Normalizing action of exendin-4 and GLP-1 in the glucose metabolism of extrapancreatic tissues in insulin-resistant and type 2 diabetic states

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Abstract

Exendin-4 (Ex-4) mimics glucagon-like peptide-1 (GLP-1 or GCG as listed in the HUGO database), being anti-diabetic and anorectic, in stimulating glucose and lipid metabolism in extrapancreatic tissues. We studied the characteristics of Ex-4 and GLP-1 action, during prolonged treatment, on GLUTs expression (mRNA and protein), glycogen content (GC), glucose transport (GT), glycogen synthase *a* (GSA), and kinase (PI3K and MAPKs) activity, in liver, muscle, and fat of insulin-resistant (IR, by fructose) and type 2 diabetic (T2D, streptozotocin at birth) rats compared with normal rats. In both IR and T2D, the three tissues studied presented alterations in all measured parameters. In liver, GLP-1 and also Ex-4 normalized the lower than normal *Glut2* (*Slc2a2*) expression and showed a trend to normalize the reduced GC in IR, and GLP-1, like Ex-4, also in T2D, effects mediated by PI3K and MAPKs. In skeletal muscle, neither GLP-1 nor Ex-4 modified *Glut4* (*Slc2a4*) expression in either experimental model but showed normalization of reduced GT and GSA, in parallel with the normalization of reduced PI3K activity in T2D and MAPKs in both models. In adipose tissue, the altered *GLUT4* expression in IR and T2D, along with reduced GT in IR and increased GT in T2D, and with hyperactivated PI3K in both, became normal after GLP-1 and Ex-4 treatment; yet, MAPKs, that were also higher, became normal only after Ex-4 treatment. The data shows that Ex-4, as well as GLP-1, exerts a normalizing effect on IR and T2D states through a distinct post-receptor mechanism, the liver being the main target for Ex-4 and GLP-1 to control glucose homeostasis.

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Introduction

Insulin resistance (IR) leads to hypertension, cardiovascular disease, dyslipidemia, and type 2 diabetes (T2D; Hotamisligil 2000, Zierath *et al.* 2000); it is also associated with obesity and hyperinsulinemia.

Glucagon-like peptide-1 (GLP-1 or GCG as listed in the HUGO database), an incretin hormone with anti-diabetic properties, exerts insulin-like effects upon glucose transport (GT) and metabolism in the liver, skeletal muscle, and fat of humans and rats (Valverde *et al.* 1994, Villanueva-Peñacarrillo *et al.* 1994, 2001, Sancho *et al.* 2005), tissues where its effects are dependent on protein kinase (Redondo *et al.* 2003, Acitores *et al.* 2004, Sancho *et al.* 2005). The regulatory action of GLP-1 in glucose and lipid metabolism in the liver, muscle, and fat in normal and T2D situations is, out of doubt, due to its neurotrophic and cardioprotective properties (Ossum *et al.* 2009, Bak *et al.* 2011). In addition, a recent study has shown that in mesenchymal cells, GLP-1 promotes cellular proliferation and

cytoprotection and prevents cell differentiation into adipocytes (Sanz *et al.* 2010).

Exendin 1–39 amide (Ex-4), a naturally occurring non-mammalian (Chen & Drucker 1997) peptide that has partial sequence homology with GLP-1 (Eng *et al.* 1992), has several similar glucoregulatory effects on mammals as GLP-1; however, Ex-4 has a much longer circulating half-life than GLP-1 – it is resistant to degradation by dipeptidyl peptidase IV – which makes it suitable as an anti-diabetic agent (Nielsen *et al.* 2004); in fact, as such it is being used either alone or in combination with oral anti-diabetic drugs (Fineman *et al.* 2003).

Ex-4 mimics GLP-1 in stimulating GT and metabolism in rat liver, skeletal muscle and fat, in an insulin-independent manner (Alcántara *et al.* 1997, Morales *et al.* 1997, Sancho *et al.* 2005), as well as lipogenesis and lipolysis in the latter cells (Sancho *et al.* 2005). In rat muscles, Ex-4, like GLP-1 (Villanueva-Peñacarrillo *et al.* 2001), stimulates the *in vivo* expression of corresponding major glucotransporter in normal and T2D states

(Arnés *et al.* 2009). Similar to GLP-1 (Willms *et al.* 1996), Ex-4 lowers gastric emptying (Talsania *et al.* 2005) due to its anorectic properties (Barrera *et al.* 2009). The reported effects of Ex-4 on extrapancreatic tissues are dependent on an increase in the activity of several kinases (González *et al.* 2005a, Sancho *et al.* 2005, Arnés *et al.* 2008), some of them are also involved in GLP-1 and insulin actions (Acitores *et al.* 2004, González *et al.* 2005a, Sancho *et al.* 2005). The GLP-1-like glucoregulatory actions of Ex-4 (Nielsen *et al.* 2004) are thought to be exerted, at least in part, not only through the pancreatic GLP-1 receptor (Tourel *et al.* 2002) but also by interacting with GLP-1 receptors in the liver and muscle (Delgado *et al.* 1995, Villanueva-Peñacarrillo *et al.* 1995, Arnés *et al.* 2008), which seem to be structurally and/or functionally distinct from that in the pancreas (Thorens 1992). Thus, in the former tissues, neither GLP-1 nor Ex-4 increases cAMP content, although both stimulate inositol phosphoglycan production (Villanueva-Peñacarrillo *et al.* 1994, Alcántara *et al.* 1997, Luque *et al.* 2002).

Very recently, an osteogenic effect of GLP-1 and also Ex-4 has been noted in three experimental rat models, IR, T2D, and hyperlipidemic where both peptides promote toward normalization, bone formation (Nuche-Berenguer *et al.* 2009, 2010a,b, 2011), and action of GLP-1 that has been proposed to occur through a specific receptor (Nuche-Berenguer *et al.* 2010a,b) other than the pancreatic cAMP-associated GLP-1 receptor.

At present, little is known about the effect of a sustained action of GLP-1 on the glucose metabolism in the main tissues participating in the hexose homeostasis in the IR state, similar to that of Ex-4 in the same situation of altered insulin secretion and also in diabetes. Here, we have studied the possible *in vivo* modulating effect of both Ex-4 and GLP-1, during prolonged treatment, on the characteristics of parameters related to the liver, muscle, and fat/glucose uptake and metabolism, in an IR and in a T2D model, compared with the normal state.

Materials and methods

Reagents

Human GLP-1 (7–36) amide (GLP-1) and exendin (1–39) amide (Ex-4) were purchased from Bachem AG (Bubendorf, Switzerland); rat insulin was purchased from Linco (St Charles, MO, USA) and pork insulin from Novo Biolabs (Bagsvaerd, Denmark); streptozotocin from Sigma–Aldrich; Alzet 1003D osmotic pumps from Alza (Palo Alto, CA, USA); Rainbow markers and ECL-Western blotting kit and Hyperfilm ECL were from Amersham Pharmacia Biotech; glucose reagents from Beckman Instruments, Inc. (Palo Alto, CA, USA); collagenase P of

Clostridium histolyticum from Roche Diagnostics; transfer semidry system from Trans-blot SD semidry transfer cell, Bio-Rad; TRI REAGENT for RNA isolation from Sigma–Aldrich Química S.A.; Taqman Universal PCR master mix and high-capacity cDNA reverse transcription kit, probes and primers for rat *Glut2* (Rn00563565_m1), *Glut4* (Rn00562597_m1), eukaryotic 18s (4319413E), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, 4331182) were from Applied Biosystems (Woolston, Warrington Cheshire, UK); mouse anti-total, rabbit anti-total, and anti-phosphorylated form of p44/42MAP kinase were from Cell Signalling Technology (New England Biolabs, Beverly, MA, USA); rabbit anti-PI3-kinase p85 from Upstate Biotechnology (Lake Placid, NY, USA); 2-deoxy-D-[1,2-³H(N)]glucose (40 Ci/mmol) from Moravek Biochemicals (Brea, CA, USA); [U-¹⁴C]sorbitol (320 mCi/mmol) from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA); UDP-[¹⁴C]D-glucose (302 mCi/nmol) from Perkin-Elmer (Boston, MA 02118, USA); [γ -³²P]ATP (30 Ci/nmol) and Ultima Gold scintillation liquid were from Packard (Gröninger, The Netherlands). All other commonly used chemicals, unless otherwise stated, were purchased from Sigma–Aldrich Química or Merck Pharma Química.

Animals

Male Wistar rats, maintained on a standard pellet diet (UAR Panlab, Barcelona, Spain) and tap water made available *ad libitum*, were used. The IR model was prepared in normal rats by chronic feeding standard chow combined with D-fructose dissolved in drinking water at 20% concentration (Cancelas *et al.* 2008) for 8 weeks. The T2D model was obtained in normal rats by a single dose of streptozotocin dissolved in 0.9% NaCl-saline (100 μ g/g of body weight), intraperitoneally administered on the day of birth (Portha *et al.* 1979, Iwase *et al.* 1987); at the age of 8 weeks, those rats showing a glucose disappearance constant (K) below 2.5×10^{-2} /min after an i.v. glucose tolerance test (0.05 mg glucose/g of body weight in 30 s) were selected. All rats belonging to the same three groups were of the same age (12–13 weeks old) by the time applying the experimental protocol of the study. Animal housing and protocols were approved by the Animal Use Committee of the Fundación Jiménez Díaz, Madrid, Spain.

Experimental design

Normal (N), IR, and T2D rats were subjected to a 3-day treatment (Villanueva-Peñacarrillo *et al.* 2001, Arnés *et al.* 2008) with GLP-1 and Ex-4 at a dose of 0.86 and 0.1 nmol/kg per h respectively, dissolved in saline solution, by continuous infusion through

a subcutaneously implanted osmotic pump. Treatment of rats from the three groups was initiated at 0900 h. As respective control, rats of the three groups were treated only with saline solution. Just before and after the treatment (72 h), blood samples were collected from all rats in fed condition for plasma glucose and insulin determinations; afterwards, the animals were stunned and killed by a sharp blow to the head, and soleus muscles, epididymal fat pads, and liver of the animals, quick-frozen at -70°C , were used to prepare solubilized membranes (Villanueva-Peñacarrillo *et al.* 2001) for the analysis of major respective glucose-transporter gene expression and the activity of cell signaling enzymes (Villanueva-Peñacarrillo *et al.* 2001, Redondo *et al.* 2003, Acitores *et al.* 2004, Sancho *et al.* 2005); additionally, the liver glycogen content (GC) was measured by the anthrone method (Chun and Yin 1998), the soleus muscle glycogen synthase *a* (GSA) activity as described previously by Luque *et al.* (2002) and glucose transport and the isolated adipocytes glucose uptake by Sancho *et al.* (2005).

Plasma measurements

Glucose was measured by the glucose oxidase method (Glucose analyzer 2; Beckman, Galway, Ireland), and insulin was measured by RIA (Herbert *et al.* 1956) using rat insulin as a standard and guinea pig serum anti-insulin (GP-25) that was developed in our laboratory (Valverde *et al.* 1988).

Glucose transport

The soleus muscles in rats were individually attached by the tendon to a steel clip (Valverde *et al.* 1994) and were incubated in the absence of added peptides (control), for 60 min at 37°C , in 3 ml Krebs–Henseleit (KH) buffer containing 15 μmol 2-deoxy-D-[1,2- ^3H (N)] glucose (Sp. Act: 40 nCi/ μmol) and 60 μmol [U- ^{14}C] sorbitol (Sp. Act: 5 nCi/ μmol) 5 and 20 mM in the incubation media. During the incubation period, an atmosphere of O_2/CO_2 (95/5) was maintained in the vials sealed with a rubber stopper. After, the extracellular radioactivity was removed by quick washing at 4°C in KH, the muscle was treated with 1 ml 1 M NaOH for 10 min at 70°C . The solubilized product was placed in 5 ml scintillation liquid for determining the ^3H and ^{14}C content by double channel counting; an aliquot volume (2 μl) had previously been taken from all solubilized samples for the measurement of total protein (Bradford 1976). To calculate the cellular D-glucose uptake in each muscle sample (pmol/mg protein per min), the total 2-deoxy-D-[1,2- ^3H]glucose accumulated was corrected taking into account the extracellular space, as

determined by the [^{14}C]sorbitol uptake value, following a method previously described in Dohm *et al.* (1988).

Adipocytes were isolated at 37°C by enzymatic digestion with collagenase P from the rat epididymal fat pads (Rodbell 1964). Then, cells (10^5) were incubated for 15 min at 37°C in 400 μl KRB, 10.9 mM HEPES, 500 U/ml Trasylol, and 2% BSA, pH 7.4. This was followed by a 3 min incubation in the additional presence of 0.26 μCi (6.5 pmol) 2-deoxy-D-[1,2- ^3H (N)]glucose (final concentration 16.3 nM 2-DOG). Adipocytes, after being separated at 7200 *g* in 100 μl dioctyl phthalate, were added to 3 ml scintillation liquid for β -counting. The total D-glucose content was corrected for the unspecific D-glucose uptake value, obtained in cell samples from each experiment treated in parallel with 0.175 mM cytochalasin B (Perea *et al.* 1997).

GSa activity

The soleus muscle samples were homogenized in a medium containing 100 mM NaCl, 35 mM EDTA, 50 mM glycylglycine, and 0.5% glycogen (w/v) at pH 7.4, as already described in Villanueva-Peñacarrillo *et al.* (1994), and maintained at -70°C until their enzymatic activity was analyzed. The frozen tissue homogenates were thawed at 4°C , and the enzymatic activity was analyzed, at least in duplicate, by the incorporation of UDP-[^{14}C]D-glucose into glycogen, for 15 min, as previously described in detail in Villanueva-Peñacarrillo *et al.* (1994). For each experimental rat, the mean value of the replicates corresponding to the muscles of rats treated with saline was used as the control value.

Isolation of total RNA and real-time PCR

Total RNA was extracted from the muscle, fat, and liver by the TRI REAGENT isolation method, according to the manufacturer's protocol. Total RNA concentration and purity were estimated spectrophotometrically from the absorption at 260 and 280 nm respectively, in an aliquot volume of each sample (Arnés *et al.* 2008). For quantitative real-time PCR, the first-strand cDNA was synthesized from 4 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit. Samples were subjected to quantitative amplification using the TaqMan probe and primer sets for rat *Glut4* (*Slc2a4*) and *Glut2* (*Slc2a2*) PCR amplification was carried out in triplicate for each sample and was performed in a total volume of 20 μl containing 400 ng cDNA, 900 nM each primer, 500 nM of the respective probe, and 10 μl TaqMan Master Mix. The conditions for amplification and detection – 40 cycles/110 min – were described in Ascencio *et al.* (2004). For each rat, gene expression was normalized with that of the house-keeping gene *18S* or *Gapdh* and expressed as $2^{-\Delta\Delta\text{C}_t}$.

Kinase activity

Tissue samples were homogenized at 4 °C in 1.25% Triton X-100 containing 250 mM sucrose, 20 mM Tris/HCl, 2.5 mM MgCl₂, 50 mM β-mercaptoethanol, 1.2 mM EGTA, 1 mM Na₃VO₄, 5 mM Na₄P₂O₇, 50 mM NaF, 2 μM leupeptin, 2 μM pepstatin, pH 7.4, and 2 mM PMSE, then maintained at 4 °C for 30 min, and finally centrifuged at 15 000 g (Dohm *et al.* 1988). The supernatant (tissue lysate), containing cytosol and solubilized membranes, was kept at –70 °C until needed. An aliquot volume was taken from all tissue lysate samples for determining the protein content (Valverde *et al.* 1988). MAP kinase activity (p44/p42) was measured by immunoblotting (Redondo *et al.* 2003, Acitores *et al.* 2004, and Sancho *et al.* 2005). PI3K activity was measured directly in p85 immunoprecipitates obtained by treating the muscle, fat, and liver lysates with anti-PI3 kinase p85 and subsequent coupling to protein A-agarose (Phung *et al.* 1997, Redondo *et al.* 2003, Acitores *et al.* 2004, Sancho *et al.* 2005); the immunoprecipitates were incubated for 20 min at room temperature with 20 μM [γ -³²ATP] (5 μCi/nmol) containing 6.25 mM HEPES, 5 mM MgCl₂, and 0.25 mM EGTA, and in the presence of 0.25 mg/ml phosphatidylinositol/phosphatidylserine as a substrate; the reaction was interrupted by addition of 400 μl chloroform/methanol/HCl (1:2:1, v/v), 150 μl chloroform, and 150 μl HCl. After centrifugation (10 000 g), the organic phase was treated with an equal volume of methanol/100 mM HCl/2.5 mM EDTA (1:1:1, v/v), and the new organic phase was separated by centrifugation and speed-vac dried. The lipidic extract, redissolved in chloroform, was spotted, together with PIP₃ standard, on a silica gel TLC plate and developed in *n*-propanol/acetic acid/H₂O (66:2:33, v/v). Plates were dried, and radioactive PIP₃ was subsequently visualized by autoradiography and analyzed by densitometric scanning.

Immunoblotting

Equal amounts of tissue lysate from each sample were subjected to SDS-PAGE (Laemmli 1970), in parallel with molecular weight markers, on an 8% 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, a western blotting kit was used following the manufacturer's instructions, using total and phosphorylated p44/42 MAPK antibodies, GLUT2 (for liver), and GLUT4 (for muscle and fat) respective antibodies and anti-α-tubulin, and a HRP-conjugated donkey anti-rabbit immunoglobulin secondary antibody, with detection by the enhanced chemiluminescence method and quantization by densitometric scanning of the autoradiography (Villanueva-Peñacarrillo *et al.* 2001, Redondo *et al.* 2003, Acitores *et al.* 2004,

Sancho *et al.* 2005). The densitometric value of the band corresponding to GLUT2 and GLUT4 protein was normalized, when required, with respect to that of the α-tubulin, and the values obtained from the rat treated with saline solution were used as control values.

Statistical analysis

Results are expressed as mean ± S.E.M., together with the number of observations. The statistical significance ($P < 0.05$) of the increments was assessed by either one-way ANOVA, followed by the least significant differences test for *post hoc* multiple comparisons, using the Statistical Package for the Social Science (SPSS Inc, Chicago, IL, USA) software, or Student's *t*-test.

Results

Plasma measurements

Plasma glucose value in the IR rats (91 ± 3 mg/dl, $n = 24$ rats) was slightly lower than that of the normal rats (102 ± 5 mg/dl, $n = 25$), whereas higher in the T2D group (141 ± 8 , $n = 24$, $P < 0.001$); however, the insulin level in IR was higher (3.0 ± 4 mg/ml, $P < 0.02$) than that in N (1.7 ± 0.2), while lower in T2D (0.7 ± 0.1 , $P < 0.05$ vs N). No significant differences were observed in either parameter, glucose or insulin, after GLP-1 or Ex-4 treatment. It should be taken into account that the plasma variables were measured in the rats in fed condition, as they had free access to food and water throughout the 3-day experimental period.

Liver GLUT2 expression and GC

In the IR-control group (Fig. 1), the liver *Glut2* mRNA was much lower ($P < 0.01$, $n = 17$ rats) than that in the N group, and hence it was the protein ($-44 \pm 8\%$ Δ N-control, $P < 0.01$). Both GLP-1 and Ex-4 increased not only the *Glut2* mRNA (both, $n = 5$; $P < 0.01$) to a level even higher than that in the normal group (GLP-1: 2.8 ± 0.4 times N-control and Ex-4: 4.0 ± 0.4 ; both $P < 0.05$) but also the protein (GLP-1: $147 \pm 18\%$ N-control; Ex-4: $187 \pm 11\%$ N-control; both $P < 0.05$).

In the IR-control rats (Fig. 1), GC in the liver (193 ± 20 μg/mg protein, $n = 8$) was lower ($P < 0.01$) than that in the N rats (395 ± 35 μg/mg protein, $n = 6$), and so it was in T2D rats (225 ± 20 μg/mg protein, $n = 5$; $P < 0.01$), the latter confirming the previous data from our laboratory (Arnés *et al.* 2009); GLP-1 treatment increased the concentration in the IR group (295 ± 14 μg/mg protein, $P < 0.01$ vs IR-control, $n = 11$) and also in the T2D rats (561 ± 57 μg/mg, $P < 0.01$ vs T2D-control, $n = 5$), the value reached in the latter group being even

Figure 1 Effect of GLP-1 and Ex-4 treatment on *Glut2* expression ($n=7-15$ rats) and glycogen content (GC, $n=5-12$) in the liver of insulin-resistant (IR) and type 2 diabetic (T2D) rats. Data (mean $\pm$ s.e.m.) are expressed as relative amounts (*Glut2* mRNA) or percentage (GLUT2 protein and GC) of the normal (N) or respective control group (saline treated). * $P<0.05$, or lower, vs N-control; # $P<0.05$, or lower, vs respective control value, IR or T2D.

above ($P<0.05$) than that in the liver of N rats. Ex-4 also increased the GC in the liver of IR rats to a value ($338 \pm 27 \mu\text{g}/\text{mg}$ protein, $n=12$; $P<0.01$ vs IR-control) indistinguishable from that in the N rats. Tables 1 and 2 show the qualitative changes in the abovementioned parameters and correspond to the data contained in Fig. 1; those that are already being published are not shown in the figures but have been included in the tables for comparison.

Muscle GLUT4 expression, GT, and GSa activity

In IR rats, *Glut4* mRNA ($P<0.01$ vs N-control; Fig. 2) as well as protein ($143 \pm 39\%$ Δ N-control, $n=5$ rats; $P<0.05$) was overexpressed; treatment with both GLP-1 and Ex-4 increased the mRNA even further (both, $n=5$; $P<0.001$ vs IR-control) as well as the protein (GLP-1:

$283 \pm 59\%$ Δ IR-control, $n=5$, $P<0.01$; Ex-4: $76 \pm 27\%$ Δ , $n=5$, $P<0.02$).

In the skeletal muscle, the net glucose uptake (GT) in the IR group (0.192 ± 0.04 nmol/mg protein per min, $n=7$) was much lower ($P<0.05$) than that in the N group (0.418 ± 0.09 , $n=5$); a 3-day treatment with GLP-1 increased ($P<0.001$) the value in the IR-control rats ($268 \pm 13\%$, $n=10$) to levels even higher than those in the N-control rats ($P<0.001$), and the same increasing effect was observed by Ex-4 ($392 \pm 9\%$ IR-control, $n=10$; $P<0.001$), although to a higher ($P<0.001$) extent than that by GLP-1. In T2D rats, GT in the muscle was indistinguishable from that in N rats, confirming the previous results (Arnés *et al.* 2009); this GT value was not altered after GLP-1 treatment, in comparison with the previous observations on Ex-4 treatment in the skeletal muscle of this same experimental diabetic model, by which an increase was detected after treatment (Arnés *et al.* 2009).

In the IR group (Fig. 2), the GSa activity in the muscle was lower (0.41 ± 0.09 U/g protein, $n=5$; $P<0.01$ vs N-control) than that in the N group (1.80 ± 0.14 U/g protein, $n=5$); treatment with both GLP-1 or Ex-4 induced a clear increase in this enzyme activity (GLP-1: $461 \pm 22\%$ IR-control, $n=4$; Ex-4: $181 \pm 24\%$, $n=6$; both, $P<0.001$), which in the case of GLP-1 reached normal values. In the T2D rats, the GSa activity (1.08 ± 0.08 U/g protein, $n=5$), although higher than that in the IR rats, was lower than in the normal rats ($P<0.001$ vs N-control). Although GLP-1 ($n=5$) failed to modify the enzyme activity in this diabetic group, Ex-4 induced a trend toward its normalization (1.57 ± 0.15 U/g protein, $n=5$, $P<0.05$ vs N-control).

Tables 1 and 2 show the qualitative changes in the abovementioned parameters and correspond to the

Table 1 Qualitative changes in *GLUT2* ($n=7-15$ rats) and *GLUT4* ($n=5-8$) expression in insulin-resistant (IR) and type 2 diabetic (T2D) rats relative to normal (N) animals, and effect of GLP-1 and Ex-4 treatment in relation to their respective control value (saline treated)

	N mRNA/ protein	IR mRNA/ protein	T2D mRNA/ protein
Liver: GLUT2			
Control	-/-	↓/↓	↓/↑
GLP-1	↓/↓	↑/↑	↓/-
Ex-4	↑/↑	↑/↑	↑/↑
Muscle: GLUT4			
Control	-/-	↑/↑	-/↓
GLP-1	↑/↓	↑/↑	↑/↑
Ex-4	↓/↑	↑/↑	-/-
Fat tissue: GLUT2			
Control	-/-	↓/-	↑/↓
GLP-1	↓/↓	-/↑	↓/↑
Ex-4	↑/-	↑/-	-/↑

—: equal to control; ↓ and ↑ slightly higher and lower, respectively, than control; ↑ and ↓: significantly higher and lower, respectively, than control.

Table 2 Glycogen content (*n*=5–12 rats), glucose transport (GT, *n*=5–12), and glycogen synthase *a* (GSa, *n*=6) in insulin-resistant (IR) and type 2 diabetic (T2D) models compared with normal rats (N), and qualitative changes after GLP-1 and Ex-4 treatment with respect to their own control group (saline treated)

	N	IR	T2D
Liver glycogen			
Control	—	↓	↓
GLP-1	—	↑	↑
Ex-4	—	↑	↑
Muscle GT			
Control	—	↓	—
GLP-1	—	↑	—
Ex-4	—	↑	↑
Muscle GSa			
Control	—	↓	↓
GLP-1	↓	↑	—
Ex-4	↓	↑	↑
Fat tissue GT			
Control	—	↓	↑
GLP-1	↑	↑	↓
Ex-4	—	↑	↓

—: equal to control; ↑ and ↓: significantly higher and lower, respectively, than control.

data contained in Fig. 2; those not shown in the figures, as for being already published, have been included in tables for comparison.

Fat GLUT4 expression and GT

Glut4 mRNA in IR (Fig. 3) was lower (*P*<0.001) than in normal rats (*n*=8) while no changes were observed in the protein value; however, in the T2D group (*n*=8), the mRNA was not significantly different from that in the N group, while the GLUT4 protein was lower (*P*<0.05 vs N), the latter confirming the previous results (Villanueva-Peñacarillo *et al.* 2001). Treatment of IR rats with GLP-1 did not alter the initially reduced mRNA value, but increased (*P*<0.02) than that of the protein (26±5% Δ IR–control, *n*=5) to levels even higher than those in the N rats (149±6% N–control; *P*<0.001); in comparison with GLP-1, Ex-4 (*n*=5) induced an increase in the *Glut4* mRNA (*P*<0.05) to a normal expression (109±9% N–control).

The GT value in isolated adipocytes (Fig. 3) from the IR rats (8.10±0.49 fmol/10⁵ cells, *n*=6) was lower than that in the N rats (15.1±1.6 fmol/10⁵ cells *n*=12), while in the T2D group (*n*=9) it was much higher (45.0±2.7 fmol/10⁵ cells, *P*<0.001 vs N), the latter confirming the previous observations (Sancho *et al.* 2005). In the IR rats, both GLP-1 and Ex-4 treatment increased (*P*<0.05) the GT (GLP-1: 157±11% IR–control *n*=8; Ex-4: 161±11% *n*=6); however, in T2D group, both GLP-1 and Ex-4 exerted a significantly reducing effect (*P*<0.02) on GT levels (GLP-1: 14.5±0.9 fmol/10⁵ cells *n*=5, and Ex-4:

20.9±1.7 *n*=5), by which, in the case of GLP-1, normal values were reached.

Tables 1 and 2 show the qualitative changes in the abovementioned parameters and correspond to the data contained in Fig. 3; those not shown in the figures, have been included in tables for comparison.

Effect on kinases activity

Table 3 contains the activity values of PI3K (PIP₃) and p44/42 MAPKs (phosphorylated form) in the liver, muscle, and fat of normal, IR, and T2D rats, treated with saline (control) or GLP-1 or Ex-4. The activity of both PI3K and p44 MAPK increased (*P*<0.02 vs N) in the liver and fat of IR–control rats, while that of p42 MAPK was higher than normal in the three tissues (*P*<0.05 vs N); the same was detected in the T2D group, except for the fact that p44 MAPK in the muscle of IR rats was not different from that of the normal rats; these observations in the muscle and fat of T2D rats coincided with previous data obtained *in vitro* (Sancho *et al.* 2005, Arnés *et al.* 2008).

In the liver of the three groups studied, both GLP-1 and Ex-4 increased the PI3K activity (overall mean: 63±10%Δ control, *n*=34 rats; *P*<0.001), and also in muscle and fat (overall mean: 57±7%Δ control, *n*=60; *P*<0.001). GLP-1 increased the initial p42 MAPK hyperactivity in the liver of IR rats further, while no effect was observed after GLP-1 treatment in the T2D group, or in the muscle and fat of the IR rats; the same was basically detected by Ex-4, except that this peptide exerted a normalizing action in the muscle and fat of IR rats (–33%Δ IR–control, *n*=11), and also on p44/42 MAPK activity in the fat tissue of T2D rats (–38%Δ T2D–control, *n*=10).

Discussion

GLUT2 and GLUT4 glucotransporter isoforms play a central role in the complex pathways mediating whole-body glucose disposal, in which dysregulation of their control mechanism can result in the pathophysiological states associated with IR and diabetes.

The liver of the IR model showed a lower than normal *Glut2* expression accompanied by a reduced hepatic GC also observed in liver of T2D model, the latter confirming the previous results (Arnés *et al.* 2009). In both experimental models, treatment with either GLP-1 or Ex-4 increased the *Glut2* expression to values even above those in normal rats; in the IR rats, this effect along with an increment in the hepatic GC to normal levels indicates an increase in the rate of glucotransporter translocation from the cytosol to the plasma membrane. GLP-1 also normalized the glycogen concentration in the T2D group, in the same manner as previously observed after Ex-4 treatment (Arnés *et al.* 2009).

Figure 2 Effect of GLP-1 and Ex-4 treatment on *Glut4* expression ($n=5$ rats), glucose transport (GT, $n=5-10$), and glycogen synthase *a* activity (GSa, $n=6$) in the muscle of insulin-resistant (IR) and type 2 diabetic (T2D) rats. Data (mean $\pm$ S.E.M.) are expressed as relative amounts (*Glut4*, mRNA) or percentage (GLUT4, protein, GT and GSa) of the normal (N) or respective control group (saline treated). * $P < 0.05$, or lower, vs N-control; # $P < 0.05$, or lower, vs respective control value, IR or T2D.

The present and previous results on Ex-4 support the idea of a direct effect, insulin independent, of Ex-4 on the glucose uptake and metabolism in the liver, as already suggested not only for Ex-4 but also for GLP-1 (Alcántara *et al.* 1997, Morales *et al.* 1997). Evidences from a report (Valh *et al.* 2007) demonstrating the presence of GLP-1 receptor in the terminal nerves of the portal vein should be taken into account; although the first step in the action of Ex-4 in the liver remains unknown, because of its homology with GLP-1, the possibility that its effects could be mediated and regulated by the central nervous system should not be discarded. These impaired values of GLUT2 and GC in the liver of IR and T2D rats were accompanied by a hyperactivation in PI3K, as a consequence, perhaps, of the development of a correcting mechanism for the former alterations; both GLP-1 and Ex-4 increased this enzyme activity even more. The latter not only confirms the importance of PI3K in the glucose metabolism of the liver (Redondo *et al.* 2003) but also suggests its role in restoration of the normal conditions of this tissue. In the case of MAP kinases, hyperactivated in IR and T2D, the effect of GLP-1 differed from that of Ex-4, as each showed to be targeting a different MAPK isoform, indicating a distinct mechanism of action. In fact, the insulinotropic and glucoregulatory effect of Ex-4 has been attributed to occur through the pancreatic GLP-1 receptor (Göke *et al.* 1993), and based on the results obtained in a GLP-1 receptor knockout animal model, it was suggested that the pancreatic GLP-1 receptor is involved in the incretin/insulinotropic action of Ex-4 and GLP-1 and also in the hepatic portal glucose sensor action of the latter (Edwards *et al.* 1999, Burcelin *et al.* 2001). Yet, it is also known that Ex-4 displaces the GLP-1 binding to its receptors in plasma membranes of extrapancreatic tissues involved in the glucose homeostasis (Alcántara *et al.* 1997, Arnés *et al.* 2008), which are other than those in the pancreas (Thorens *et al.* 1993). The present observations of the liver indicate that Ex-4 can control the glucotransporter expression process and also glycogenesis, not only in normal but also in IR and T2D states, similar to GLP-1 in this study and also in a previous study on normal and diabetic rats (Villanueva-Peñacarrillo *et al.* 2001). The results also suggest that although other tissues could be participating, the liver seems to represent an important target organ in the glucoregulatory action of Ex-4 in states of impaired glucose homeostasis.

The observed GLUT4 overexpression in the muscle of IR rats further increased after treatment with both GLP-1 and Ex-4 and accompanied by an increase in the lower than normal glucose uptake and GSa activity values, Ex-4, although stimulating the enzyme activity shown to be less efficient than GLP-1. In T2D rats, GT in the muscle was apparently indistinct from that in normal rats; yet, in a previous work performed on this

Figure 3 Effect of GLP-1 and Ex-4 treatment on fat *Glut4* expression ($n=8$ rats) and glucose transport (GT, $n=5-12$) in the isolated adipocytes of insulin-resistant (IR) and type 2 diabetic (T2D) rats. Data (mean $\pm$ S.E.M.) are expressed as relative amounts (*Glut4* mRNA) or percentage (GLUT4, protein and GT) of the normal (N:) or respective control group (saline treated). * $P<0.05$, or lower, vs N-control; # $P<0.05$, or lower, vs respective control value, IR or T2D.

same experimental model (Arnés *et al.* 2009), a slightly lower than normal value was detected, coinciding with the observations in muscle cells from T2D patients (González *et al.* 2005b); nevertheless, the muscle GSa activity also showed to be reduced. These altered values in the muscle of T2D had shown along with an apparently higher than normal levels of *Glut4* mRNA, and with a slightly lower protein levels

(Villanueva-Peñacarrillo *et al.* 2001, Arnés *et al.* 2009). In the present T2D group, GLP-1 did not alter the values of GT or GSa activity, while an increase in the *Glut4* expression after GLP-1 treatment was previously noted (Villanueva-Peñacarrillo *et al.* 2001). *In vitro* experiments exploring the action of Ex-4 in the skeletal muscle showed no effect on GT or GSa in this same T2D model (Arnés *et al.* 2008), *Glut4* expression (Arnés *et al.* 2009), while in normal rats Ex-4, like GLP-1, stimulated the uptake of glucose (Arnés *et al.* 2008). Yet, it was noted that maintained treatment with Ex-4 induces a significant increment in glucose uptake in the muscle in T2D but not in normal animals (Arnés *et al.* 2009). These alterations in the muscles of the two experimental models of this study were accompanied by a lower than normal PI3K activity in the case of T2D rats, which was increased in both IR and T2D after GLP-1 or Ex-4 treatment. Also, the activity of MAPKs studied was impaired in IR and T2D, the latter confirming the previous observations (Arnés *et al.* 2008), each peptide, GLP-1 and Ex-4, shown to target a different isoform. The reduced PI3K activity in the muscles of T2D rats differs from previous results obtained in myocytes from T2D patients (González *et al.* 2005b), in which a higher than normal enzyme activity was detected, perhaps due to species differences. In fact, results from an *in vitro* study performed on skeletal muscle of normal rats (Acitores *et al.* 2005), and by using specific inhibitors for different kinases, confirmed that both PI3K and MAPKs are participating in the insulin-induced glucose uptake; yet, the role of PI3K in this same action of GLP-1 did not seem to be evident; it was demonstrated that an increment in PI3K is determinant for GLP-1, and also for Ex-4, to exert its stimulatory effect on GT and GSa in rat muscles (Acitores *et al.* 2004, Arnés *et al.* 2008) and liver (Redondo *et al.* 2003), and also in human muscle cells (González *et al.* 2005a). Taken into consideration all these observations it can be said that although the skeletal muscle could be participating in the glucoregulatory action of Ex-4, the role of this tissue in diabetic state would not be determinant.

In the fat tissue of the IR model, the downregulated *Glut4* mRNA was not modified after GLP-1 treatment, while it increased the protein value above the normal, in comparison with Ex-4 which stimulated the transcriptional process without affecting the translational process; yet, both GLP-1 and Ex-4 treatment induced a normalization of the lower than normal GT. In the T2D group, Ex-4 did not alter the *Glut4* mRNA value but normalized the initially reduced protein, as previously observed after GLP-1 treatment in this same T2D model (Villanueva-Peñacarrillo *et al.* 2001). This altered glucotransporter expression resulted in a decrease in glucose uptake in the IR rats and an increase in the T2D rats, the latter confirming the previous results (Sancho *et al.* 2005); treatment with either GLP-1 or Ex-4

Table 3 Effect of GLP-1 and Ex-4 on kinase activity in liver, muscle, and fat from normal, insulin-resistant (IR), and type 2 diabetic (T2D) rats. Results are presented as mean $\pm$ S.E.M. (n)

	p13K			p42 MAPK			p44 MAPK		
	Liver	Muscle	Fat	Liver	Muscle	Fat	Liver	Muscle	Fat
Normal	100 $\pm$ 3 (5)	100 $\pm$ 8 (5)	100 $\pm$ 8 (5)	100 $\pm$ 9 (5)	100 $\pm$ 12 (5)	100 $\pm$ 3 (5)	100 $\pm$ 4 (5)	100 $\pm$ 11 (10)	100 $\pm$ 4 (6)
IR	419 $\pm$ 49 (5) [†]	109 $\pm$ 3 (5)	269 $\pm$ 10 (5) [†]	188 $\pm$ 16 (5) [†]	197 $\pm$ 23 (6) [†]	312 $\pm$ 38 (6) [†]	159 $\pm$ 18 (6) [†]	101 $\pm$ 8 (5) [†]	346 $\pm$ 59 (6) [†]
T2D	720 $\pm$ 58 (5) [†]	69 $\pm$ 7 (5) [†]	172 $\pm$ 7 (5) [†]	234 $\pm$ 18 (5) [†]	173 $\pm$ 15 (5) [†]	146 $\pm$ 8 (5) [†]	162 $\pm$ 20 (5) [†]	125 $\pm$ 4 (5) [†]	140 $\pm$ 9 (5) [†]
Normal	100 $\pm$ 3 (5)	100 $\pm$ 4 (5)	100 $\pm$ 5 (5)	100 $\pm$ 5 (10)	100 $\pm$ 6 (5)	100 $\pm$ 5 (11)	100 $\pm$ 3 (10)	100 $\pm$ 5 (12)	100 $\pm$ 7 (8)
Control	116 $\pm$ 11 (5) [†]	262 $\pm$ 47 (5) [*]	147 $\pm$ 7 (5) [*]	60 $\pm$ 3 (6) [*]	83 $\pm$ 6 (8) [*]	54 $\pm$ 4 (7) [*]	83 $\pm$ 4 (8) [*]	165 $\pm$ 8 (8) [*]	180 $\pm$ 10 (8) [*]
GLP-1	135 $\pm$ 2 (5) [*]	173 $\pm$ 2 (5) [*]	151 $\pm$ 6 (5) [*]	81 $\pm$ 6 (6) [*]	74 $\pm$ 5 (8) [*]	122 $\pm$ 12 (5)	84 $\pm$ 8 (6) [*]	147 $\pm$ 11 (6) [*]	102 $\pm$ 10 (5)
IR	100 $\pm$ 4 (5)	100 $\pm$ 12 (6)	100 $\pm$ 10 (8)	100 $\pm$ 3 (10)	100 $\pm$ 6 (10)	100 $\pm$ 8 (5)	100 $\pm$ 3 (10)	100 $\pm$ 7 (10)	100 $\pm$ 4 (9)
Control	159 $\pm$ 15 (5) [*]	143 $\pm$ 8 (5) [*]	157 $\pm$ 13 (5) [*]	230 $\pm$ 5 (5) [*]	60 $\pm$ 13 (6) [*]	86 $\pm$ 8 (6)	117 $\pm$ 10 (5) [*]	116 $\pm$ 11 (5) [*]	100 $\pm$ 9 (5)
GLP-1	129 $\pm$ 8 (6) [*]	146 $\pm$ 8 (5) [*]	195 $\pm$ 15 (5) [*]	161 $\pm$ 18 (5) [*]	98 $\pm$ 12 (6)	125 $\pm$ 10 (8)	102 $\pm$ 2 (5)	73 $\pm$ 5 (6) [*]	61 $\pm$ 5 (5) [*]
T2D	100 $\pm$ 16 (5)	100 $\pm$ 3 (6)	100 $\pm$ 4 (5)	100 $\pm$ 8 (8)	100 $\pm$ 3 (12)	100 $\pm$ 8 (5)	100 $\pm$ 5 (8)	100 $\pm$ 8 (5)	100 $\pm$ 6 (7)
Control	228 $\pm$ 26 (8) [*]	115 $\pm$ 5 (5) [*]	114 $\pm$ 2 (5) [*]	97 $\pm$ 8 (7)	135 $\pm$ 3 (5) [*]	85 $\pm$ 7 (8)	126 $\pm$ 6 (8) [*]	300 $\pm$ 3 (6) [*]	95 $\pm$ 6 (5) [*]
GLP-1	181 $\pm$ 17 (5) [*]	172 $\pm$ 2 (5) [*]	114 $\pm$ 3 (5) [*]	106 $\pm$ 13 (8)	94 $\pm$ 3 (6)	67 $\pm$ 10 (5) [*]	90 $\pm$ 5 (8)	274 $\pm$ 16 (6) [*]	57 $\pm$ 6 (5) [*]
Ex-4									

**P* < 0.05 or lower vs respective control value (saline treated). [†]*P* < 0.05 or lower vs normal-control.

normalized the GT in both experimental models, Ex-4 being less potent in the T2D rats. Nevertheless, the observed apparent discrepancy in the fat tissue of the T2D rats, in which GLP-1 restores close to normal values of the hexose transport despite increasing the gluco-transporter protein, suggests the participation of factor(s) other than GLUT4, e.g. supply of glucose as a source of metabolites for the re-esterification of fatty acids, in the regulation of GT into adipocytes. In the T2D group, the lower than normal *Glut4* expression was seen along with a higher PI3K activity; in the IR group, the hyperactivity of this enzyme was accompanied by a slightly diminished glucose uptake; in both experimental models, the MAPKs activity was increased. By using specific inhibitors, the participation of PI3K and MAPKs in the stimulating effect of GLP-1, and also Ex-4, on GT and metabolism, as well as on lipid metabolism, has already been demonstrated in the adipose tissue of normal and diabetic rats (Sancho *et al.* 2005), and also in normal subjects (Sancho *et al.* 2007); in fact, an increase in the activity of both enzymes is needed not only for insulin but also for GLP-1 and Ex-4 to stimulate GT in normal human (Sancho *et al.* 2007) and obese patients (Sancho *et al.* 2006). In agreement with the latter, treatment of IR and T2D with either GLP-1 or Ex-4 clearly increased PI3K activity. The lack of effect of GLP-1 on the altered MAPK activity in either experimental model indicates that the normalizing action of GLP-1 upon *Glut4* expression and GT in the adipose tissue of the T2D model is not mediated by these enzymes, and thus other mechanism could be activated in this pathological situation. In fact, it has been demonstrated that GLP-1, like insulin, Ex-4, and also its truncated form Ex-9 all stimulate not only PI3K activity but also MAPKs and p70s6k in the adipocytes of normal rats, but not PKB, which is only increased by insulin; yet, the adipocytes of the T2D rats apparently develop a mechanism by which an increment in the activity of PKB is clearly visible when incubated with GLP-1, Ex-4, and Ex-9 (Sancho *et al.* 2005).

In summary, considering the results of this study as a whole, it can be asserted that the three selected extrapancreatic tissues from the IR and T2D rats were involved to a higher or lower extent in the control of glucose homeostasis exhibiting alterations in parameters related to the hexose uptake and metabolism. Also, the data demonstrate that treatment with GLP-1 or Ex-4 has a positive effect upon some of them, which results in a restoration of the normal situation in many cases, and it could justify the demonstrated glucoregulatory action of both peptides. Although Ex-4 and GLP-1 appear to share certain glucose-lowering actions, not all actions of Ex-4 are predictable based on the known pharmacology of GLP-1 (Idris *et al.* 2002), suggesting that Ex-4 may exert some of its effects through a functionally different receptor that is yet to be identified.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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