■), insulin (▨), or saline (□) as control on GLUT2 protein in rat liver. (**A**) Dot blot and (**B**) a representative Western blot of 5 and 25–50 µg, respectively, total membrane protein, in normal rats ($n = 6$ to 7) and type 2 ($n = 4$ to 5) and type 1 ($n = 6$ to 7) diabetic models are shown. The results of densitometric scanning, calculated using autoradiographic exposures within the linear range, are expressed in percentage relative to normal control rats (±SEM) and were obtained from experiments done comparatively within blots. *$p < 0.001$ vs normal control.

Effect of GLP-1 on GLUT4 Expression in Normal and Diabetic Rats

Dot-blot analysis of the skeletal muscle showed (Fig. 3) that GLUT4 protein content in the type 2 diabetic group was no different than that in normal animals (76 ± 9% normal control), whereas it was clearly lower in the type 1 diabetic rats (55 ± 4% normal control; $p < 0.01$). Three days of GLP-1 treatment slightly reduced, although not to a statistically significant degree, the GLUT4 protein content in normal rats, but it increased the levels in type 2 (147 ± 26% normal control; $p < 0.05$ vs type 2 control) and also normalized those of the type 1 group (96 ± 10% normal control; $p < 0.01$ vs type 1 control). Insulin treatment exerted

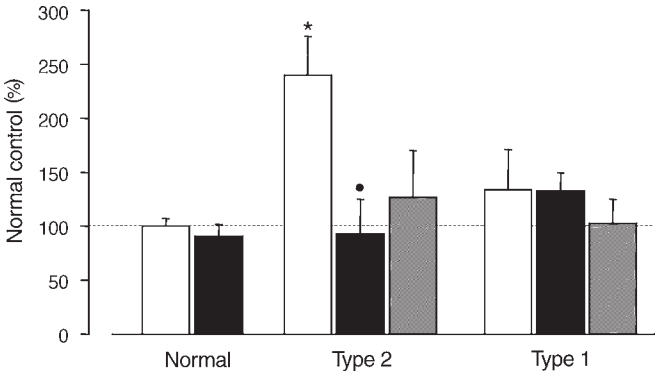

Fig. 2. Effect of a 3-d treatment with GLP-1 (■), insulin (▨), or saline (□) as control on GLUT2 mRNA expression in rat liver. Northern blot of 20 µg total RNA isolated from normal rats (n = 6) and type 2 (n = 5 to 6) and type 1 (n = 5–7) diabetic models is shown. The results of densitometric scanning are expressed in percentage relative to normal control rats (±SEM) and are normalized to β-actin for variations in gel loading. *$p < 0.05$ or lower vs normal control; •$p < 0.05$ or lower vs respective group control.

the same effect as GLP-1 on the type 2 (179 ± 16% normal control; $p < 0.001$ vs type 2 control) and type 1 groups (82 ± 5% normal control; $p < 0.01$ vs type 1 control). By Northern blot analysis, it was observed (Fig. 4) that GLUT4 mRNA expression was lower in the type 2 (42 ± 5% normal control; $p < 0.001$) and higher in the type 1 model (133 ± 8% normal control; $p < 0.01$), as compared with normal rats. GLP-1 treatment did not alter the glucotransporter gene expression levels in normal animals but normalized those in the type 2 group (79 ± 13% normal control; $p < 0.05$ vs type 2 control) and lowered, to slightly below normal, those in type 1 (70 ± 17% normal control; $p < 0.01$ vs type 1 control). With insulin, the same effect as that with GLP-1 treatment was observed in the type 2 diabetic model (101 ± 26% normal control), but a tendency to increase the gene expression was detected in type 1 diabetic rats.

In adipose tissue (Fig. 5), GLUT4 protein content was shown to be initially lower in the type 2 (58 ± 13% normal control; $p < 0.05$) and type 1 (53 ± 5% normal control; $p < 0.001$) diabetic models, as compared with normal animals; GLP-1 treatment induced in both diabetic groups a rise toward normal glucotransporter protein expression ($p < 0.05$, vs type 2 and type 1 control, respectively), whereas in normal animals a slight reduction (82 ± 5% normal control; $p < 0.05$) was detected. After 3 d of insulin treatment, a normalization of the GLUT4 protein levels in both diabetic groups (type 2: 88 ± 19% normal control; type 1: 111 ± 18% normal control; $p < 0.05$ vs type 1 control) was also observed. Northern blot analysis (Fig. 6) of the samples showed a higher, although not statistically significant, mean GLUT4 mRNA expression value in the type 2 diabetic group (187 ± 34% normal control), whereas a significantly reduced level in type 1 (64 ± 6% normal control; $p < 0.001$) was observed. GLP-1 treatment significantly lowered the glucotransporter mRNA expression levels in the

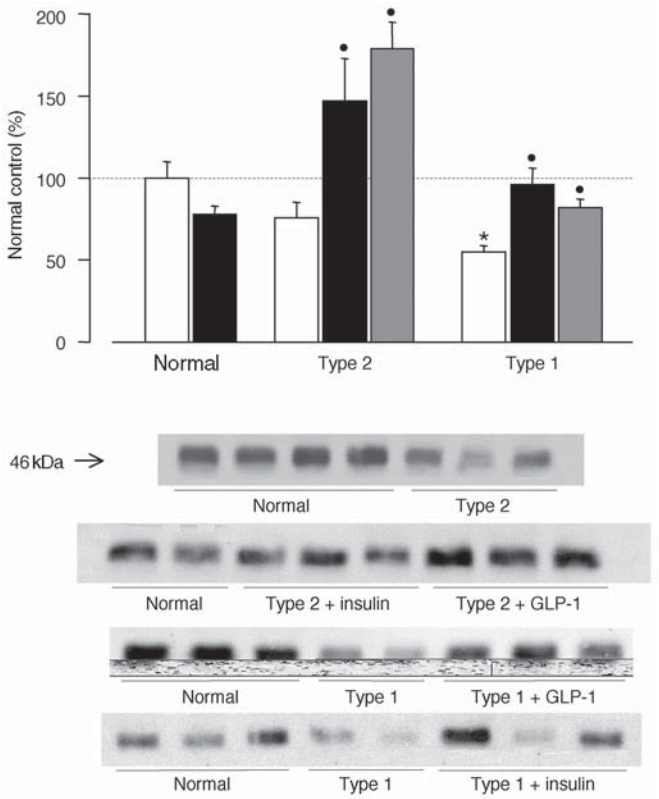

Fig. 3. Effect of a 3-d treatment with GLP-1 (■), insulin (▨), or saline (□) as control on GLUT4 protein in rat skeletal muscle. (**A**) Dot blot and (**B**) a representative Western blot of 5 and 25–50 µg, respectively, total membrane protein, in normal rats (n = 6) and type 2 (n = 6 to 7) and type 1 (n = 6 to 9) diabetic models are shown. The results of densitometric scanning, calculated using autoradiographic exposures within the linear range, are expressed in percentage relative to normal control rats (±SEM) and were obtained from experiments done comparatively within blots. *$p < 0.01$ or lower vs normal control; •$p < 0.05$ or lower vs respective group control.

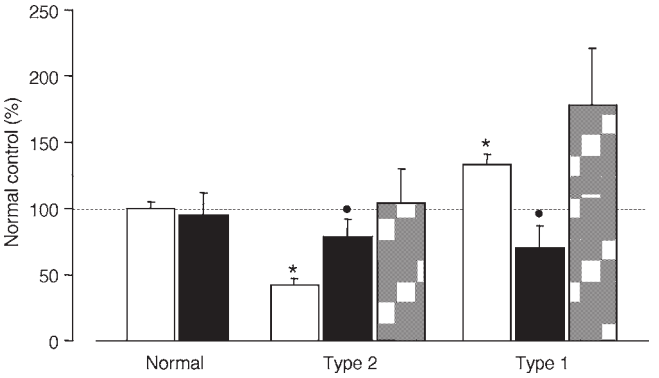

Fig. 4. Effect of a 3-d treatment with GLP-1 (■), insulin (▨), or saline (□) as control on GLUT4 mRNA expression in skeletal muscle. Northern blot of 20 µg total RNA isolated from normal rats (n = 9) and type 2 (n = 4 to 8) and type 1 (n = 6 to 9) diabetic models is shown. The results of densitometric scanning are expressed in percentage relative to normal control rats (±SEM) and are normalized to β-actin for variations in gel loading. *$p < 0.05$ or lower vs normal control; •$p < 0.05$ or lower vs respective group control.

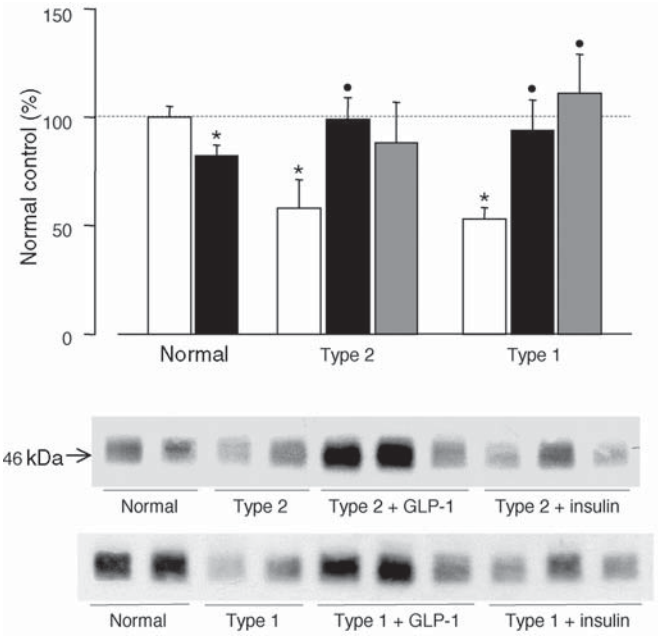

Fig. 5. Effect of a 3-d treatment with GLP-1 (■), insulin (▨), or saline (□) as control on GLUT4 protein in rat adipose tissue. (**A**) Dot blot and (**B**) a representative Western blot of 5 and 25–50 µg, respectively, total membrane protein, in normal rats ($n = 6$) and type 2 ($n = 5$ to 6) and type 1 ($n = 5$ to 7) diabetic models are shown. The results of densitometric scanning, calculated using autoradiographic exposures within the linear range, are expressed in percentage relative to normal control rats (±SEM) and were obtained from experiments done comparatively within blots. *$p < 0.05$ vs normal control; •$p < 0.05$ vs respective group control.

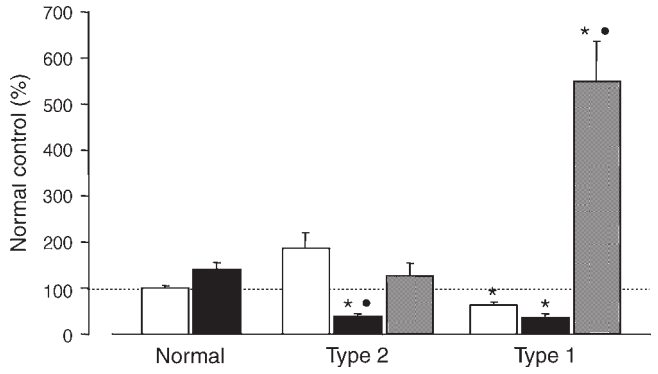

Fig. 6. Effect of a 3-d treatment with GLP-1 (■), insulin (▨), or saline (□) as control on GLUT4 mRNA expression in rat adipose tissue. Northern blot of 20 µg total RNA isolated from normal rats ($n = 5$) and type 2 ($n = 5$ to 6) and type 1 ($n = 4$ to 7) diabetic models is shown. The results of densitometric scanning are expressed in percentage relative to normal control rats (±SEM) and are normalized to β-actin for variations in gel loading. *$p < 0.05$ or lower vs normal control; •$p < 0.05$ or lower vs respective group control.

type 2 group (39 ± 5% nor-mal control; $p < 0.001$ vs both normal and type 2 control), whereas no effect was observed in either normal or type 1 diabetic animals. In contrast to GLP-1, insulin treatment did not alter the GLUT4 mRNA values in type 2 diabetic rats but highly increased those of the type 1 group (549 ± 88% normal control; $p < 0.05$ vs both normal and type 1 control).

Table 1
GLUT2 and GLUT4 in Normal and Diabetic Rats Before and After 3-d Treatment with Saline, GLP-1, or Insulin in Relation to Normal Saline-Treated Animals (control) [a]

	Normal	Type 2	Type 1
Liver GLUT2 protein/mRNA			
Saline	—/—	↓/↑	↑/—
GLP-1	↓/—	—/—	—/—
Insulin		—/—	—/—
Muscle GLUT4 protein/mRNA			
Saline	—/—	↓/↓	↓/↑
GLP-1	↓/—	↑/↓	—/↓
Insulin		↑/—	—/↑
Fat GLUT4 protein/mRNA			
Saline	—/—	↓/↑	↓/↓
GLP-1	↓/—	—/↓	—/↓
Insulin		—/—	—/↑

[a] —, Equal to control; ↑ and ↓, significantly higher and lower, respectively, than control; —↑ and ↓—, slightly higher and lower, respectively, than control.

Table 1 summarizes the relative changes in GLUT2 and GLUT4 expression in the three groups of rats studied—normal and type 1 and 2 diabetic models—after GLP-1 or insulin treatment.

Discussion

The glucotransporter isoforms GLUT2 and GLUT4 play central roles in the complex pathways mediating whole-body glucose disposal, in which disregulation of their controlling mechanism can result in the pathophysiologic states associated with diabetes. The two diabetic animal models used in this study, streptozotocin-treated at birth (type 2) and at adult age (type 1), showed, in general, liver, muscle, and fat glucotransporter expression levels in agreement with those reported by others in various diabetic animal models *(8,11,40–43)*. Increased GLUT2 protein level in the liver of diabetic Zucker fa/fa rats and viable yellow mouse *(44)*, and a reduced GLUT4 mRNA value in the muscle of type 1 diabetic rats up to 4 wk after streptozotocin injection *(18,45)*, were reported. These discrepancies with our results could be attributed to differences in the diabetic model used, or to the severity of the diabetic state, since opposite changes have been observed in liver GLUT2 mRNA and protein depending on the time elapsed after streptozotocin injection *(3)*.

In normal rats, GLP-1 seems to act at the glucotransporter posttranslational level in liver, muscle, and fat. In these tissues, an eventual depletion of the membrane glucotransporter protein could occur owing to the sustained stimulus exerted on it, since a decrease in the corresponding protein values, with no accompaning changes in their

Table 2
Plasma Glucose, GLP-1, and Insulin in Normal and in Type 2 and Type 1 Diabetic Rats Before and Finishing 3-d Treatment with GLP-1 or Insulin[a]

	Pretreated	GLP-1 treated	Insulin treated
Normal			
Glucose (mg/dL)	121 ± 4 (9)	112 ± 3 (9)	
Insulin (ng/mL)	1.8 ± 0.3 (9)	2.2 ± 0.3 (9)	
GLP-1 (ng/mL)	0.32 ± 0.03 (9)	0.91 ± 0.29 (9)[b]	
Type 2			
Glucose (mg/dL)	140 ± 8 (16)	112 ± 2 (9)[b]	93 ± 30 (7)
Insulin (ng/mL)	1.6 ± 0.1 (16)	1.9 ± 0.2 (9)	3.1 ± 0.1 (3)[b]
GLP-1 (ng/mL)	0.15 ± 0.02 (16)	0.63 ± 0.06 (3)[b]	0.13 ± 0.02 (4)
Type 1			
Glucose (mg/dL)	650 ± 76 (15)	538 ± 14 (6)	437 ± 27 (9)[b]
Insulin (ng/mL)	0.5 ± 0.2 (15)	0.4 ± 0.1 (6)	1.8 ± 0.5 (4)[b]
GLP-1 (ng/mL)	0.46 ± 0.04 (15)	0.97 ± 0.03 (4)[b]	0.31 ± 0.01 (4)

[a]Data presented are the mean ±SEM. Numbers in parentheses are the number of rats.
[b]$p < 0.05$ or lower vs respective pretreated group control.

mRNA expression, was detected after treatment. Although in this group of experiments no significant increase in plasma insulin was observed in a sample taken at the end of the treatment (Table 2), its levels during the study could have been elevated, and, therefore, a participation of endogenous insulin in the variations of glucotransporter levels cannot be excluded.

In type 2 diabetic rats, GLP-1, as well as insulin treatment, seems to act in liver GLUT2 and fat GLUT4 expression at the posttranscriptional level, by stimulating the translational process toward normalization of glucotransporter protein values; in the muscle, the effect of GLP-1 on GLUT4 expression, like that of insulin, seems to be exerted at the transcriptional as well as translational level. In addition, although in this diabetic group no major change in plasma insulin concentration was detected at the end of GLP-1 treatment (Table 2), an insulin-mediating participation in the GLP-1 effect cannot be ruled out, because we have observed, in another study in the same diabetic model, a clear increase in insulin secretion after an acute administration of GLP-1 *(46)*.

In type 1 diabetic rats, GLP-1 treatment, like insulin, seems to only slightly reduce the liver GLUT2 mRNA and protein. Nevertheless, in diabetic and nondiabetic rats, it has been shown that hyperglycemia partially prevents the lowering effect of insulin on liver GLUT2 expression, suggesting that the glucotransporter could be regulated in opposing manners by insulin and glucose *(3,5)*. In the present study, although GLP-1, as well as insulin treatment, reduced the plasma glucose, its level still remained much above the normal value (Table 2). In muscle, and also in fat, where insulin seems to activate transcription as well as the translational process—effects also observed by others *(18,47)*—the action of GLP-1 seems to be only posttranscriptional, since an increase in GLUT4 protein was accompanied by a decrease in the mRNA. This lack of a stimulating effect of GLP-1 on GLUT-4 mRNA in fat and muscle tissues could indicate that perhaps insulin is required to stimulate gene transcription; in fact, no significant increase in plasma insulin was observed at the end of the GLP-1 treatment (Table 2), and, moreover, a GLP-1 bolus injection did not increase insulin secretion in this type 1 diabetic model (data not shown).

In conclusion, the present data show that exogenous ad-ministration of GLP-1 exerts a control on the glucotransporter expression in normal and diabetic rats. Its action in extrapancreatic tissues participant in the overall glucose homeostasis—liver, muscle, and fat—seems to be directed only toward the translational or posttranslational level, with the presence of insulin, in muscle and fat, possibly being required for GLP-1 to activate the transcriptional process. The stimulating action of GLP-1 on GLUT2 and GLUT4 expression, mRNA or protein, could be a mechanism by which, at least in part, the peptide exerts its lowering effect on blood glucose.

Materials and Methods

Animals

Male Wistar rats, kept on a standard pellet diet (UAR, Panlab, Barcelona, Spain) and tap water ad libitum, were used. The type 2 diabetic model was obtained by a single dose of streptozotocin (Sigma-Aldrich Química S.A., Madrid, Spain) dissolved in 0.9% NaCl-saline (100 µg/g of body wt dissolved in 25 µL of a citrate buffer, 0.05 *M*, pH 4.5), intraperitoneally administered on the day of birth *(48)*. At the age of 6 to 7 wk, those rats showing a glucose disappearance constant below 2.5×10^{-2}/min during an iv glucose tolerance test (0.5 mg of glucose/g of body wt in 30 s) were selected. The type 1 diabetic model was induced in

the adult rat (≈200 g of body wt) by a single dose of streptozotocin (60 μg/g of body wt), intraperitoneally administered *(49)*; four days later, those animals showing a glucosuria >1 g/L were selected for the study.

Experimental Design

The two groups of diabetic rats were subjected to a 3-d treatment with GLP-1 (Bachem AG, Bubendorf, Switzerland) or insulin (Novo Nordisk Pharma S.A., Bagsvaerd, Denmark), at doses of 2.5 and 19 pmol/min, respectively, while the normal group was treated only with GLP-1. The hormones were dissolved in saline and administered by continuous infusion through a subcutaneously implanted osmotic pump (Alzet 1003D; Alza, Palo Alto, CA). As respective controls, rats of the three groups were treated only with saline solution. In a preliminary test, these hormone infusions reflected an increase in plasma GLP-1 and insulin, during the respective treatments, of 103 ± 19 and 245 ± 72 pmol/L ($n = 3$ in both groups). Just before and on finishing the treatment (70 h), blood samples were collected from all rats for plasma glucose and hormone determinations (Table 2); samples were taken from some rats on the second day of treatment, and hormone values (data not shown) were not different from those of the last day. Afterward, the animals were stunned and killed by decapitation, and the gastrocnemius muscle, liver, and epididymal fat pads were quick-frozen at −70ºC for later RNA extraction (Northern blot study) and for membrane isolation (Dot and Western blot analyses). All chemicals and reagents, unless otherwise stated, were purchased from either Sigma-Aldrich Quimica or Merck Farma Química S.A. (Barcelona, Spain). Animal housing and protocols were approved by the Animal Use Committee of the Fundación Jiménez Díaz, Madrid, Spain.

Isolation of Membrane

Plasma membranes were obtained from the liver, by sucrose gradient centrifugation *(50)*. Approximately 5 g of the whole organ previously minced with scissors and pooled were resuspended in cold HES (25 m*M* HEPES; 4 m*M* EDTA; 0.25 *M* sucrose, pH 7.4), homogenized, and centrifuged at 15,000*g*; the pellet was resuspended in HES and sedimented at 750*g*. The supernatant was adjusted to 44% Brix with sucrose, and an equal volume of 42.3% Brix sucrose was placed on top of it and then centrifuged at 70,000*g*. The interface was collected, washed with HES, sedimented at 27,000*g*, and finally resuspended in HES-1 (HES, 1 U/mL of Trasylol, 0.2 m*M* phenylmethylsulfonyl fluoride, 25 m*M* benzamidine, and 1 μ*M* leupeptin). Muscle membranes were isolated by a combination of two previously reported processes *(51,52)*: one of the two gastrocnemius muscles from each rat, visually separated and frozen, was placed in HES; minced with scissors; pestle homogenized; and sedimented at 750*g*. The supernatant was then centrifuged at 183,000*g*, and the sediment was collected in HES-1. For fat membranes, one of the epididymal fat pads was homogenized in HES and centrifuged at 200,000*g*; the sediment was treated for 1 h with HES-1 containing 1% Triton X-100, and the solubilized membrane proteins were separated by centrifugation at 200,000*g*. Aliquot samples of each membrane preparation were taken for protein content determination *(53)*, before storing at −70ºC until required for analysis.

Dot and Western Blotting

For dot-blot analysis, 5 μg of membrane protein per sample resuspended in 200 μL of 20 m*M* Tris and 500 m*M* NaCl (pH 7.4), was applied to a nitrocellulose membrane in a commercial system (BIO-DOT SF microfiltration apparatus; Bio-Rad, Munich, Germany) and subjected to the process according to the manufacturer's instructions. For Western blot analysis, 25–50 μg of membrane protein per sample was subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis *(54)* in parallel with molecular weight markers (Rainbow Markers; Amersham, Buckinghamshire, England) on a 10% resolving gel; the separated proteins were then transferred to a nitrocellulose membrane in a semidry system (Trans-blot SD semidry transfer cell; Bio-Rad). For immunodetection, an electrochemiluminescence–Western blotting kit was used, following the manufacturer's instructions (Amersham), using C-terminal GLUT-2 and GLUT-4 rabbit polyclonal antibodies (Wak-Chemie Medical GmbH, Bad Soden, Germany) and a horseradish peroxidase–conjugated donkey antirabbit immunoglobulin second antibody (Amersham). Detection was done by the enhanced chemiluminiscence method and quantitation by densitometric scanning of the autoradiographic GLUT-2 and GLUT-4 signals.

Isolation of RNA and Northern Blot Analysis

Quick-frozen muscle, epididymal fat pad—in both cases, one of the pair—or aliquot portions of the whole liver previously minced and pooled were separately powdered in a cold steel mortar and pestle. Approximately 100–200 mg of each tissue was separately homogenized, using a polytron, in denaturing solution (4 *M* guanidinium thiocyanate; 25 m*M* Na-citrate, pH 7.0; 0.5% *N*-laurylsarcosin; 0.1 *M* 2-mercaptoethanol), and centrifuged at 12,000*g*. To 500 μL of the supernatant, 100 μL of 2 *N* Na-acetate (pH 8.0), 500 μL of H_2O-saturated phenol, and 22 μL of chloroform/isoamylic alcohol (49/1 [v/v]) were added. The mixture was cooled on ice and centrifuged at 10,000*g*. The aqueous phase was mixed with isopropanol (1/1 [v/v]) and kept at −20ºC overnight, and the precipitated RNA was separated at 10,000*g*. The pellet was then redissolved in denaturing solution, and the RNA was reprecipitated with isopropanol (1/1 [v/v]) and sedimented by centrifugation. The pellet was washed with 70% ethanol, then separated at 10,000*g*, and resuspended in diethylpyrocarbonate-treated H_2O. RNA concentrations were calculated spectrophotometrically from the absorption at 260 nm in an aliquot volume removed from each sample *(55)*. The RNA (20 μg/sample) was denatured;

stained (0.04 μg/μL of ethidium bromide); and size-fractionated on a 1.2% agarose, 0.5 *M* formaldehyde gel. RNA was transferred to a Hybond-N membrane (Amersham), ultraviolet crosslinked, and prehybridized at 42ºC for 1–5 h in a solution of 50% deionized formamide, 5X saline sodium citrate (SSC) (1X SSC = 0.15 *M* NaCl; 0.015 *M* sodium citrate, pH 7.0), 50X Denhardt's reagent (1% each of polyvinylpyrrolidone, Ficoll, and bovine serum albumin), 75 m*M* sodium phosphate (pH 6.5), 0.1% SDS, and 100 μg/mL of salmon sperm DNA. Hybridizations were carried out overnight in the hybridizing solution at 42ºC using the appropriate cDNA probe at 10^6 cpm/mL; all cDNA probes, i.e., full-length GLUT-4, GLUT-2 (ATCC, Manassas, VA), and β-actin (CLONTECH, Palo alto, CA), were labeled with [α-^{32}P]dCTP (Amersham) by the nick translation system (Boehringer Mannheim GmbH, Barcelona, Spain). The resulting Northern blots were visualized by autoradiography and quantified using laser densitometry.

Plasma Glucose, Insulin, and GLP-1 Analyses

Glucose levels were determined by the glucose oxidase method (Glucose analyzer 2; Beckman, Galway, Ireland); insulin was measured by radioimmunoassay *(56)* using rat insulin (Linco, St. Charles, MO) as standard and a guinea pig antiinsulin serum (GP-25) developed in our laboratory. GLP-1 was previously extracted from each plasma sample with 60% ethanol *(57)* and then measured by radioimmunoassay *(58)* using anti-GLP-1 #2135, kindly provided by Dr. J. J. Holst (Denmark).

Statistical Analyses

Data were analyzed for statistical significance among experimental groups using one-way analysis of variance, or Student's *t*-test, paired or unpaired, with significance at $p < 0.05$.

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