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Synergistic Effect of Glucagon-Like Peptide 2 (GLP-2) and of Key Growth Factors on the Proliferation of Cultured Rat Astrocytes. Evidence for Reciprocal Upregulation of the mRNAs for GLP-2 and IGF-I Receptors

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Abstract The aim of this work was to determine whether the stimulating effect of glucagon-like peptide (GLP)-2 on astrocyte proliferation could be reinforced by proliferating substances, including growth factors such as EGF, platelet-derived growth factor, insulin-like growth factor type I (IGF-I) or a hormone such as insulin. Both DNA synthesis and astrocyte density, as well as the expression of c-Fos, Ki-67, proliferating cell nuclear antigen and glial fibrillary acidic proteins, were found to be higher in the presence of GLP-2 than in its absence. In an attempt to get a better understanding of this process, intracellular cyclic adenosine monophosphate (cAMP) production, extracellular signal-regulated kinase (ERK) 1/2 phosphorylation and the expression of GLP-2R and IGF-I receptor (IGF-IR) mRNAs were studied in response to growth factors. Our results indicate that, in the presence of different growth factors, GLP-2 does not increase cAMP production but raises ERK 1/2 phosphorylation. In addition, GLP-2R mRNA expression was increased by IGF-I, whilst mRNA expression of IGF-IR was higher in cells incubated with GLP-2 than in control cells. These results suggest for the first time that GLP-2 and several growth factors show synergistic effects on the proliferation of rat astrocytes, a process in which an enhanced expression of GLP-2R and IGF-IR may be involved, providing additional insights into the physiological role of this novel neuropeptide, specially during astroglial regeneration.

Keywords GLP-2 · Growth factors · Insulin · Astrocytes · Proliferation · Gene expression

Introduction

Glucagon-like peptide 2 (GLP-2) is a 33-amino-acid peptide derived from the differential posttranslational processing of proglucagon in intestinal endocrine L cells and in the brain [1]. It has been reported that GLP-2 stimulates both small intestinal mucosal growth [2] and the proliferation of rat astrocytes [3], which has opened new insight into the physiological role of this peptide.

The actions of GLP-2 are mediated via a G-protein-coupled receptor (GLP-2R), which is linked to the activation of the adenylate cyclase pathway [4]. The localisation of GLP-2R has mainly been reported in subsets of enteric neurons [5], in enteroendocrine cells [6], in vagal afferents of the nodose ganglia [7], and in several regions of the central nervous system (CNS) [8]. However, a detailed understanding of the mechanism of action of GLP-2 has been hampered by a lack of suitable physiological models for the study of GLP-2R activation. Although it has recently been reported that cultured hela cells [9], CCD-18Co intestinal myofibroblasts and foetal human colon

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cells express a functional GLP-2R endogenously [10], this receptor is not expressed in most intestinal epithelial cell lines [6, 11]. Nevertheless, a direct proliferative response to treatment with GLP-2 in Caco-2 and T84 cells, but not in IEC-6 cells, has been established in vitro [12]. In any case, the most detailed characterisation of the GLP-2R intracellular signalling pathways has been reported in studies addressing GLP-2R-transfected cell lines [4, 12, 13], and it may be concluded that GLP-2R activation functions via divergent signalling pathways [14].

To date, two reports using primary cultured cells have demonstrated the presence of native GLP-2R expression, one occurring in cultured rat intestinal mucosal cells [15] and the other in cultured rat cortical astroglial cells [3]. In both cell types, the GLP-2R expressed proved to be functional, as judged by GLP-2-induced cyclic adenosine monophosphate (cAMP) production and [^{3}H]-thymidine incorporation. In astrocytes, GLP-2 also induces cell cycle progression and c-Fos mRNA expression.

The aim of the present study was to determine whether or not the previously reported effect of GLP-2 on astrocyte proliferation can be enhanced by other growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin-like growth factor type I (IGF-I), or by insulin. This was analysed by [^{3}H]thymidine incorporation and cell density studies and by the expression of glial fibrillary acidic protein (GFAP), c-Fos, proliferating cell nuclear antigen (PCNA) and Ki-67 (pKi-67). In an attempt to gain a better understanding of the synergistic effects of GLP-2 with other growth factors on astrocyte proliferation, mechanisms of GLP-2 signalling, such as cAMP production and ERK 1/2 phosphorylation, were determined. In addition, the possible existence of a reciprocal control of both the GLP-2R and IGF-I receptor (IGF-IR) genes by their ligands was studied.

Materials and Methods

Materials

Rat GLP-2 and exendin (9–39) were obtained from Bachem (St. Helens, UK). Recombinant IGF-I, PDGF and EGF were purchased from PreproTech EC Ltd. (London, UK). Recombinant human insulin Arg^+ Zn was obtained from Calbiochem (La Jolla, CA, USA). [Methyl-^{3}H]-thymidine (60–80 Ci/mmol) was from NEN Life Science Products, Inc. (Boston, MA, USA). The cAMP enzyme immunoassay (EIA) system was from Amersham Pharmacia Biotech (Little Chalfont Bucks, UK). Foetal bovine serum (FBS), heat-inactivated foetal bovine serum (hiFBS), bovine serum albumin (BSA), diprotin A, forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma (Poole, Dorset, UK). All other chemicals were of reagent grade or molecular biology grade.

Rat Astrocyte Cultures and Treatments

All procedures involving animals were approved by the appropriate institutional review committee and met the guidelines for the care of animals specified by the European Community. Primary rat astroglial cell cultures were prepared from cerebral hemispheres of 1-day-old Wistar rats as previously described [3, 16]. Immunocytochemical analysis of the cultures revealed that at least 95% of the cells were positive for the astrocyte-specific marker, glial fibrillary acidic protein. Astrocytes were grown in 12- and 24-well (for [^{3}H]-thymidine incorporation, crystal violet assays and cAMP measurements) or in 100×20-mm (for Western blot and gene expression analyses) tissue culture dishes. Different treatments were carried out after the cell cultures had been shifted to serum-free medium for 24–48 h. GLP-2 stimulation experiments were carried out in Dulbecco's modified Eagle's medium/F-12 medium and 15 mM Hepes, supplemented with FBS, hiFBS or BSA. When diprotin A, growth factors (EGF, IGF-I, PDGF), insulin or forskolin were used, they were simultaneously added to GLP-2. Exendin (9–39) was added 10–60 min before GLP-2. At the indicated times, the reactions were stopped by removing the supernatants. Then, the cells were washed with phosphate-buffered saline (PBS) and harvested for the different analyses, as described below.

[^{3}H]-Thymidine Incorporation into DNA

The astrocyte DNA synthesis rate was estimated by quantification of the amount of tritium incorporated into the acid-precipitable form after [^{3}H]-thymidine incubation, as previously described [3]. [^{3}H]-Thymidine (5 μCi/ml, final concentration) was present during the last 4 h of the different treatments. Results are expressed as disintegrations per minute of [^{3}H]-thymidine incorporated per well.

Crystal Violet Staining

The relative number of cells was measured as described previously [3], using a method based on the staining of cells with crystal violet. Optical density at 560 nm (OD_{560}) was determined on 96-well plates (Varioskan Thermo Electron Corporation, Software ScanIt 2.0.91). The results were expressed as absorbance per well.

Western Blotting Analyses

After the treatments, the cells were lysed on ice for 30 min in ice-cold lysis buffer containing PBS, pH 7.5,

0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate, 1% NP-40, 10 μM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin and 10 μg/ml leupeptin. The protein concentration of cleared lysates was determined with the Bradford reagent (Bio-Rad Laboratories S.A., Madrid, Spain), using BSA as standard. Proteins (25–40 μg) were loaded on 4% stacking/6–14% separating SDS polyacrylamide gels (Bio-Rad). The resolved proteins were electrotransferred onto a 0.45-μm polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA, USA) for Western blot analyses. Blots were blocked with 5% non-fat milk in Tris-buffered saline (20 mM Tris, 150 mM NaOH, pH 7.4) containing 0.1% Tween-20 and were incubated with rabbit polyclonal anti-c-Fos (1:500, Calbiochem, La Jolla, CA, USA), rabbit polyclonal anti-Ki-67 (1:500, Chemicon International, Temecula, CA, USA), mouse monoclonal anti-PCNA (1:1,000, Chemicon International), mouse monoclonal anti-GFAP (1:8,000, Sigma), rabbit polyclonal anti-Phospho-Erk1/Erk2 (1:3,000, MBL Woburn, MA, USA) or rabbit polyclonal anti-Erk1/Erk2 (1:15,000, Calbiochem) overnight at 4ºC. After extensive washing, horseradish-peroxidase-linked secondary antibodies were added (1:5,000, Calbiochem). Chemiluminescence detection was performed with a Western blotting ECL system (Amersham) and exposed to X-ray films according to the manufacturer's instructions. The autoradiographs were analysed by densitometry (Model GS-800, Bio-Rad) and quantified (Quantity One software, Bio-Rad). Blots were routinely stripped (Re-Blot western Blot Recycling, Chemicon International) and re-probed with rabbit polyclonal anti-cyclophilin-A (1:5000, Calbiochem) or mouse polyclonal anti-alpha-tubulin (1:10,000, Sigma) to monitor the levels of an invariant control.

Intracellular cAMP Measurement

Intracellular cAMP was measured using the commercial protocol for the non-acetylation EIA system with some modifications [3]. Reactions were stopped by removing the supernatants. Then, cold ethanol was added to extract cAMP which was evaporated in a speed vac system. cAMP was dissolved in lysis reagent and harvested at −80ºC until use. Proteins were dissolved after in 0.5 N NaOH. Results are expressed as picomole per milligramme protein.

RNA Preparation and Real-Time Quantitative RT-PCR of GLP-2R and IGF-IR

Total cellular RNA was isolated using a modified guanidinium thiocyanate procedure [17]. First-strand cDNA was synthesised from 1 μg of total RNA in 20 μl using a commercial Kit (RETROscript™, cat num. 1710, Ambion®, Austin, TX, USA). Real-time polymerase chain reaction (PCR) was performed using the TaqMan Universal PCR Mix (Applied Biosystems TaqMan Universal Master Mix), using the FAM-labelled TaqMan gene expression assays according to the manufacturer's standard protocol (Applied Biosystems, Warrington, UK). Real-time quantitative reverse-transcribed (RT)-PCR analyses for the GLP-2R and IGF-IR genes were performed starting with 75–110 and 4 ng of RT total RNA, respectively. The PCR was carried out in 96-well plates using the 7300 Real-Time PCR System (Applied Biosystems). Data were analysed using 7300 System SDS software, version 1.2.3 (2004). The relative amounts of GLP-2R mRNA and IGF-IR mRNA were calculated using the comparative $\Delta\Delta C_T$ method. Cyclophilin mRNA was used as the invariant endogenous control for all studies.

Statistical Analysis

Values are reported as means ± SD; P values from <0.05 were considered statistically significant. For statistical comparisons between two independent variables presenting a normal distribution with equal variances, Student's unpaired t test was used.

Results

GLP-2 Enhances the Effects of EGF, PDGF and IGF-I or Insulin on the Proliferation of Cultured Rat Astrocytes

We have reported previously that GLP-2 stimulates the proliferation of cultured rat astrocytes [3]. To determine whether the effect of GLP-2 on cell proliferation could be modified by the presence of another proliferate agent, the experiments were first carried out in the presence of serum, as a source of unknown growth factors, but also in the presence of EGF, PDGF, IGF-I or insulin, all of which have significant effects on astrocyte proliferation. As shown in Fig. 1a, when the experiments were performed in the presence of diprotin A, a dipeptidyl peptidase IV (DPPIV) inhibitor, the radioactivity incorporated into DNA was 30–40% higher in cells containing 1 μM GLP-2 than in 1% FBS control cells. In another set of experiments, an hiFBS was used as a possible source of a DPPIV activity-deficient serum. Although hiFBS produced between twofold and fivefold lower [^{3}H]-thymidine incorporation into DNA than normal serum at the same concentration (data not shown), the stimulating effect of GLP-2 on the radioactivity incorporated into DNA was always highest with respect to that in the control cells (Fig. 1a) and which was also dose dependent on the concentration of GLP-2 added (Fig. 1b). In addition, GLP-2 induction of [^{3}H]-thymidine incorporation was associated with a scheduled DNA synthesis and

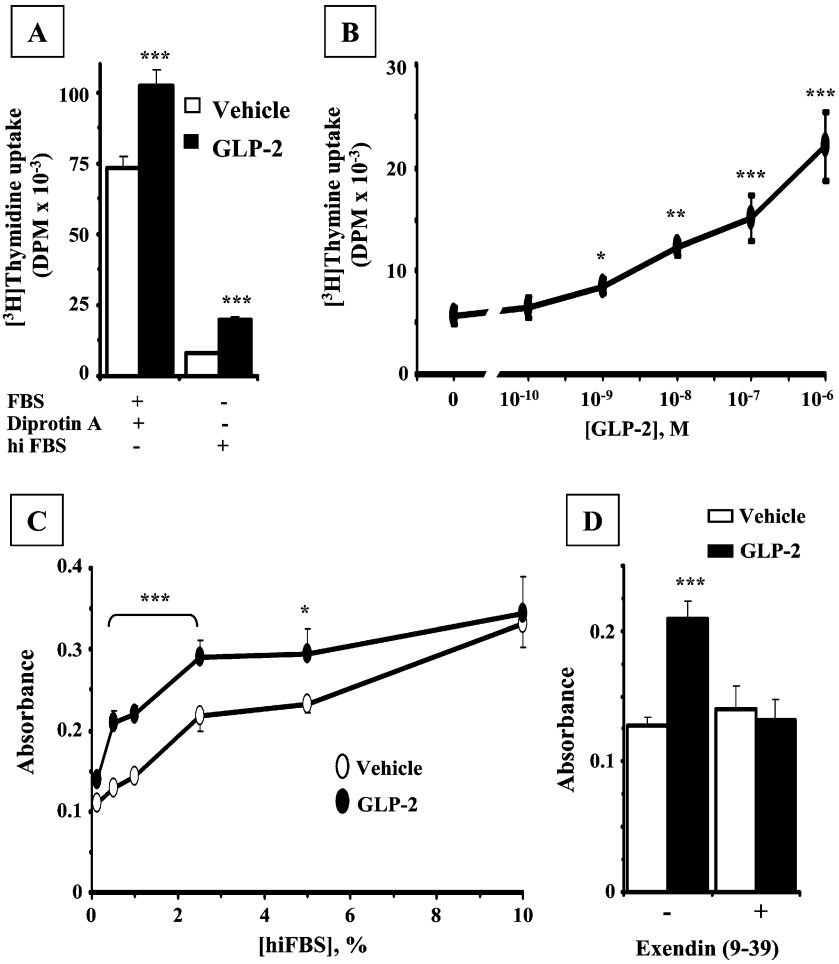

Fig. 1 Effect of GLP-2 on [^{3}H]thymidine incorporation into DNA (**a**, **b**) and on cell density (**c**, **d**) of rat astrocyte cultures. Astrocytes were incubated for 20 h with media containing 1% FBS plus 50 μM diprotin A or 1% hiFBS in the absence or in the presence of 1 μM GLP-2 (**a**) or for 24 h with 0.5% hiFBS-containing media supplemented with the indicated concentrations of GLP-2 (**b**). The amount of tritium incorporated into an acid precipitate form was measured by scintillation counting. Results are expressed as disintegrations per minute per well. Data represent means ± SD of n=2–3 independent experiments carried out four times each in 12-well plates. In **c** and **d**, astrocytes were incubated for 24–26 h with the media containing different concentrations of hiFBS (**c**) or with 0.5% hiFBS supplemented or not with 200 nM exendin (9–39) (**d**), in the absence and in the presence of 10 nM GLP-2. The absorbance of the crystal-violet-stained cells was measured at 560 nm and determined in each well. Data represent means ± SD of n=3 independent experiments carried out six times each in 24-well plates. *P<0.05; **P<0.01; ***P<0.001, on comparing GLP-2-treated cells with their respective controls (Student's unpaired t test)

not only with DNA reparation, as judged by 10 nM GLP-2 producing a significant increase in cellular density even at concentration of hiFBS higher than 5% (Fig. 1c). Under these experimental conditions, the effect of GLP-2 on the number of the cells was completely abolished by the pre-incubation with exendin (9–39) (Fig. 1d).

To assess whether the effects of GLP-2 on proliferation were related to an increased expression of regulatory proteins involved in the process of cell growth and cell differentiation, rat astrocytes were incubated for different periods of time in the absence and in the presence of this peptide, after which the expression of c-Fos, pKi-67, PCNA and GFAP proteins was measured by Western blotting. Figure 2a shows that c-Fos expression was 2.5-fold to fourfold higher in GPL-2-treated cells than in the control ones. As observed in Fig. 2b, two isoforms with molecular weights higher than 300 kDa were immunostained with Ki-67 antibody. Although the expression of the smaller isoform was time-course-decreased in the control cells, GLP-2 produced a significant increase in pKi-67 expression at all times studied. Likewise, although PCNA and GFAP expression was time-course-increased in the control cells, GLP-2 produced an additional and significant increase at all the times studied (Fig. 2c, d).

In an attempt to check whether GLP-2 and key growth factors presented synergistic effects on the astrocyte proliferation, the [^{3}H]-thymidine incorporation into the DNA was determined after cells were incubated with

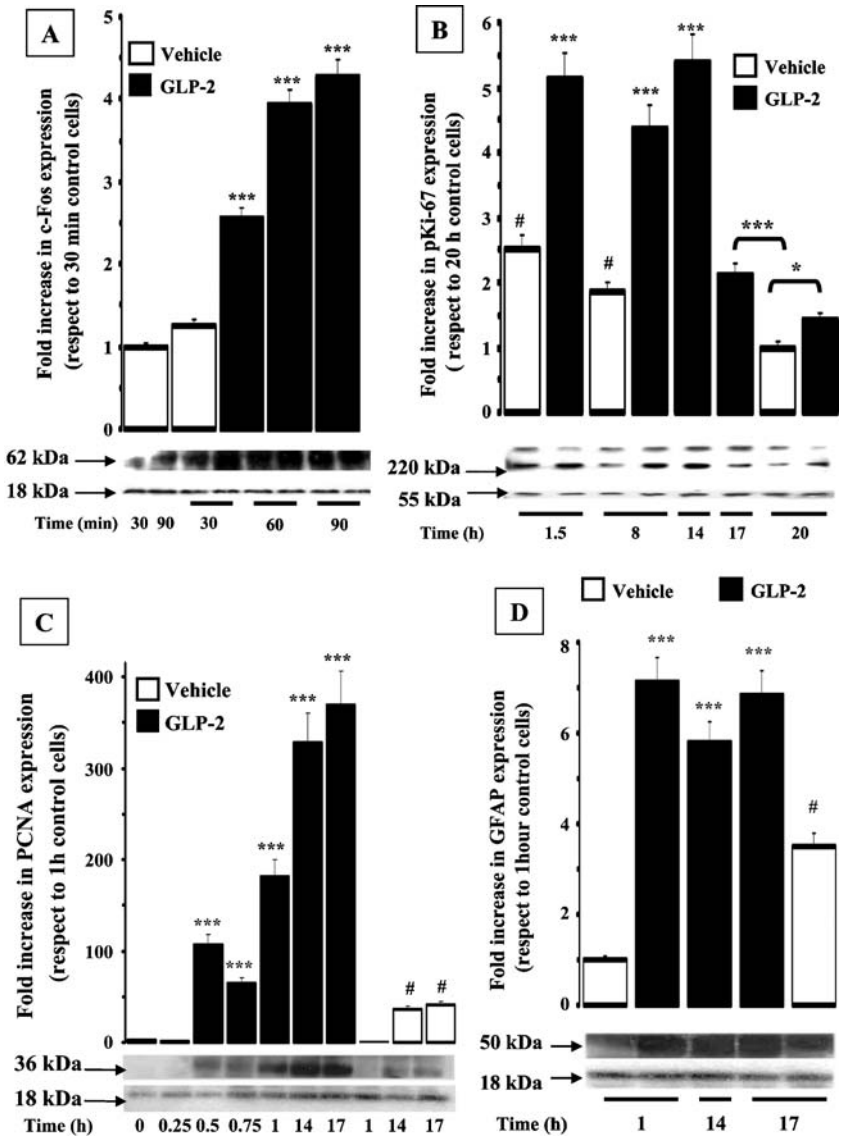

Fig. 2 Time course of the effects of GLP-2 on the expression of c-Fos (**a**), pKi-67 (**b**), PCNA (**c**) and GFAP (**d**) in cultured rat astrocytes. Astrocytes were incubated for the times indicated in the figures with media containing 0.5% hiFBS in the absence and in the presence of 50 nM GLP-2. Figures show densitometric analysis (of the smaller band, in **b**) from $n=3$ independent experiments carried out two times each and representative bands from Western blot analyses in which cyclophilin A (18-kDa band, in **a**, **c** and **d**) or α-tubulin (55-kDa band, in **b**) were used as a loading control. *$P<0.05$; ***$P<0.001$, on comparing GLP-2 treated and untreated cells. #$P<0.001$, on comparing with 20-h (**b**) or 1 h (**c**, **d**) control cells (Student's unpaired t test)

GLP-2 plus EGF, PDGF, IGF-I or insulin. Figure 3a shows that [^{3}H]-thymidine incorporation was significantly higher in EGF-stimulated cells than in control cells. Similarly, PDGF-AA, IGF-I and insulin also afforded significant increases with respect to control cells. As shown in the same figure, the presence of 1 μM GLP-2 produced an additional and significant increase in the uptake of [^{3}H]-thymidine in all the situations studied, the values being between 1.6- and 2.9-fold higher than those obtained in the absence of GLP-2. As expected, the [^{3}H]-thymidine incorporation was associated with a scheduled DNA synthesis. Figure 3b shows that the presence of EGF, PDGF, IGF-I or insulin elicited a significant increase in cell numbers, which were 1.5-fold to threefold higher than in control cells. The simultaneous addition of 100 nM GLP-2 produced a new and significant increase in cellular density with respect to those obtained with the growth factors or insulin alone.

Effect of GLP-2 on cAMP Production and ERK 1/2 Phosphorylation in the Presence of EGF, PDGF, IGF-I or Insulin

In an attempt to uncover which processes at a post-receptor level were involved in the synergistic effect of GLP-2 and growth factors or insulin on astrocyte proliferation, we firstly studied the effect of GLP-2 of cAMP production in the presence of growth factors or insulin. As shown in Fig. 4a, EGF, PDGF, IGF-I or insulin did not modify the levels of cAMP obtained by control cells, and, under these experimental conditions, the addition of GLP-2 did not induce significant changes of the basal levels of cAMP.

Because changes in cAMP signalling pathway could not be responsive of the described synergistic effects on cell proliferation, we studied the effect of GLP-2 on the

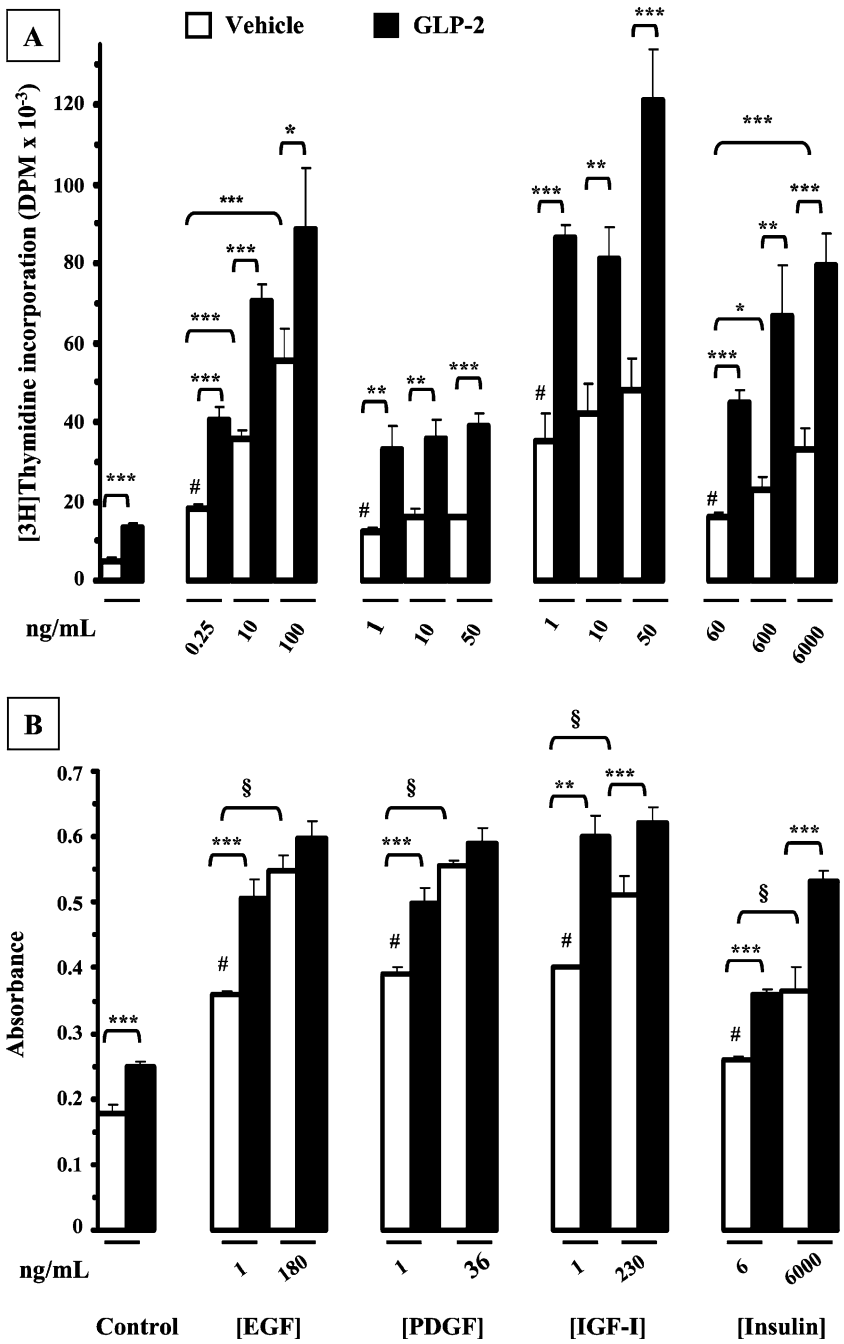

Fig. 3 Effect of GLP-2 on [^{3}H]thymidine incorporation into DNA (**a**) and on cell density (**b**) of rat astrocytes incubated with different concentrations of EGF, PDGF, IGF-I or insulin. Astrocytes were incubated for 20 h (**a**) or 26 h (**b**) with media containing 0.1% hiFBS and supplemented with the indicated concentrations of EGF, PDGF, IGF-I or insulin in the absence and in the presence of 1 μM (**a**) and 100 nM (**b**) GLP-2. [^{3}H]Thymidine incorporation and crystal violet staining were determined as described in the legend of Fig. 1. Data represent means ± SD of n=3 independent experiments carried out four times each in 12-well plates. *P<0.05; **P<0.01; ***P<0.001, on comparing GLP-2 treated cells with their respective controls (**a** and **b**) and on comparing cells incubated with media containing different concentrations of EGF, PDGF, IGF-I or insulin (**a**). Also, #P<0.001 on comparing cells incubated with the lowest concentration of EGF, PDGF, IGF-I or insulin and those incubated with hiFBS alone (**a** and **B**) and $^{§}P$<0.001 on comparing between them the two concentrations of EGF, PDGF, IGF-I or insulin used (**b**; Student's unpaired t test)

mitogen-activated protein signalling pathway by determining the ERK 1/2 phosphorylation ratio in the presence of growth factors or insulin. Figure 4b shows that 100 nM GLP-2 induced a significant time course activation of this signal transduction pathway, which was maximal at 5 min of incubation. At this same time period, insulin, IGF-I and EGF also produced significant increases of the ERK 1/2 phosphorylation, and the addition of 10 nM GLP-2 induced a new increase of the phosphorylated form of ERK 1/2 (Fig. 4c). Likewise, as it can be observed in Fig. 4d, the stimulating effect of GLP-2 on ERK 1/2 phosphorylation was completely reverted in cells pre-incubated with exendin (9–39).

IGF-I Upregulates GLP-2 Receptor mRNA Expression Whereas GLP-2 Increases IGF-I Receptor mRNA Expression in Rat Astrocyte Cultures

In an attempt to uncover which other processes could be involved in the synergistic effect of GLP-2 and key growth factors on astrocyte proliferation, we studied whether GLP-2R or IGF-IR expression was regulated by the presence of

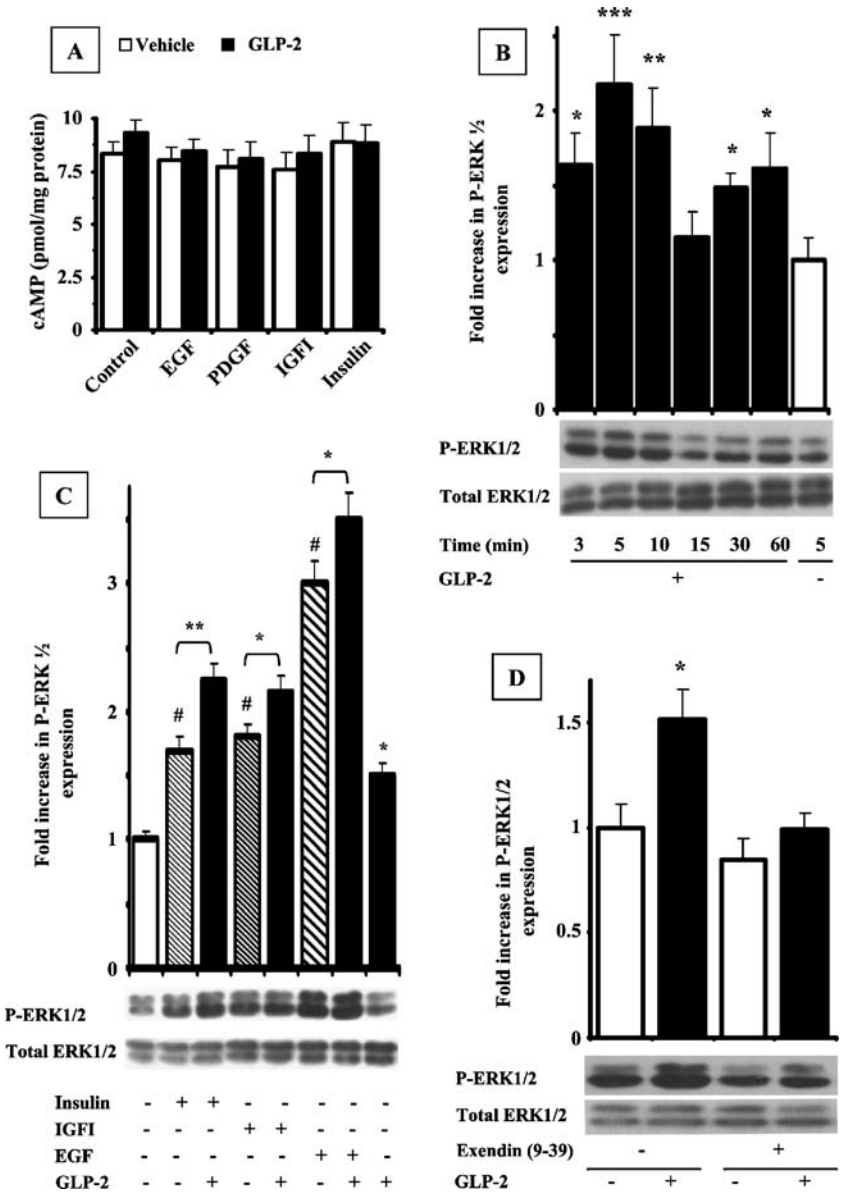

Fig. 4 Effect of GLP-2 on intracellular cAMP production and on the phosphorylation of the ERK 1/2 in cultured rat astrocytes. **a** Astrocytes were incubated for 30 min with 0.1% hiFBS alone or supplemented with 10 nM EGF, 1 nM PDGF, 10 nM IGF-I or 100 nM insulin in the absence and in the presence of 100 nM GLP-2. IBMX (100 μM) was present since 1 h before the different treatments and 20 μM forskolin (221.8±20.2 pmol cAMP per milligramme protein) was used as a positive control. Intracellular cAMP was measured by an enzyme immunoassay system. Data represent means ± SD of $n=2$ independent experiments carried out three times each in 24-well plates. **b**, **c**, **d** Astrocyte cultures were incubated for the times indicated with 0.1% hiFBS in the absence or presence of 100 nM GLP-2 (**b**); for 5 min with media supplemented with 100 nM insulin, 5 nM IGF-I or 5 nM EGF in the absence or presence of 10 nM GLP-2 (**c**); and for 5 min with media supplemented with 100 nM exendin (9–39) in the absence or presence of 10 nM GLP-2 (D) and ERK 1/2 phosphorylation was determined by Western blots analyses. Figures show densitometric analysis from $n=3$ independent experiments carried out two times each, and representative bands from Western blot analyses in which total ERK 1/2 (42–44 kDa bands) was used as a loading control. $*P<0.05$; $**P<0.01$; $***P<0.001$, on comparing GLP-2 treated with their respective controls. Also, $\#P<0.001$, on comparing insulin, IGF-I or EGF treated cells with control cells (Student's unpaired t test)

growth factors (such as IGF-I) or GLP-2. To do so, we first designed experiments in which the GLP-2R mRNA expression was upregulated. Figure 5a shows that hiFBS produced a significant time course increase in GLP-2R mRNA expression, which was maximal at 6 h of incubation, and that the addition of 10 nM GLP-2 induced a slight but significant decrease in the level of mRNA expression of its own receptor. As shown in Fig. 5b, hiFBS also produced a dose-dependent increase in GLP-2R mRNA expression, the maximum effect being observed with 1% hiFBS. In another

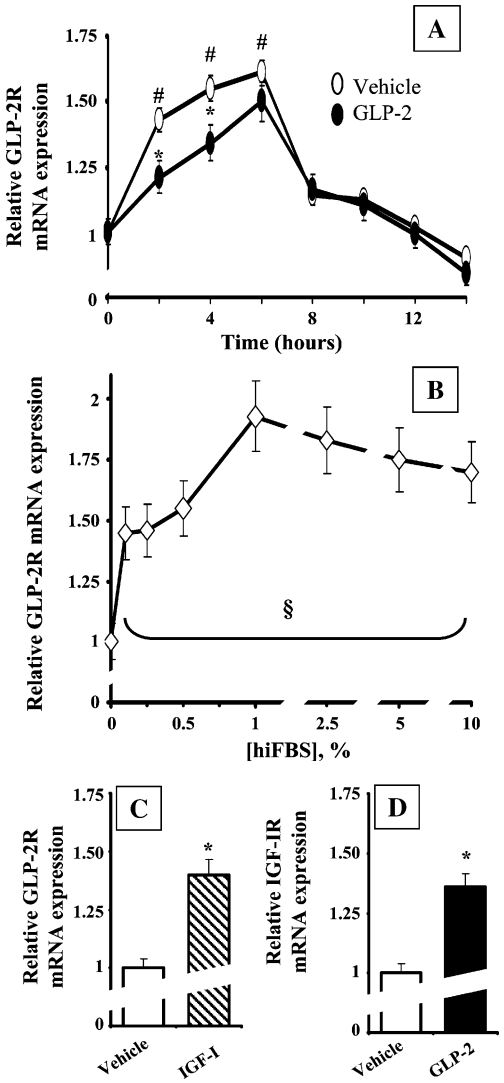

Fig. 5 Regulation of the GLP-2 receptor mRNA (**a**, **b** and **c**) and IGF-I receptor mRNA (**d**) expression. Astrocyte cultures were incubated for the times indicated with media containing 0.5% hiFBS in the absence or presence of 10 nM GLP-2 (**a**); for 4 h with the indicated concentrations of hiFBS (**b**) and for 4 h with 0.1 nM IGF-I (**c**) or 10 nM GLP-2 (**d**) in 0.5% hiFBS-containing media. Analyses of mRNA for GLP-2R (**a**, **b** and **c**) and mRNA for IGF-I (**d**) were performed using real-time quantitative RT-PCR as indicated in "Materials and Methods". Results were obtained from $n=3$ independent experiments carried out two times each. *$P<0.05$; on comparing GLP-2-treated (**a** and **d**) or IGF-I-treated (**c**) and untreated cells. Also, #$P<0.001$, on comparing with zero-time control cells (**a**), and §$P<0.001$, on comparing with cells in the absence of hiFBS (**b**; Student's unpaired t test)

set of experiments, we studied the effect of IGF-I as a possible growth factor involved in the regulation of GLP-2R mRNA expression. Figure 5c shows that 0.1 nM IGF-I led to a slight but significant increase in mRNA GLP-2R expression. Similarly, we studied whether GLP-2 might elicit a similar effect on IGF-IR mRNA expression and, as shown in Fig. 5d, 10 nM GLP-2 also produced a discrete but significant increase in IGF-IR mRNA expression.

Discussion

We have previously reported that GLP-2R is present in cultured rat astrocytes, through which GLP-2 induces a transduction of signals that stimulate cell proliferation [3]. Taking into account that astrocytes are the targets of several growth factors that stimulate their proliferation, the main aim of this study was to ascertain whether this proliferative activity of GLP-2 could be enhanced by other growth factors. These findings should emphasise the importance of astrocytes as the main glial cell population of the CNS of relevance in nutritional support and the development of neurones. Since astrocytes were obtained from cerebral hemispheres of 1-day-old pups, the age when these cells are immature in the rat, this would condition the responsiveness of this type of cells to any of the assayed growth factors. However, we used astrocytes 30–40 days after their isolation and at this time 95% of the cells were positive for GFAP, the main cytoskeletal protein of differentiated astrocytes.

The experiments presented here were carried out in the presence of hiFBS as a source of DPPIV-deficient serum instead of using a serum supplemented with a DPPIV inhibitor, such as diprotin A [18], because the increase of the astrocyte proliferation (measured as [^{3}H]thymidine incorporation into DNA and crystal violet staining) induced by GLP-2 was significantly higher in hiFBS-incubated cells than in cells incubated with FBS plus diprotin A at all the concentrations of serum studied (data not shown) but also because the concentration of diprotin A used (50 μM) does not inhibit efficiently enough all dipeptidyl peptidases present in the serum [19], and our cells seemed to be sensitive to higher doses of diprotin A. On the other hand, the effect of GLP-2 on [^{3}H]thymidine uptake and cell density observed in the presence of hiFBS was twofold to sixfold higher than in those previously reported by us [3], in which experiments were carried out in the presence of BSA. This apparent synergistic effect between GLP-2/hiFBS as compared with GLP-2/BSA suggests that GLP-2 enhances the proliferative effects of some thermostable substance present in hiFBS.

Under our experimental conditions, GLP-2 was also able to induce protein expression of proliferation and differentiation markers, such as c-Fos, pKi-67, PCNA and GFAP. Although we have previously reported that the levels of c-Fos mRNA are increased by GLP-2 in astrocytes [3], the upregulation of c-Fos protein has not yet been described. Here, we show that GLP-2 produces a fourfold increase in c-Fos expression, which is stronger than that reported in jejunum tissue extracts from newborn piglets [14] but similar to those measured in the neurones of the rat solitary tract nucleus [7] or in the neurones expressing vasoactive intestinal peptide of the rat submucosal plexus [20]. The

expression of pKi-67 is strictly associated with cell proliferation [21]. Our results indicate that astrocytes display at least two isoforms of the Ki-67 protein that are expressed throughout the active phases of the cell cycle, including the early G1 phase. GLP-2 only elicited a significant increase in the smaller isoform, indicating that pKi-67 can be considered a marker of proliferation but also suggesting a different role for each isoform during the cell cycle. These results are in agreement with those reported for the small intestine of mice [22], piglet [23] and rats [24]. PCNA is an auxiliary protein of DNA polymerase-δ that plays a role in both DNA replication and repair [25]. Our results demonstrate that GLP-2 induces a strong increase in PCNA in cultured astrocytes, which is consistent with the findings on crypt cell proliferation rate in mice [26, 27] but unlike that reported for the jejunal mucosa of total parenteral-nutrition-maintained rats [28]. Expression of the astrocyte-specific marker GFAP is regulated by several hormones and growth factors, but the protein is also expressed in response to inflammation [29]. Although GFAP phosphorylation was fourfold increased by receptor agonists that increase cAMP levels within 20–30 min after drug administration [30], significant changes in the total cell content of GFAP were only observed over a 2-h treatment period with 1 mM dibutyryl cAMP [31]. However, our results demonstrate that the GFAP cell content was increased sevenfold after 1-h incubation with GLP-2, which seems to indicate that GLP-2 upregulates GFAP expression by means of a cAMP-independent mechanism, because in our experimental conditions GLP-2 did not modify the intracellular cAMP content.

Our results indicate that EGF, PDGF, IGF-I and insulin administered alone also induce significant and dose-dependent increases in both [^{3}H]thymidine incorporation into DNA and cellular density of cultured astrocytes. In addition, when these substances were administered in combination with GLP-2, the values obtained were greater than the sum of the two individual ones, which denotes synergistic rather than additive effects between GLP-2 and EGF, PDGF, IGF-I or insulin. Experiments conducted to determine the interactions between growth factors and GLP-2 have been carried out in vivo. No synergistic changes were observed when the effects of EGF, IGF-I, IGF-II and GH, either alone or in combination with GLP-2, on the control of intestinal growth were studied in mice [32]. Nevertheless, a synergistic effect between GLP-2 and EGF on cellular proliferation in the small intestine was observed in parenterally fed rats [33].

Our results indicate that the synergistic effect of GLP-2 and EGF, PDGF, IGF-I or insulin on astrocyte proliferation is independent of the cAMP production but dependent of signalling through mitogen-activated protein kinase, as demonstrated by the increase in phospho-ERK 1/2 expression both in the absence and in the presence of growth factors or insulin. These results are in concordance with those in foetal rat intestinal cell (FRIC) cultures in which cAMP levels were not affected by the addition of IGF-I in the presence or absence of GLP-2 [22] and those in Caco-2 cells in which GLP-2 increases cell proliferation in association with decreased cAMP production but increased ERK 1/2 phosphorylation [11]. Whether GLP-2-induced ERK 1/2 phosphorylation in astrocytes is independent of protein kinase C activation as what occurs in GLP-2R hela-transfected cells [34] has to be determined.

In Caco-2 cells, neither the putative GLP-2R protein expression nor GLP-2R mRNA was detected [11], which raised the possibility of a diversity of receptors mediating the biological actions of GLP-2. However, endogenous GLP-2R mRNA has been detected in astrocytes by RT-PCR [3]. In addition, we propose that the biological effects of GLP-2 on astrocyte proliferation are mediated through its own receptor because when cells were pre-incubated with the GLP-1R antagonist exendin (9–39), the effect of GLP-2 on cell density and on phospho-ERK 1/2 expression was inhibited, which indicates that, at least in our experimental conditions, exendin (9–39) is also a GLP-2R functional antagonist. The biological effects of GLP-2 in the presence of exendin (9–39) have been little studied, although with opposite results. Thus, whereas exendin (9–39) did not inhibit the stimulating effect of GLP-2 on cAMP production both in baby hamster kidney cells expressing the cloned GLP-2R [8] and in dispersed neonatal rat brainstem [35], it completely inhibited the anorexic effects of GLP-2 in rats [36]. We think that this apparent paradox may be due to possible different GLP-2R isoforms or to distinctive cell types because there are several lines of experimental evidence supporting that GLP-1 receptors are insensitive to GLP-2 [36].

Our current findings provide evidence demonstrating that hiFBS upregulates GLP-2R mRNA expression and that GLP-2 partially antagonises this effect, which seems to be in accordance with those reported for newborn pigs, in which the introduction of enteral feeding transiently increases plasma GLP-2 concentrations and decreases small intestinal GLP-2R mRNA levels during development [37]. Although the effects of GLP-2 on GLP-2R desensitisation, endocytosis and G-protein-dependent effector activation have already been reported in GLP-2R-transfected cells [34, 38], experiments conducted to study the regulation of GLP-2R expression have not been made yet.

In an attempt for gaining a better knowledge of the role of GLP-2/GLP-2R in astrocytes, the effect of GLP-2 on IGF-IR mRNA expression and the effect of IGF-I on GLP-2R mRNA expression were studied. Our results demonstrate that GLP-2 and IGF-I induce a reciprocal upregulation of the expression of mRNAs for the corresponding receptors, which

may in part explain the synergistic effects of GLP-2 and growth factors on cell proliferation. Whether GLP-2 and the other growth factors or insulin might elicit a reciprocal upregulation in the expression of their receptors or even whether the effects of GLP-2 on astrocyte proliferation might occur through the transactivation of a growth factor receptor, as has been reported for GLP-1 and EGF receptors for pancreatic β-cell proliferation [39], should be investigated. However, the results indicating that mice treated with gefitinib (an EGFR tyrosine kinase inhibitor) displayed a growth inhibition of the small intestine that was completely prevented by simultaneous treatment with GLP-2 [40] seem to be in disagreement with the notion of a direct interaction between GLP-2 and the EGF receptor.

Recent studies indicate that the activation of GLP-2R induces the release of several growth factors that may be responsible for some of the effects of GLP-2 on the intestine [20, 41–44]. Since astrocytes express, secrete and respond to a broad range of different growth factors and cytokines, our results could also be related to an indirect mechanism that would mediate the biological actions of GLP-2, either by stimulating the release of some growth factors with paracrine and/or autocrine effects or by modulating the activity of its own receptors. Locally produced IGF-I seems to play an important paracrine role in regulating cell growth in several tissues. Thus, it has been reported that GLP-2 controls the mRNA expression and both cellular and medium content of IGF-I in FRIC cultures. Likewise, a dependent relationship between GLP-2 and IGF-I has been confirmed in intestine using igf1 knockout mice [22]. In addition, long-term treatment of mice with GLP-2 produced an approximately twofold increase in the expression of ileal IGF-I mRNA transcripts. However, there is no relationship between GLP-2 and liver IGF-I because GLP-2 administration does not result in increased levels of circulating IGF-I, and additionally IGF-I is not required for the intestinotrophic actions of GLP-2 in piglet [23]. These results are in accordance with those obtained in mice [32], showing that the administration of GH fails to increase intestinal growth either alone or in combination with GLP-2.

The present results suggest for the first time that the proliferation of rat astrocytes is synergistically stimulated by GLP-2 and several growth factors, which may be modulated at least through the expression of some receptors, i.e. IGF-1R and GLP-2R. These findings could afford additional insight into the pathophysiological role of GLP-2 in the CNS and emphasise the importance of astrocytes in such processes.

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