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Presence of a Functional Receptor for GLP-1 in Osteoblastic Cells, Independent of the cAMP-Linked GLP-1 Receptor

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Glucagon-like peptide 1 (GLP-1) controls glucose metabolism in extrapancreatic tissues through receptors other than the pancreatic cAMP-linked GLP-1 receptor; also, GLP-1 induces an insulin- and PTH-independent bone anabolic action in insulin-resistant and type-2 diabetic rats. Here we searched for the presence and characteristics of GLP-1 receptors in osteoblastic MC3T3-E1 cells. [^{125}I]-GLP-1 specific binding to MC3T3-E1 cells was time- and temperature-dependent, reaching maximal value at 30 min at 25°C; in these conditions, [^{125}I]-GLP-1 binding was dissociable, and displaced by GLP-1, partially by GLP-2, but not by exendin-4 (Ex-4), exendin-9 (Ex-9), glucagon or insulin; Scatchard analysis of the unlabeled GLP-1 data showed high and low affinity binding sites; cross-linking of GLP-1 binding revealed an estimated 70 kDa band, almost undetectable in the presence of 10^{-6} M GLP-1. GLP-1, Ex-9, insulin or glucagon failed to modify cellular cAMP content, while GLP-2 and Ex-4 increased it. However, GLP-1 induced an immediate hydrolysis of glycosylphosphatidylinositols (GPIs) generating short-lived inositolphosphoglycans (IPGs), and an increase in phosphatidylinositol-3 kinase (PI3K) and mitogen activated protein kinase (MAPK) activities; Ex-4 also affected GPIs, but its action was delayed with respect to that of GLP-1. This incretin was found to decrease Runx2 but increased osteocalcin gene expression, without affecting that of osteoprotegerin or the canonical Wnt pathway activity in MC3T3-E1 cells which do not express the pancreatic GLP-1 receptor. Our data demonstrate for the first time that GLP-1 can directly and functionally interact with osteoblastic cells, possibly through a GPI/IPG-coupled receptor.

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Parenteral feeding is associated with reduced bone mass, suggesting that hormones released after nutrients absorption—as it is the case of incretins—could have a role on bone turnover (Henriksen et al., 2003; Clowes et al., 2005). In fact, it has been demonstrated not only the presence of glucose-dependent insulinotropic (GIP peptide) receptor, coupled to the adenylate cyclase-cAMP system, in bone and osteoblastic cell lines (Bollag et al., 2000), but also that mice overexpressing GIP have an increased bone mass (Xie et al., 2007). With respect to the other well known incretin, glucagon-like peptide 1 (GLP-1)—a glucose-dependent insulinotropic hormone that has insulin-independent antidiabetic properties (Creutzfeldt, 2001)—it has recently been demonstrated its insulin-independent bone anabolic action—by continuous infusion of the peptide for 3 days—in both insulin-resistant and type 2 diabetic rat models, in which GLP-1 exerts a normalizing effect on their impaired bone structure (Nuche-Berenguer et al., 2009). Yet, the mechanisms by which GLP-1 is acting on bone turnover are unknown. A previous study showed that genetic disruption of the pancreatic GLP-1 receptor in mice results in an increased bone resorption associated with decreased thyroid calcitonin expression (Yamada et al., 2008); however, the possibility that GLP-1 might act directly on bone cells through this receptor or other uncharacterized receptors is not conclusive from that data.

GLP-1 has direct actions upon glucose and lipid metabolism in extrapancreatic tissues such as liver, skeletal muscle and fat (Valverde et al., 1994; Villanueva-Peñacarrillo et al., 1994; Sancho et al., 2005), which are exerted through specific receptors (Valverde et al., 1993; Delgado et al., 1995; Villanueva-Peñacarrillo et al., 1995). However, the GLP-1 receptor, at least in liver and muscle, seems to be different in structure and/or function to that reported in the pancreas (Thorens, 1992), as GLP-1 does not promote cAMP formation in these tissues or cells (Delgado et al., 1995; Villanueva-Peñacarrillo et al., 1995; Yang et al., 1998; Nishizawa et al., 2000); instead, it induces inositolphosphoglycan (IPG) generation (Márquez et al., 1998; Luque et al., 2002). Moreover, in rats, GLP-1, by prolonged treatment, exerts a control on GLUT-2 and GLUT-4 expression in normal as well as in type 2 and type 1 diabetic states (Villanueva-Peñacarrillo et al., 2001). No previous attempts have currently been made to characterize the presence of putative GLP-1 receptors or their signaling in bone cells.

In the present study, we have searched for the presence and characteristics of GLP-1 receptors in a well characterized osteoblastic cell line. In an attempt to evaluate the function of these receptors, we also assessed in these cells the possible interaction of GLP-1 with osteoblastic genes which are involved

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in bone formation and remodeling. Among them, osteoprotegerin (OPG), which binds to the receptor activator of NF-κB ligand (RANKL) and thus inhibits osteoclast-mediated resorption (Glass et al., 2005; Jackson et al., 2005; Spencer et al., 2006), and Runt-related transcription factor 2 (Runx-2), are Wnt pathway-regulated genes (Glass and Karsenty, 2007, review). Thus, we also explored the putative activation of the latter pathway by GLP-1 in osteoblastic cells.

Materials and Methods

Cells

MC3T3-E1 osteoblastic cells were grown in α-MEM supplemented with 1% penicillin–streptomycin, 2 mM glutamine and 10% fetal bovine serum (FBS), as previously described (Lozano et al., 2009).

Chemicals

Human GLP-1(7-36)amide (GLP-1), exendin(1-39)amide (Ex-4) and GLP-2 (Bachem AG, Bubendorf, Switzerland); pork insulin (Novo Biolabs, Bagsvaerd, Denmark); exendin(9-39)amide (Ex-9, kindly supplied by Dr. John Eng, VAMC, NY); crystalline pork glucagon (Elly Lilly Co., Indianapolis, IN); Na[^{125}I] (580–600 MBq/μg, Hartmann Analytic GmbH, Braunschweig, Germany); polyethilenglycol 6000 (PEG), dimethylsulphoxide (DMSO) and Triton X-100 (Merck, Darmstadt, Germany); bacitracin, phenylmethylsulphonylfluoride (PMSF), leupeptin, pepstatin A, protease inhibitor cocktail P8340, trypsin, HEPES, glutamine, penicillin–streptomycin, chloramine-T, glycerol, isobuthylmethylxantine (IBMX), human PTH (1-34), wortmannin and forskolin (Sigma Chemical Co., St. Louis MO); fetal bovine serum (FBS, Biochrom, Cambridge, UK); α-MEM (Gibco® Invitrogen, Barcelona, Spain); sodium dodecyl sulphate (SDS, Scharlau S.A, Barcelona, Spain); human γ-globulin (Behring, Hoechst Ibérica S.A., Barcelona, Spain); disuccinyl suberate and Perfect Western Dura West (Pierce, Rockford, IL); protein assay kits (Bio-Rad protein assay); nitrocellulose membranes, Tween-20, acrylamide, and bis-acrylamide (Bio-Rad Laboratories, Munich, Germany); Fixer liquid, Developer liquid and X-OMAT Films (Kodak, La Hulpe, Belgium); Rianen cyclic AMP [^{125}I]RIA kit (Perkin Elmer, Waltham, MA); Rainbow markers, and myo-[^{3}H]inositol (Amersham International, Aylesbury, Buckinghamshire, UK); Ultima Gold scintillation liquid (Packard, Groningen, Holland); EN^{3}HANCE (Perkin Elmer); RNAeasy extraction kit (Qiagen, Germantown, MD); β-catenin monoclonal antibody (Millipore, Billerica, MA) horseradish peroxidase-conjugated rabbit anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA); cDNA reverse transcription kit, Taqman MGB probes and TaqMan Universal PCR Master Mix^{TM} (Applied Biosystems, Foster City, CA); Syber Premix Ex Taq^{TM} (Takara, Shiga, Japan); DNA TOPFLASH/renilla mixture (SABiosciences, Frederick, MD); fugene 6 (Roche, Indianapolis, IN); passive lysis buffer and dual luciferase kit (Promega, Madison, WI); Berthold tube luminometer FB/12 Sirius (Berthold Technologies, Bad Wildbad, Germany). All other commonly used chemicals, unless otherwise stated, were from Sigma–Aldrich or Merck (Barcelona, Spain).

[^{125}I]-GLP-1

[mono-^{125}I]GLP-1(7-36)amide (70 MBq/nmol) was prepared by the chloramine-T method using 5 μg of the peptide, 29.6 MBq Na[^{125}I] and 4 μg chloramine-T, in a total volume of 48 μl 0.3 M phosphate buffer, pH 7.4, for 10–20 sec at 25°C, to yield 45–60% [^{125}I] incorporation, and then purified by reversed phase HPLC on a μBondapak C_{18} column, with a linear gradient of 28–49% acetonitrile in 0.1% trifluoroacetic acid (Delgado et al., 1995).

Binding studies

Osteoblastic MC3T3-E1 cells (2×10^5/well) were incubated, at 37 or 25°C, in serum-depleted culture medium, containing Trasylol 500 KIU (kalicrein inhibitor units)/ml, 10 mM $MgSO_4$ and 50 mM Hepes (binding medium), and 20 fmol [^{125}I]-GLP-1 (70 MBq/mol) as tracer, for increasing time periods (0–120 min), in the absence and presence of GLP-1, GLP-2, Ex-4, Ex-9, or insulin (10^{-10}–10^{-6} M). After that, the incubation medium was removed, and in order to eliminate the excess of radioactivity, the cells were washed two times, at 4°C, with the same medium, except for the absence of [^{125}I]-GLP-1. The cells were then recovered from the wells with 0.5 N KOH, and their gamma-radioactivity was measured. The specific binding was calculated by subtracting the non-specific binding (10^{-6} M unlabeled peptide) from each total binding value.

Dissociation study

Cells (7.5×10^6 in 2.5 ml binding medium) were first exposed for 30 min at 25°C to [^{125}I]-GLP-1 (46 fmol), then quickly washed two times at 4°C, resuspended in radioactive free binding medium (10 times initial volume), and then maintained at 25°C for up to 120 min; cell samples treated in the same manner, but first exposed to the additional presence of 10^{-6} M GLP-1, were also included in parallel (for estimation of the corresponding non-specific binding). At different time periods, aliquot volumes (3×1.5 ml) were taken, and cells were pelleted and γ-counted. Data were expressed in relative percent of the specific [^{125}I]-GLP-1 binding, obtained in cell samples (3×150 μl) taken before the rest was subjected to the dilution procedure.

Degradation study

After incubating the cells with [^{125}-I]-GLP-1 at different testing conditions, the radioactive medium was collected and treated with 10% trichloroacetic acid (TCA); the radioactivity of the resulting pellet was measured to determine the extent of peptide damage during the binding procedure.

Isolation of osteoblast membranes

Cells grown in 75 cm^2 flasks were detached with 2% trypsin and 0.02% EDTA in PBS. After centrifugation (1,200*g*, 10 min at 4°C), the cell pellet was resuspended in 50 mM HEPES, pH 7.4, containing 150 mM NaCl, 0.01% bacitracin, 0.01 mM PMSF, 2 μM leupeptin, 2 μM pepstatin and 1% Triton X-100, to a final protein concentration of 2–4 mg/ml (Bradford, 1976), and stirred continuously for 1 h at 4°C (Hedo and Simpson, 1984). The undissolved material, pelleted by centrifugation at 100,000*g* for 1 h at 4°C, was discarded. To remove the detergent in the dissolved membranes, proteins were precipitated with 37.5% PEG in 50 mM Hepes and 10 mM $MgSO_4$, pH 7.5, at 4°C for 30 min (Marshall et al., 1985). The pellet, representing 20–25% of the total protein content in the initial untreated membranes, was reconstituted in 50 mM Hepes pH 7.4, containing 150 mM NaCl, 0.01% bacitracin, 0.01 mM PMSF, 2 μM leupeptin, 2 μM pepstatin, and stored at −70°C.

Cross-linking study

GLP-1 binding was performed in osteoblastic membranes (60 μg protein) incubated with 5×10^{-11} M mono-^{125}I-GLP-1 for 30 min at 25°C, in the absence or presence of unlabeled 10^{-6} M GLP-1, in a total volume of 100 μl 50 mM Hepes, pH 7.4, containing 10 mM $MgSO_4$, 4% BSA, 0.2% bacitracin and 1,000 KIU Trasylol/ml. At the end of the incubation period, the samples were placed in an iced water bath, and the receptor bound peptide was separated from the free peptide by precipitation, after the addition of 500 μl ice-cold 0.11% human γ-globulin with 500 μl PEG (final concentration 12.5%); after 10 min at 4°C, the cells were pelleted at 12,000*g* for 5 min, the supernatants were then removed, and the pellets resuspended in a total volume of 100 μl of the same medium as above. For cross-linking reaction, DSS dissolved in DMSO was added to a final concentration of 1 mM; after 15 min in an iced water bath, 62.5 mM Tris-HCl, pH 7.4, containing 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.003% bromophenol blue was added, and the samples were boiled for 3 min (Delgado et al., 1995).

SDS-PAGE was carried out in a 4% stacking gel and 7.5% resolving gel, following the procedure of Laemmli (1970); a control sample of mono-^{125}I-GLP-1, without membranes, followed the whole process. Analysis of the radioactive bands was performed by autoradiography and subsequent densitometry; for apparent M_r estimation, standard markers with M_r in the range 225,000–17,000 were used.

Adenylate cyclase assay

MC3T3-E1 cells (10^6/well) were incubated for 30 min at 37°C in α-MEM supplemented with 1 mM glutamine and 1% penicillin/streptomycin additionally containing 500 KIU/ml Trasylol and 1 mM IBMX—a phosphodiesterase inhibitor—in the absence (control) or presence of 10^{-7} and 10^{-9} M GLP-1, GLP-2, Ex-4, Ex-9, insulin or glucagon; an equal volume of the medium, as that of the peptides, was added for the control samples. As positive controls, PTH 1-34 (10^{-7} and 10^{-9} M) and 40×10^{-6} M forskolin, were also tested. The reaction was stopped with ethanol (65%, final); the resulting cell extracts were sonicated and centrifuged (at 4°C), and the final supernatants were dried and reconstituted to determine their cyclic AMP content (Luque et al., 2002).

Assay of IPGs

Inositolphosphoglycans (IPGs) were measured indirectly (Saltiel et al., 1986) as the hydrolysis of [^{3}H]-labeled glycosylphosphatidylinositols (GPIs). Thus, MC3T3-E1 cells (9×10^5 cells per well) were pre-labeled overnight at 37°C with 0.2 μCi myo-[^{3}H]inositol, in 0.75 ml α-MEM with added 0.1% BSA, 1% glutamine and 1% penicillin/streptomycin; the radioactive medium was then removed and cells were incubated in fresh α-MEM for different time periods (0.5–10 min) in the absence (control) and presence of tested peptides (10^{-9} M); an equal volume of this medium, as that of the peptides, was added for the control samples. Incubation was stopped by addition of 10% TCA at 4°C, and the radioactive GPI content was extracted and determined in the respective precipitates, as previously described in detail (Márquez et al., 1998).

Kinase activity measurements

Cells were incubated during 3 min at 37°C in the aforementioned culture medium without FBS, in the absence (control) and presence of 10^{-9} M GLP-1, alone or combined with 10^{-5} M wortmannin (a PI3K activity inhibitor); GLP-1 and wortmannin were pre-dissolved in the same media as above, and an equal volume of this medium was added for the control samples. Cells were then scrapped and plasma membranes were isolated (González et al., 2005). For p44/p42 MAPKs, an equal amount of membrane extract from each cell sample and condition tested was subjected to 8% polyacrylamide-SDS gels under reducing conditions (Laemmli, 1970), in parallel with molecular weight markers; after transferring to a nitrocellulose membrane, immunodetection and subsequent quantification by densitometric scanning were performed as previously described in detail (Villanueva-Peñacarrillo et al., 2001). PI3-kinase activity was measured directly in p85 immunoprecipitates obtained in MC3T3-E1 cell membranes, according to a previously reported protocol (González et al., 2005).

Gene expression of osteoblastic genes

MC3T3-E1 osteoblastic cells were treated for 6 h with GLP-1 (10 nM) alone, or in combination with Rp-cAMPS (25 μM), a specific PKA inhibitor. Total RNA from MC3T3-E1 cells was extracted by the RNAeasy extraction kit, following manufacturer's instructions. RNA retrotranscription was carried out with 0.5 μg of total RNA with the cDNA High capacity cDNA reverse transcription kit. Detection of pancreatic GLP-1 receptor was carried out through reverse transcription PCR, with the following primers: 5′-GGAGTGTGAAGAGTCTAAGC-3′ (*forward* GLP-1 receptor) and 5′-GCAGTACTGCATGAGCAGGA-3′ (*reverse* GLP-1 receptor), accordingly with a previous publication (Noguchi et al., 2009). Bands were resolved and visualized in a 2% agarose gel.

Changes in gene expression of low density lipoprotein receptor-related protein 5 (LRP5), low density lipoprotein receptor-related protein 6 (LRP6), axin1 and 18S rRNA were quantified by real time PCR with Syber Premix Ex Taq™, with the following primers: LRP5, *forward* 5́-CAACGTGGACGTGTTTTATTCTTC-3́, *reverse* 5́-ACTGGTGCTGTAGTCA-3; LRP6, *forward*, 5́-AGATCCATC-AAGTGGGTTCATGTA-3́, *reverse* 5́-AAGCGACTTGAGCCAT-CCA-3́; axin1, *forward* 5́-CGAAGGGAGATGCAGGAGAGTA-3́, *reverse* 5-AGTGCGAGGAATGTGAGGTAGAG-3́ and 18S rRNA (housekeeping gene), *forward* 5́-TGCTCTTAGCTGAGGTGCC-CG-3́, *reverse* 5-ATTCCTAGCTGCGGTATCCAGG-3́. Osteocalcin (OC), Runx-2, and OPG were quantified with specific primers and Taqman MGB probes, and TaqMan Universal PCR Master Mix™. At least four PCR reactions per experimental condition were carried out, and gene expression was normalized with that of the housekeeping gene 18S. Gene expression was calculated using comparative threshold cycle method ($2^{-\triangle\triangle Ct}$), as previously described (Nuche-Berenguer et al., 2010).

β-Catenin Western analysis

Serum-depleted (1% FBS) MC3T3-E1 cells were treated with 10^{-8} M GLP-1 for 24 h. Total protein was then extracted with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, supplemented with the protease inhibitor cocktail P8340. Protein extracts (10 μg) were separated on 8% polyacrylamide-SDS gels under reducing conditions. After electrophoresis, samples were transferred onto nitrocellulose membranes, followed by blocking with 5% defatted milk in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl with 0.05% Tween-20. Immunodetection was done with a monoclonal antibody raised against β-catenin, at 1/1,000 dilution, and horseradish peroxidase-conjugated rabbit anti-mouse IgG. Perfect Western Dura West was used as developing reagent.

TOPFLASH luciferase assay

MC3T3-E1 cells (10^4/cm^2) in 12-well plates were transfected with 0.5 μg of the DNA TOPFLASH/renilla mixture (40:1 ratio) and Fugene 6, following manufacturer's instructions. After 24 h of transfection, cells were treated without and with 10^{-8} M GLP-1. As positive control for TOPFLASH activity, 25% (v/v) of conditioned medium (CM, 2% FBS), obtained from L-cells stably transfected with a plasmid coding for Wnt3a was used (Willert et al., 2003). After 24 h, the cells were lysed with passive lysis buffer, and luciferase/renilla activity was quantified with a dual luciferase kit using a Berthold tube luminometer FB/12 Sirius. Renilla activity was used as internal control in order to normalize the luciferase values. Experiments were carried out in triplicate.

Statistical analysis

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

Results

GLP-1 binding

In a preliminary set of experiments, we found that the [^{125}I]-GLP-1 binding to MC3T3-E1 cells (2×10^5/well) for 30 min at 25°C represented 3.34 ± 0.38% of the total radioactivity (n = 10 separated experiments). The time course of the tracer binding is shown in Figure 1 (upper part). At 25°C, binding was detectable at minute 10 (1.6 ± 0.1% total tracer

radioactivity, n = 4), reaching the maximal value after 30–45 min (overall mean: 2.65 ± 0.07%, n = 10), and decreasing thereafter (at 120 min: 1.60 ± 0.18%, n = 3); the non-specific binding did not change with time (1.01 ± 0.02%, n = 20). At 25°C, degradation of [^{125}I]-GLP-1, measured in the incubation medium as non-precipitated TCA, represented 14.0 ± 0.4% total tracer radioactivity (n = 3) at minute 30—being 5.0 ± 1.0% (n = 3) in the absence of cells—, and increased to 30% after 120 min incubation; meanwhile, at 37°C, degradation of [^{125}I]-GLP-1 represented 31 ± 1% (n = 3) after 30 min. The tracer specific binding obtained at 25°C during 30 min was dissociable (Fig. 1, lower part), decreasing to 50.1 ± 2.5% and 19.2 ± 1.6% (n = 3), at 30 and 120 min incubation, respectively; the non-specific binding in these experiments, measured in cells pre-incubated with 10^{-6} M GLP-1, and then treated in the same manner, did not significantly change (data not shown). Further characterization of [^{125}I]-GLP-1 binding was performed at 25°C for 30 min.

The tracer specific binding at 25°C during 30 min (0.33 ± 0.04% total, n = 11) was displaced by increasing concentrations of GLP-1 (Fig. 2A), with a 50% inhibition dose (ID_{50}) of 3×10^{-9} M. A displacement of the GLP-1 tracer binding by GLP-2 was detected only at 10^{-6} M of the peptide which, at this concentration, was similar to that obtained by 10^{-6} M GLP-1; neither Ex-4 nor Ex-9, glucagon or insulin competed with the [^{125}I]-GLP-1 binding (data not shown). The Scatchard analysis of the unlabeled GLP-1 data (Fig. 2A, inset) revealed the presence of high affinity binding sites, with a dissociation constant (Kd) of 1.46 nM, an affinity constant (Ka) of 6.87×10^{7} L/mol, and a binding capacity of 4 fmol/10^{6} cells; low affinity binding sites were also present. As shown in Figure 2B, no expression of the pancreatic GLP-1 receptor was detected by reverse transcription-PCR in MC3T3-cells.

Cross-linking studies

SDS-PAGE of the MC3T3-E1 membrane binding activity, cross-linked to mono-^{125}I-GLP-1, revealed a single radioactive band with an apparent M_r of 70,000 Da (Fig. 2A, inset), whose intensity was much lower in the membrane samples incubated in the additional presence of 10^{-6} M unlabeled GLP-1.

Adenylate cyclase activity

As shown in Table 1, in the presence of 1 mM IBMX, neither 10^{-9} M GLP-1 nor Ex-4 or Ex-9 modified the cyclic AMP control value (27.1 ± 1.7 pg/2 × 10^{5} cells, n = 6 individual

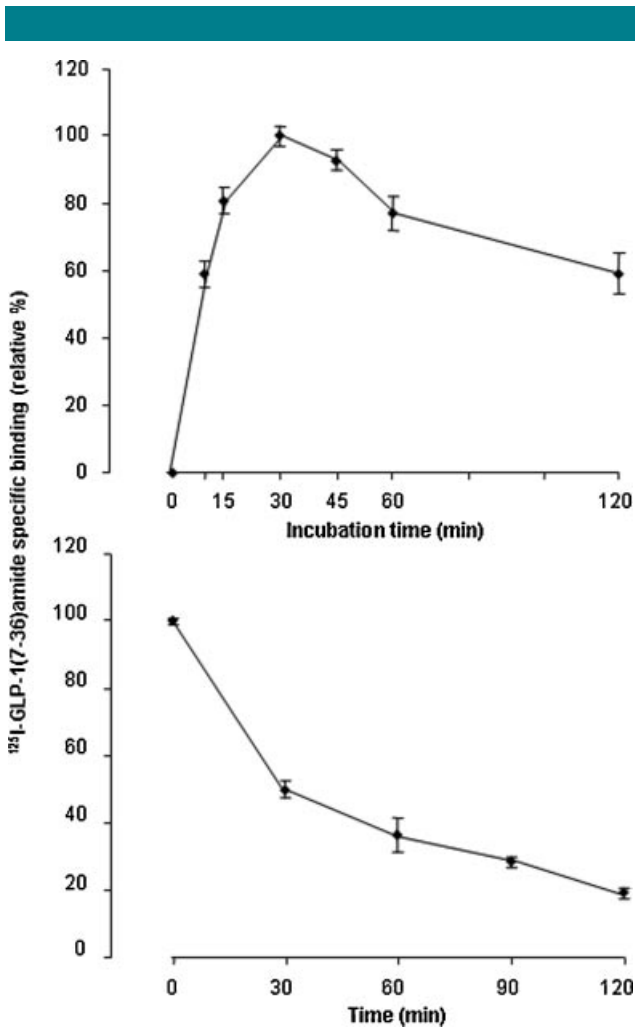

Fig. 1. GLP-1 binding at 25°C to osteoblastic MC3T3-E1 cells as a function of incubation time (upper part), an its dissociation rate (lower part). Mean ± SEM, both n = 3–10 separated experiments.

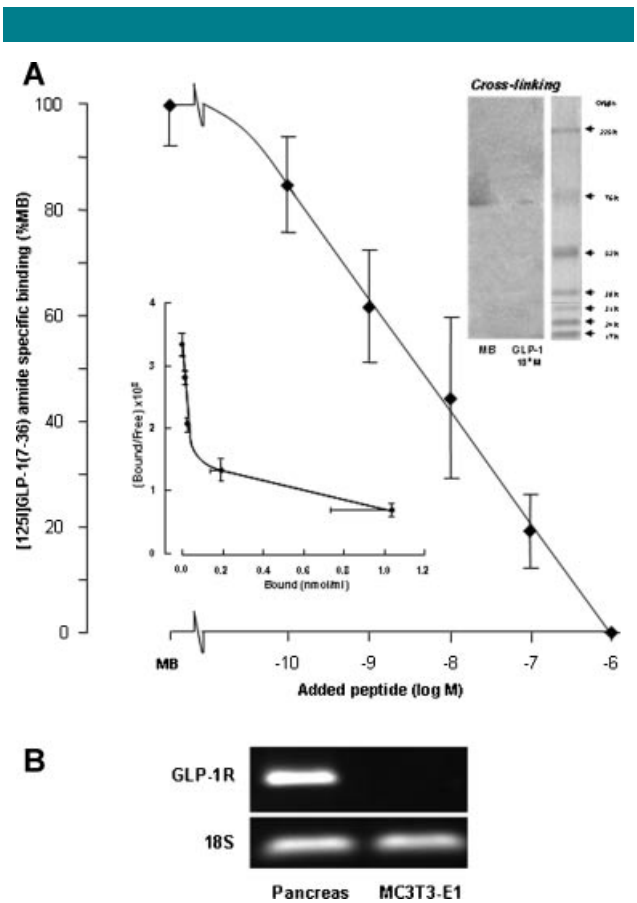

Fig. 2. A: Displacement by unlabeled GLP-1 of the [^{125}I]-GLP-1 specific binding to MC3T3-E1 cells, at 25°C during 30 min. Insets: Scatchard analysis, and representative autoradiogram of [^{125}I]-GLP-1 cross-linked to partially purified osteoblast membranes, in the absence (MB) and presence of 10^{-6} M unlabeled GLP-1, together with molecular weight markers; data are expressed as mean ± SEM, and correspond to 11 individual experiments. B: Representative reverse transcription-PCR showing the absence of expression of the pancreatic GLP-1 receptor (GLP-1R) in MC3T3-E1 cells, and its presence in the mouse pancreas (as positive control).

TABLE 1. Effect of GLP-1, Ex-4, Ex-9, and GLP-2 on the cAMP production, in osteoblastic MC3T3-E1 cells [mean ± SEM, n = number of individual experiments]

	% Control
Control	100 ± 7
GLP-1	
10^{-9} M	108 ± 6
10^{-7} M	104 ± 5
Ex-4	
10^{-9} M	96 ± 5
10^{-7} M	154 ± 16*
Ex-9	
10^{-9} M	93 ± 10
10^{-7} M	101 ± 8
GLP-2	
10^{-9} M	142 ± 3*
10^{-7} M	151 ± 8*
PTH	
10^{-9} M	239 ± 38*
10^{-7} M	418 ± 31*
Forskolin	
4×10^{-5} M	1,147 ± 37*

% Control value (27.1 ± 1.7 pg/2 × 10^5 cells, n = 6).
*$P < 0.05$, or lower, versus control (no added peptide).

experiments) obtained in cells incubated in the absence of peptides (GLP-1, Ex-4, and Ex-9 overall mean: 95.1 ± 5.3% control, n = 4–6); neither glucagon nor insulin at 10^{-9} M induced any change (overall mean: 111.5 ± 6.1% control, n = 3), while a clear increase occurred with 10^{-9} M PTH, 10^{-9} M GLP-2 and 4×10^{-5} M forskolin. At 10^{-7} M, GLP-1, Ex-9 and glucagon also failed to modify the cAMP control value (overall mean: 105 ± 3% control, n = 4–6); yet, at this same concentration, Ex-4 induced a moderate but clear cAMP stimulation, while that by PTH was even higher ($P < 0.03$) than that observed with 10^{-9} M of the hormone; no further increment in cAMP cell content was detected with 10^{-7} M GLP-2 than that obtained at 10^{-9} M of the peptide.

Analysis of IPGs

In the absence of added peptide (control), GPI content in MC3T3-E1 cells was unchanged up to 10 min, indicative of no IPGs release (Saltiel and Cuatrecasas, 1986; Saltiel et al., 1986); thus, the mean value at different times within this period was used as control (Fig. 3A). GLP-1, at 10^{-9} M, induced a rapid disappearance of the radioactive GPIs, already detected at 30 sec, and statistically significant at 1 and 2 min (n = 4 individual experiments, $P < 0.05$); this reduction was followed by a recovery, reaching the control value at minute 10. Ex-4 (n = 4) also showed to have a decreasing action on the GPI content, its initial effect though being less potent and delayed with respect to that of GLP-1.

Kinase activities

As shown in Figure 3B, GLP-1, at 10^{-9} M, induced a clear increase in the phosphorylation of both p44 and p42 MAPKs in MC3T3-E1 cells (*p*-p44: 515 ± 86% control, $P < 0.01$; and *p*-p42: 157 ± 12%, $P < 0.01$; both n = 3 individual experiments). GLP-1 also increased PI3K activity, measured as PIP3 generation (156 ± 9% control, n = 3 individual experiments, $P < 0.05$), this effect being inhibited by 10^{-5} M wortmannin (a PI3K inhibitor) to a value even below that of the control (54 ± 8% control, $P < 0.05$).

GLP-1 effect on the expression of various osteoblastic genes

We previously demonstrated that infusion of GLP-1 for 3 days into normal or diabetic rats increased bone OC mRNA levels (Nuche-Berenguer et al., 2009). Thus, we aimed to explore

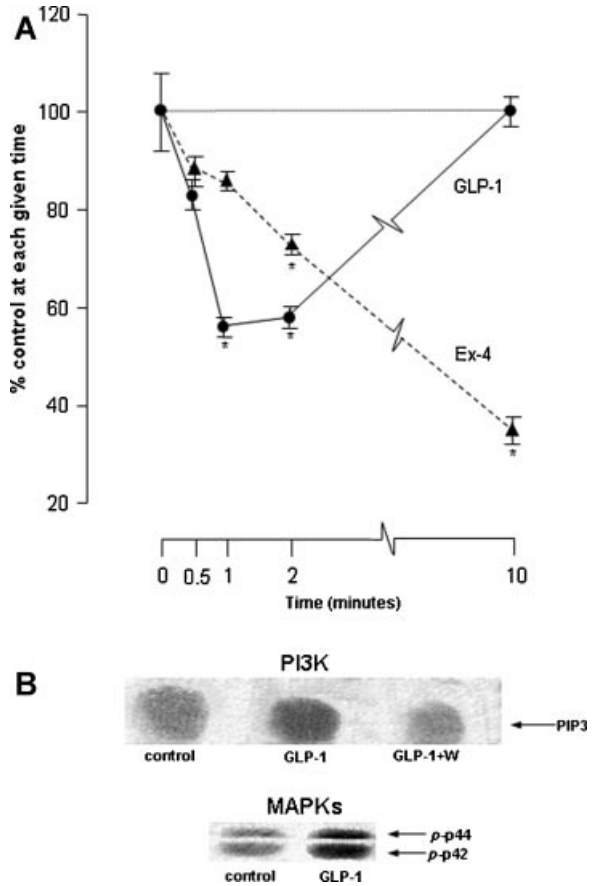

Fig. 3. A: Effect of 10^{-9} M GLP-1 or Ex-4 on the hydrolysis of glycosylphosphatidylinositols (GPIs) in osteoblastic MC3T3-E1 cells. Data (mean ± SEM, n = 4 individual experiments) are relative to the overall mean of the respective paired control, obtained in cells incubated in the absence of peptide at each given time. *$P < 0.05$ versus control. B: Representative autoradiogram showing changes in labeled PIP3 levels, indicating PI3K activity (upper part), in osteoblastic MC3T3-E1 cells incubated in the absence (control) and presence of 10^{-9} M GLP-1 without and with 10^{-5} M wortmannin (a PI3K activity inhibitor); phosphorylated p44/42 MAPKs by 10^{-9} M GLP-1 (lower part); protein loading was similar in each well, as assessed by using Ponceau S staining (not shown).

whether GLP-1 interaction with MC3T3-E1 cells might influence the expression of this gene as well as that of the earlier osteoblast differentiation gene Runx-2. We found that incubation of MC3T3-E1 cells with a high dose (10^{-8} M) of GLP-1 for 24 h downregulated the latter gene expression (0.4 ± 0.1 times of control, $P < 0.01$, n = 5 separated experiments); a similar inhibitory effect (not shown) was observed by further exposure (up to 5 days) of these cells in an osteoblast differentiation medium, consisting in standard medium with 50 μg/ml ascorbic acid and 10 mM β-glycerolphosphate. OC expression, a later osteoblastic maturation marker, was slightly but significantly ($P < 0.05$ n = 5 separated experiments) upregulated (1.23 ± 0.03 times control), whereas OPG mRNA levels were unaffected (1.03 ± 0.04 times control) in this setting. Moreover, we found that the expression of the major genes of the Wnt pathway, an important modulator of OPG expression in osteoblasts (Glass et al., 2005), two main activators (LRP5 and LRP6) and the inhibitor axin, was similarly downregulated (Fig. 4A).

Consistent with an unaltered Wnt pathway by GLP-1 in MC3T3-E1 cells, β-catenin protein levels as well as its transcriptional activity, as determined by TOPFLASH luciferase, were unchanged by this incretin (Fig. 4B,C).

Discussion

It was recently demonstrated that GLP-1 has an in vivo osteogenic action on the altered bone metabolism in insulin-resistant and type 2 diabetic rats, as shown by changes in bone remodeling markers and bone architecture; and that this effect occurs in an insulin- and PTH-independent manner (Nuche-Berenguer et al., 2009). It was also previously reported that GLP-1 exerts anti-resorptive effects through a calcitonin-dependent pathway in mice with a deletion of the pancreatic GLP-1 receptor gene (Yamada et al., 2008). However, no previous attempts to characterize a GLP-1 receptor in bone have been reported.

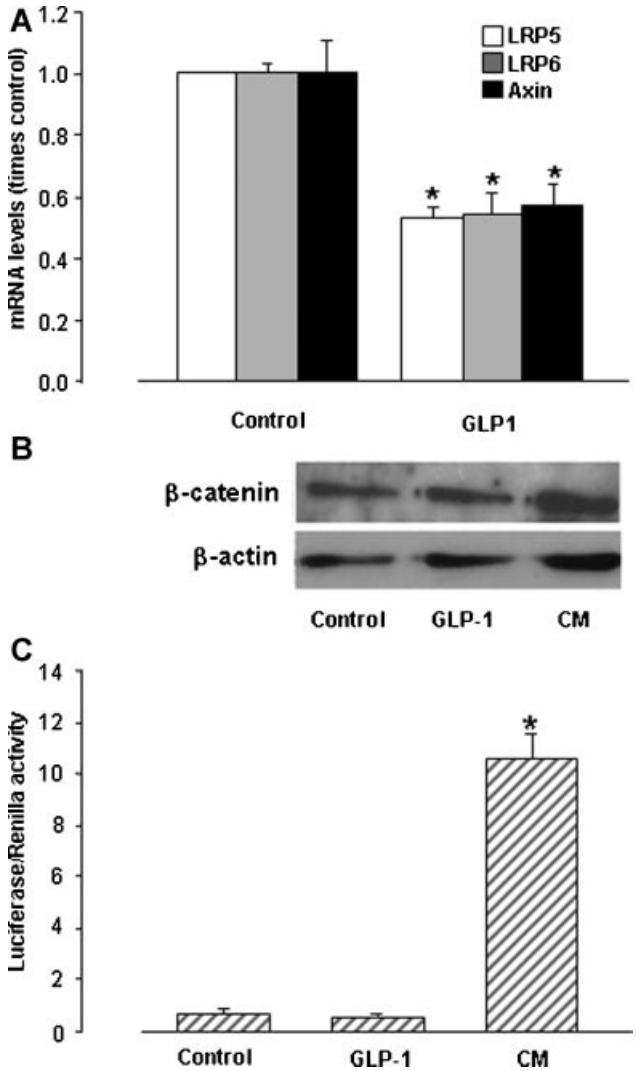

Fig. 4. GLP-1 does not affect the activity of the Wnt pathway in osteoblastic MC3T3-E1 cells. A: LRP5, LRP6, and Axin mRNA levels (assessed by real time PCR) were similarly and significantly downregulated by 10^{-8} M GLP-1 for 6 h. B: β-Catenin protein levels (Western blot) and (C) TOPFLASH luciferase activity were unchanged by 10^{-8} M GLP-1 at 24 h. A representative autoradiogram and results (mean ± SEM) from three separated experiments, each assayed in triplicate are shown in (B,C), respectively. As positive control, the conditioned medium (CM) from L-cells stably transfected with Wnt3a overexpressing plasmid, was used. *$P < 0.05$ versus corresponding control value.

The present data demonstrate that GLP-1 specifically binds to osteoblastic MC3T3-E1 cells, indicative of the presence of GLP-1 receptors which, in addition, display high- and low-affinity binding capacities; also, we show here evidence for a GLP-1-bound membrane protein in MC3T3-E1 cells, similar in size—as estimated by SDS-PAGE of the cross-linked binding activity—to that found in other extrapancreatic tissues (Delgado et al., 1995; Villanueva-Peñacarrillo et al., 1995). This potential GLP-1 receptor shows the characteristics of a classical receptor, as GLP-1 binding to these osteoblastic cells is time- and temperature-dependent, dissociable, and displaceable by GLP-1, but not by insulin, glucagon or Ex-4. GLP-2—a peptide reducing bone resorption (Henriksen et al., 2003)—seems to cross-react with this putative receptor, although in a much lower extent than GLP-1; nevertheless, while GLP-2 clearly stimulated the cAMP production in these cells, GLP-1 failed to do so. This last is in contrast to signaling through the pancreatic GLP-1 receptor (Thorens, 1992), but similar to previous observations in other extrapancreatic tissues (Valverde et al., 1994; Alcántara et al., 1997; Luque et al., 2002), and also supports the notion that GLP-1 does not interact with the yet uncharacterized GLP-2 receptor in bone cells. The existence of GLP-1 receptors in various extrapancreatic tissues and cells, different in structure and/or function from the pancreatic GLP-1 receptor, has been supported by various experimental evidences (Delgado et al., 1995; Villanueva-Peñacarrillo et al., 1995; Montrose-Rafizadeh et al., 1997; Yang et al., 1998; Nishizawa et al., 2000), although their molecular composition remains unknown since they have not been cloned. To our knowledge, the putative presence of the pancreatic GLP-1 receptor in bone cells had not been previously explored. In the present study, using reverse transcription-PCR with specific primers for the pancreatic GLP-1 receptor, we failed to detect a band in MC3T3-E1 cells as we did in the mouse pancreas. Thus, taken together, these data strongly suggest that the GLP-1 binding described in the present work cannot be accounted for by the presence of the pancreatic GLP-1 receptor in these osteoblastic cells.

In liver and muscle, the effects of GLP-1 on glucose metabolism have been characterized previously (Valverde et al., 1994; Villanueva-Peñacarrillo et al., 1994). In these tissues, GLP-1 does not stimulate cAMP but induces the rapid hydrolysis of GPIs (Márquez et al., 1998; Luque et al., 2002) related to a short-lived generation of IPGs (Saltiel and Cuatrecasas, 1986; Saltiel et al., 1986), a well characterized signaling pathway in the action of different hormones (Jarett and Seals, 1979; Larner et al., 1979; Suzuki et al., 1984a,b; Larner, 1988). In the present study, GLP-1 has shown to promote the immediate hydrolysis of GPIs in osteoblastic MC3T3-E1 cells—maximal between 1 and 2 min—and its subsequent re-synthesis thereafter, following the same pattern than that previously shown in other extrapancreatic tissues and cells (Galera et al., 1996; Márquez et al., 1998; Luque et al., 2002). These data suggest that IPGs could be acting as second messengers of the GLP-1 receptor in osteoblastic cells. In addition, this effect of GLP-1 is consistent with the GLP-1-induced stimulation upon PI3K and MAPK activities, as observed here, which has shown to be a consequence of IPGs generation in other cell types; in fact, it is known that GPI hydrolysis yields two products, diacylglycerol and IPGs, the latter, having insulin-like properties, exerts a stimulatory effect upon both enzymatic activities (see Müller et al., 1998; Jones and Varela-Nieto, 1999, for review).

A clear bone anabolic action of Ex-4—a GLP-1 homologous peptide of non-mammalian nature—has recently been reported in insulin-resistant and type 2 diabetic rat models (Nuche-Berenguer et al., 2010). Ex-4 shows GLP-1-like effects on glucose and/or lipid metabolism in liver, muscle and fat (Alcántara et al., 1997; Luque et al., 2002; Sancho et al., 2005). Although it has been suggested that the effects of Ex-4 in these extrapancreatic tissues could be exerted, at least in part, through the GLP-1 receptor (Arnés et al., 2008), it does not seem to be the case in these osteoblastic cells. In fact, Ex-4, at a high concentration (10^{-7} M), unlike GLP-1, moderately increased the cellular cAMP content in MC3T3-E1 cells; and although it induced the hydrolysis of GPIs, its effect was somewhat delayed by respect to that of GLP-1. In addition, previous reports have shown that Ex-4 does not act as an agonist of GLP-1 in some situations, where its effects are not mimicked by GLP-1 or dipeptidyl-peptidase 4 inhibitors, neither by Ex-9 (Pérez-Tilve et al., 2007; Simonsen et al., 2007).

Our present data also indicate a functional interaction of the putative GLP-1 receptor in osteoblastic cells. Hence, we show that GLP-1 downregulates Runx2 but moderately upregulates OC, suggesting a preferential effect of GLP-1 on the later stages of osteoblastic differentiation. However, this incretin failed to affect OPG; and although it similarly decreases some canonical Wnt pathway activators and an inhibitor, the final outcome of this pathway activity was unchanged by GLP-1. This was supported by the absence of changes in either β-catenin protein stabilization or its transcriptional activity triggered by GLP-1 in MC3T3-E1 cells.

The stimulatory effect of GLP-1 on OC in vitro in an osteoblastic cell line is congruent with its recently reported in vivo action in rats (Nuche-Berenguer et al., 2009). However, in contrast to our previous in vivo report, we failed here to detect any significant alteration of OPG; nevertheless, the aim of the present study was to confirm that GLP-1 can directly act on osteoblastic cells and, thus, we only tested a single dose of this peptide. It is possible that continuous infusion of GLP-1 for several hours in vivo might lead to a different outcome regarding the expression of several osteoblastic genes, as shown in our previous report (Nuche-Berenguer et al., 2009). Alternatively, the endocrine effect of GLP-1 by acting on the thyroid through its pancreatic receptor, as previously reported (Yamada et al., 2008), might highly contribute to the in vivo effects of this incretin.

In summary, the present findings point out for the first time that GLP-1 can directly and functionally interact with osteoblastic cells through a receptor, different from the cloned GLP-1 receptor in pancreas. The present data also suggest that GLP-1 signaling in osteoblastic cells involves, at least in part, the GPI/IPG system.

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