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Glucagon-Like Peptide-2 (GLP-2) Modulates the cGMP Signalling Pathway by Regulating the Expression of the Soluble Guanylyl Cyclase Receptor Subunits in Cultured Rat Astrocytes

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Abstract The aim of this work was to study the effect of glucagon-like peptide-2 (GLP-2) on the cyclic guanosine monophosphate (cGMP) signalling pathway and whether insulin or epidermal growth factor (EGF) might modulate the effects of GLP-2. GLP-2 produced a dose-dependent decrease in intracellular sodium nitroprusside-induced cGMP production. However, insulin induced an increase in the levels of cGMP that was dose-dependently decreased by the addition of GLP-2. By contrast, EGF induced a decrease in cGMP production, which was further reduced by the addition of GLP-2. To assess whether variations in cGMP production might be related with changes in some component of soluble guanylyl cyclase (sGC), the expression of the α1, α2, and β1 subunits were determined by Western blot analysis. At 1 h, GLP-2 produced a decrease in the expression of both α1 and β1 in the cytosolic fraction, but at 24 h only β1was reduced. As expected, insulin induced an increase in the expression of both subunits after 1 h of incubation; this was decreased by the addition of GLP-2. Likewise, incubation with EGF for 24 h produced a decrease in the expression of both subunits that was maximal when GLP-2 was added. In addition, incubation with insulin for 1 h produced an increase in the expression of the α2 subunit, which was reduced by the addition of GLP-2. These results suggest that GLP-2 inhibits cGMP production by decreasing the cellular content of at least one subunit of the heterodimeric active form of the sGC, independently of the presence of insulin or EFG. This may open new insights into the actions of this neuropeptide.

Keywords Astrocytes · GLP-2 · Insulin · EGF · cGMP · Guanylyl cyclase

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Introduction

Glucagon-like peptide-2 (GLP-2) is a 33-amino acid peptide derived from the differential post-translational processing of proglucagon in intestinal endocrine L-cells and in certain neurons from the nucleus of the solitary tract in the brain stem [1]. GLP-2 exerts multiple actions on the gut, through a G protein-coupled receptor (GLP-2R), which is linked to the activation of the adenylate cyclase pathway and localized in several intestinal cell types [2], although also in some osteoblastic cell lines [3], and in the rat heart [4]. Different in vitro models of heterologous cells expressing the transfected GLP-2R [5] as well as several cell types that express the endogenous GLP-2R [6] have been used to study the downstream signalling pathways of GLP-2R, and the results concerning the effect of GLP-2 on the cAMP-PKA-CREB pathway are not entirely consistent [7, 8]. In addition, the beneficial effects of GLP-2 on the gut seem to be linked to a complex network of indirect mediators, such as insulin-like growth factor (IGF)-I, IGF-II, ErbB, KGF, VIP, 5-HT, and nitric oxide (NO) [9]. GLP-2R has also been identified and GLP-2 induces biological effects in several regions of the central nervous system, including the hypothalamus, the hippocampus, and cortex [10], as well as in

cultured rat astrocytes, in which GLP-2 promotes proliferation by cAMP-dependent and independent mechanisms [11, 12].

In most tissues, NO acts as an intracellular signaling molecule and it is formed by NO synthase (NOS) from L-arginine in all brain cells, including astrocytes [13]. The major physiological receptor for NO is soluble guanylyl cyclase (sGC), an $\alpha\beta$ heterodimer that catalyses the conversion of GTP to cyclic guanosine monophosphate (cGMP), which acts as a second messenger modulating the activity of cGMP-dependent protein kinases, cyclic nucleotide phosphodiesterases, and cyclic nucleotide-gated channels [14]. cGMP is also generated by the membrane receptor guanylate cyclase, which is activated by natriuretic peptides and regulates the expression of multiple specific genes [15]. At present, four subunits of sGC have been cloned and sequenced, namely α1, α2, β1, and β2, but only $\alpha1\beta1$ and $\alpha2\beta1$ have been isolated as functional heterodimers [16]. The expression of different α and β subunits varies with the tissue examined and the distribution of α2 subunit being reported to have its major occurrence in the brain [17]. sGC activity can be controlled by different mechanisms after short- and long-term exposure to NO-generating agents [18]. In addition, a long-lasting downregulation of sGC has been reported; this would occur through NO-dependent and independent mechanisms in cultured astroglial cells [18]. Additionally, a cyclic AMP-dependent regulation of mRNA expression and stability [19] for the sGC α1 and β1 subunits has been demonstrated. Likewise, post-translational modifications, such as phosphorylation/dephosphorylation [20, 21], protein–protein interactions [22], alternative splicing [23], and proteasomal degradation [24], have been found to be responsive of variations in sGC activity.

Although the opposite effects of cAMP and cGMP are well known, including those concerning cell proliferation, the effects of GLP-2 on the cGMP signalling pathway have only been studied at the level of NOS regulation: activity, the abundance of mRNA and protein, as well as the phosphorylation of the eNOS protein [4, 25–27]. Nevertheless, no information about the effect of GLP-2 on the regulation of guanylyl cyclase activity has been reported.

The aim of the present work was to study the effect of GLP-2 on cGMP production and on the protein expression of the components of soluble guanylyl cyclase (α1, α2, and β1) in an attempt to determine whether other signalling pathways apart from the cAMP and MAP kinase routes, which induce cell proliferation in astrocytes, might be involved in the effects of GLP-2 alone or combined with insulin, IGF-I or epidermal growth factor (EGF). Here, we report for the first time that astrocytes respond to GLP-2 by inhibiting cGMP formation even in the presence of insulin and EGF, possibly due to a decrease in the abundance of at least one subunit of the heterodimeric active form of the soluble guanylyl cyclase present in these cells. This might open the door to new insights into the physiological role of this neuropeptide.

Experimental Procedures

Materials

Rat GLP-2 was from Peninsula Laboratories (St. Helens, UK). Recombinant human insulin Arg^{+} Zn was from Calbiochem (La Jolla, CA, USA). Recombinant human IGF-I and EGF were purchased from PreproTech EC Ltd (London, UK). Bovine serum albumin (BSA, 0.001 % fatty acid), 3-isobutyl-1-methylxanthine (IBMX) and sodium nitroprusside (SNP) were from Sigma-Aldrich (St. Louis, MO, USA). Exendin (9-39) was from Bachem (Bubendorf, Switzerland). The cGMP enzyme immunoassay (EIA) systems were from Amersham Pharmacia Biotech (Little Chalfont, Bucks, England) and Sigma-Aldrich. All other chemicals were of reagent or molecular biology grade.

Rat astrocyte Cultures and Treatments

Primary rat astroglial cell cultures were prepared from cerebral hemispheres of 1-day-old Wistar rats as previously described [28]. 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. Mechanically dissociated cells were plated onto 75 cm^2 tissue culture flasks at low density. Cells were grown in DMEM/F-12 medium, containing 15 mM HEPES and supplemented with 10 % (*v*/*v*) fetal bovine serum. After 21–28 days, astroglial cells were dispersed by treatment with trypsin/EDTA for 1–2 min at 37 °C. They were then replated at approximately $2.3–3\times10^4$ cells/cm^2 in 12-well (for cGMP measurements), or 100×20 mm (western blot analysis) tissue culture dishes and grown as described above. After 7–10 days, the cultures were 80–90 % confluent. Immunocytochemical analysis of these cultures revealed that at least 95 % of the cells were positive for the astrocyte-specific marker glial fibrilary acidic protein. Different treatments were carried out 24 h after cell cultures had been shifted to serum-free medium. GLP-2 stimulation experiments were carried out in serum-free medium containing 2 mg/ml bovine serum albumin. When insulin, IGF-I, or EGF is used, they were added simultaneously to GLP-2. Exendin (9–39) was present 1 h before and during the incubation with GLP-2. At the indicated times, the reactions were stopped by removing the supernatants and adding ice-cold phosphate-buffered saline (PBS) to cells. Finally, cells were washed twice with PBS and harvested for the different analyses, as described below.

Intracellular cGMP Measurements

Intracellular cGMP was measured using the commercial protocol for the acetylation EIA system described for cell culture samples, with some modifications [29]. Different treatments

were carried out to study the effects on guanylyl cyclase activity induced by 100 μM SNP during the last 3 min of incubation. All experiments were carried out in the presence of 1 mM IBMX. Reactions were stopped by removing the supernatants. cGMP was extracted with cold ethanol, which was then evaporated off in a speed vac system and dissolved in lysis reagent (Amersham) or 0.1 M HCl (Sigma) and harvested at −80 °C until use. Cells were dissolved in 0.5 N NaOH, and protein concentration was measured by the Bradford method on 96-well plates (Varioskan termo electron corporation, Software ScanIt 2.0.91). Results are expressed as picomole per milligram protein.

Western Blotting Analysis

After the treatments, cells were lysed on ice for 30 min in ice-cold lysis buffer containing phosphate-buffered saline, pH 7.5, 0.1 % sodium dodecyl sulfate (SDS), 0.5 % sodium deoxycholate, 1 % NP-40, 10 μM DTT, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 10 μg/ml leupeptin. Then, an aliquot of the cell lysate was homogenized (total fraction) and stored at −80 °C until use, and another aliquot was centrifuged at 100,000*g* for 1 h at 4 °C, and the supernatant (cytosolic fraction) was also stored at −80 °C until use [30]. The protein concentration was determined with the Bradford reagent (Bio-Rad, Uppsala, Sweden) using bovine serum albumin as standard. Proteins (25–50 μg) were loaded on 4 % stacking/10 % separating SDS-PAGE gel (Bio Rad). The resolved proteins were electrotransferred onto a 0.45 μm polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA, USA) for Western blot analyses. Equal protein loading was confirmed by Ponceau staining. 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 (TBST) at room temperature for 1 h, and incubated with rabbit polyclonal antiguanylyl cyclase α1 (Sigma-Aldrich, 1:5,000), rabbit polyclonal antiguanylyl cyclase α2 (1:500), and rabbit polyclonal antiguanylyl cyclase β1 (Santa Cruz Biotechnology, 1:500) at 4 °C overnight. 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 analyzed by densitometry (Model GS-800, Bio-Rad) and quantified (Quantity One software, Bio-Rad). Blots were routinely reprobed with mouse monoclonal anti-β actin (Sigma-Aldrich, 1:10,000) to monitor the levels of an invariant control.

Statistical Analysis

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

Results

Effect of GLP-2 in the Absence or Presence of EGF, IGF-I, or Insulin on Sodium Nitroprusside-Induced cGMP Production

By previous studies done in our laboratory, we know that intracellular cGMP was not detected after cell incubation with GLP-2, insulin, IGF-I, and EGF alone or combined (data not shown), which indicated that endogenous NO was not produced under our experimental conditions. Thus, our interest was directed to studying the effect of these peptides on cGMP production induced by the NO donor, SNP. As shown in Fig. 1, experiments were performed by incubation of cultured astrocytes for 1 and 20 h with GLP-2 in the absence or presence of insulin, IGF-I, and EGF. After 1 h incubation, GLP-2 produced a dose-dependent decrease in the intracellular cGMP content, which was 38 % lower with 1 μM GLP-2. In contrast, when cells were incubated with 1 μM insulin the amount of cGMP was 47 % higher than those incubated with BSA alone, and after the addition of GLP-2 a significant dose-dependent reduction was observed, falling to 50 % in the presence of 1 μM GLP-2. To demonstrate that the activation of GLP-2R was responsible for the inhibitory effect of GLP-2 on cGMP production, experiments in the presence of exendin (9–39) were also performed and, as observed, this completely reversed the inhibitory effect of GLP-2 on insulin-induced cGMP production. When cells were incubated with 30 nM IGF-I or 30 nM EGF, no differences were observed with respect to the control cells, and the addition of 1 μM GLP-2 only produced a slight but significant decrease in the presence of EGF. After 20 h of incubation, GLP-2 also induced a dose-dependent reduction of intracellular cGMP production. Under these conditions, neither insulin nor IGF-I produced significant changes in cGMP formation, which was not affected by the presence of GLP-2. However, the cGMP content was 33 % lower in cells incubated with EGF with respect to the controls and was even 26 % reduced in cells incubated with EGF plus 1 μM GLP-2. As observed in the same figure, exendin (9–39) abolished the effect of GLP-2 on the intracellular cGMP reduction induced by EGF.

Effect of GLP-2 on Gucy-α1 and -β1 Protein Expression

In an attempt to investigate the possible regulation pathways involved in the effect of GLP-2 on cGMP production, we performed experiments to test whether GLP-2 could modulate the expression of soluble guanylyl cyclase α1 and β1 in

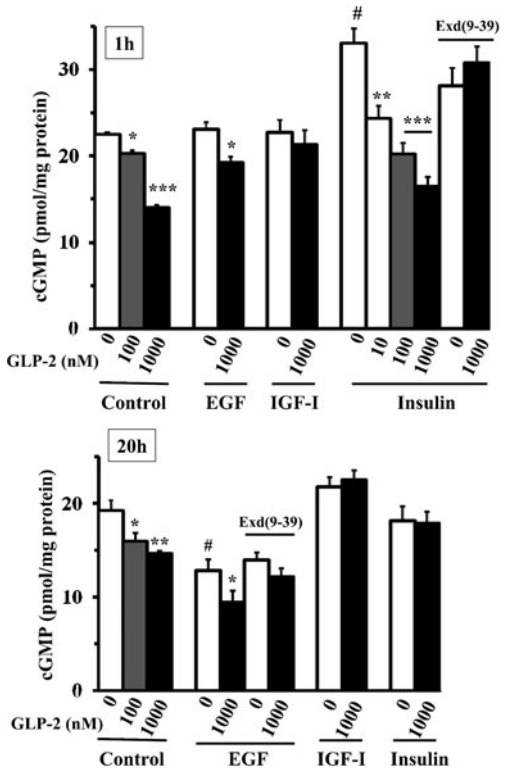

Fig. 1 Effect of GLP-2 on intracellular cGMP production in cultured rat astrocytes. Astrocytes were incubated for 1 and 20 h with the indicated concentration of GLP-2 in the absence and in the presence of 30 nM EGF, 30 nM IGF-I, and 1 μM insulin. As indicated, 1 μM exendin (9–39) was present 1 h before and during incubation with GLP-2. All experiments were carried out in the presence of 1 mM IBMX. Different treatments were carried out to study the effects on guanylyl cyclase activity induced by 100 μM SNP over the last 3 min of incubation. Intracellular cGMP was measured by an enzyme immunoassay system. Data represent means±SD of $n=3$ independent experiments carried out three times each in 12-well plates. $*p<0.1$, $**p<0.01$, $***p>0.001$ on comparing GLP-2 treated cells with their respective controls. Also, $^{\#}p<0.001$ on comparing insulin (1 h) or EGF (20 h)-treated cells with control cells (Student's unpaired t test)

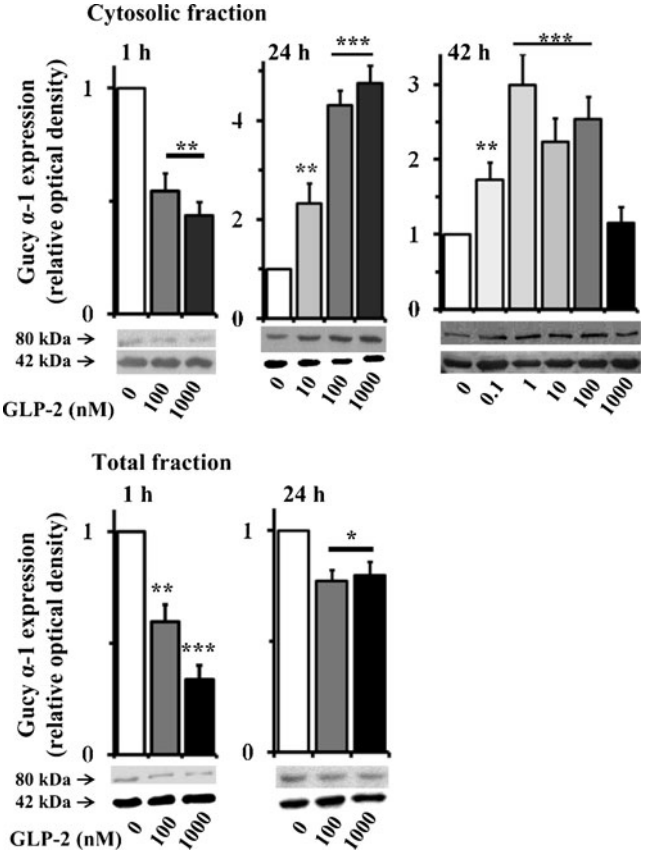

Fig. 2 Dose-dependent effect of GLP-2 on the expression of the soluble guanylyl cyclase $\alpha1$ subunit in cultured rat astrocytes. Astrocytes were incubated with the indicated concentrations of GLP-2 for 1, 24, or 42 h. Figures show densitometric analyses from $n=3$ independent experiments carried out two times and representative band (80 kDa) from Western blot analysis, performed on cytosolic (100,000g) and on total fractions, in which β-actin (42 kDa) was used as a loading control. $*p<0.1$, $**p<0.01$, $***p>0.001$ on comparing GLP-2-treated and untreated cells

both the total and cytosolic fractions. As shown in Fig. 2, after 1 h of incubation, GLP-2 induced a dose-dependent decrease in the expression of gucy-$\alpha1$ in both the cytosolic and total fractions. After 24 h of incubation, its expression in the cytosolic fraction was dose-dependent increased (until 4.75-fold), but a slight decreased (20 %) in total fraction was observed. At longer times, the expression was only studied in the cytosolic fraction, being gucy-$\alpha1$ expression 1.7-3.0-fold increased in the presence of 0.1–100 nM GLP-2 and no significant differences were observed at the highest concentrations (1 μM) of GLP-2 used.

When gucy-$\beta1$ expression was studied (Fig. 3), a slight but significant decrease in the cytosolic fraction without changes in the total fraction were found after 1 h of incubation with 1 μM GLP-2. After 24 h, gucy-$\beta1$ expression was significant decreased in both the cytosolic and the total fractions. However, at longer times, its expression was 22 and 33 % increased after incubation with 10 and 100 nM GLP-2, respectively.

Effect of Insulin and Insulin Plus GLP-2 on Gucy-$\alpha1$ and -$\beta1$ Protein Expression

To demonstrate whether the effects of insulin and insulin plus GLP-2 on cGMP production also corresponded to changes in gucy-$\alpha1$ and/or gucy-$\beta1$ expression, similar experiments to those described above were performed. Figure 4 shows that the expression of gucy-$\alpha1$ was significantly increased by 1 μM insulin in both the total (twofold) and cytosolic (1.5–3-fold) fractions and at all the times studied; however, no significant changes in the expression of gucy-$\beta1$ (Fig. 5, except for a 30 % decrease in the cytosolic fraction at 24 h) were observed. When GLP-2 was added, the expression of both gucy-$\alpha1$ and -$\beta1$ was significantly decreased in the total fraction at all times studied. However, the results obtained for

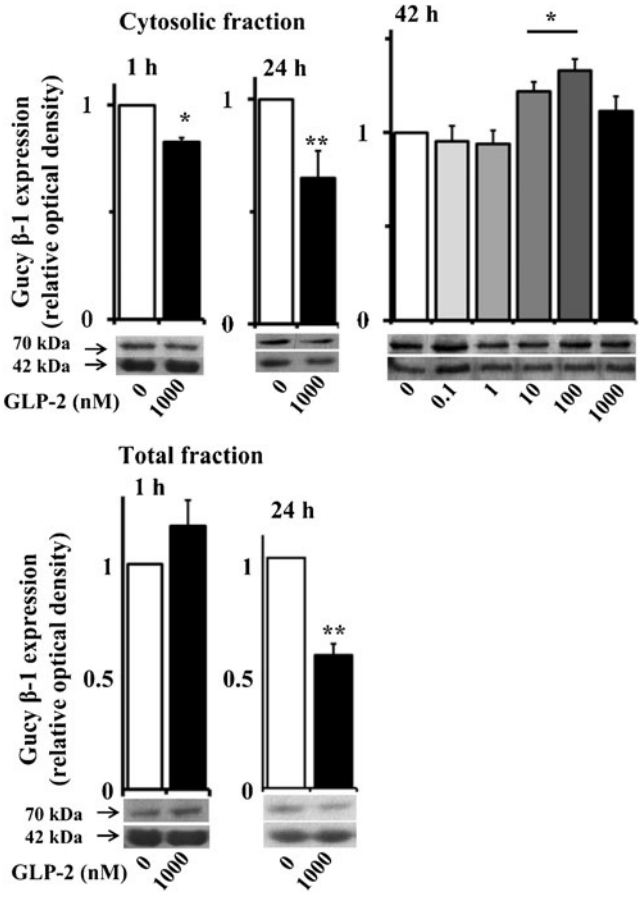

Fig. 3 Dose-dependent effect of GLP-2 on the expression of the soluble guanylyl cyclase β1 subunit in cultured rat astrocytes. Astrocytes were incubated with the indicated concentrations of GLP-2 for 1, 24, or 42 h. Figures show densitometric analyses from n=3 independent experiments carried out two times and representative band (70 kDa) from Western blot analysis, performed on cytosolic (100,000g) and on total fractions, in which β-actin (42 kDa) was used as a loading control. *p<0.1, **p<0.01 on comparing GLP-2-treated and untreated cells

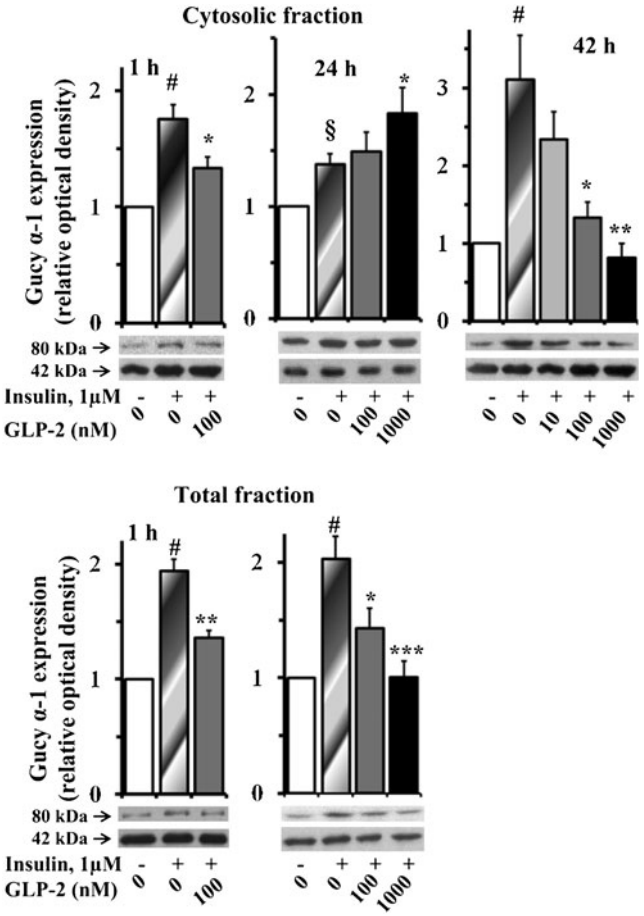

Fig. 4 Effect of insulin and insulin plus GLP-2 on the expression of the soluble guanylyl cyclase α1 subunit in cultured rat astrocytes. Astrocytes were incubated with 1 μM insulin in the absence and presence of the indicated concentrations of GLP-2 for 1, 24, or 42 h. Figures show densitometric analyses from n=3 independent experiments carried out two times and representative band (80 kDa) from Western blot analysis, performed on cytosolic (100,000g) and on total fractions, in which β-actin (42 kDa) was used as a loading control. $^{\S}p$<0.1, $^{\#}p$<0.01 on comparing insulin treated and untreated cells. *p<0.1, **p<0.01, ***p>0.001 on comparing insulin and insulin plus GLP-2-treated cells

the cytosolic fraction were different, depending on the experimental time and the sGC subunit studied. Thus, whereas the expression of gucy-α1 was 24 % lower in cells incubated with insulin plus 100 nM GLP-2 for 1 h as compared with those incubated with insulin alone, it was dose-dependent increased (until 33 %) and dose-dependently decreased (until 75 %) at 24 h and 42 h of incubation, respectively. Likewise, the expression of gucy-β1 was lower (19 %) after 1 h of incubation with GLP-2, but did not change either after 24 or 42 h in comparison with the insulin-treated control cells.

Effect of EGF and EGF plus GLP-2 on Gucy-α1 and -β1 Protein Expression

Because GLP-2 produced a significant reduction in the intracellular cGMP content in cells treated with EGF, we performed experiments to check whether EGF and EGF plus GLP-2 might modulate the expression of gucy-α1 or -β1 in the cytosolic fraction of cells incubated with EGF for 24 h. Figure 6 shows that the expression of gucy-α1 and -β1 was slightly but significantly lower (30 and 25 %, respectively) in the presence of EGF than in its absence and that GLP-2 produced an additional reduction of about 95 % for gucy-α1 and 75 % for gucy-β1.

Effect of GLP-2, Insulin, and Insulin plus GLP-2 on Gucy-α2 Protein Expression

Because $\alpha2\beta1$ may also be a heterodimeric active form of sGC and therefore responsive to cGMP production, we performed experiments to study the effect of GLP-2 on gucy-α2 expression in the both absence and presence of insulin. Our results suggested that GLP-2 induced a slight

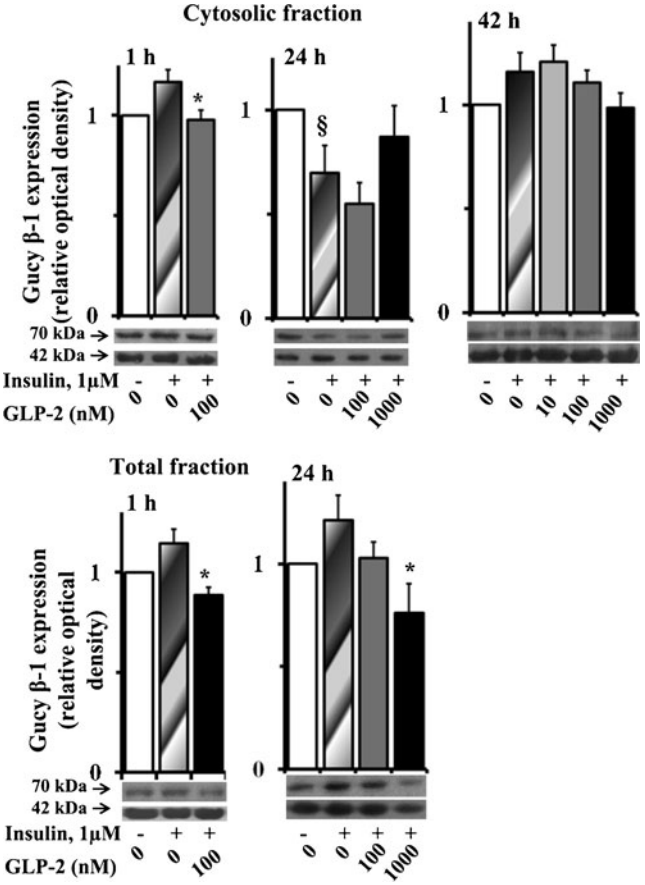

Fig. 5 Effect of insulin and insulin plus GLP-2 on the expression of the soluble guanylyl cyclase β1 subunit in cultured rat astrocytes. Astrocytes were incubated with 1 μM insulin in the absence and presence of the indicated concentrations of GLP-2 for 1, 24, or 42 h. Figures show densitometric analyses from n=3 independent experiments carried out two times and representative band (70 kDa) from Western blot analysis, performed on cytosolic (100,000g) and total fractions, in which β-actin (42 kDa) was used as a loading control. $^{\S}p<0.1$ on comparing insulin-treated and untreated cells. $^{*}p<0.1$, $^{**}p<0.01$ on comparing insulin and insulin plus GLP-2-treated cells

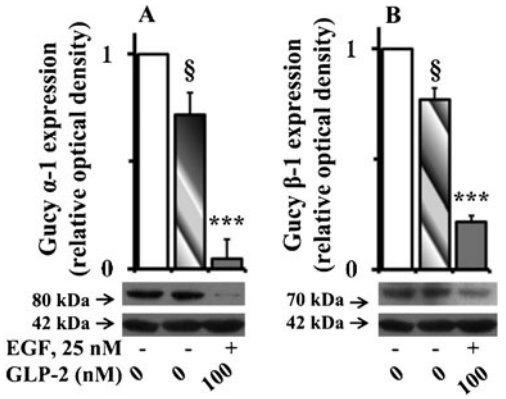

Fig. 6 Effect of EGF and EGF plus GLP-2 on the expression of the soluble guanylyl cyclase α1 (**A**) and β1 (**B**) subunits in cultured rat astrocytes. Astrocytes were incubated with 25 nM EGF in the absence and presence of 100 nM for 24 h. Figures show densitometric analyses from n=3 independent experiments carried out two times and representative bands (80 and 70 kDa, respectively) from Western blot analysis, performed on the cytosolic fraction (100,000g), in which β-actin (42 kDa) was used as a loading control. $^{\S}p<0.1$ on comparing EGF-treated and untreated cells. $^{***}p<0.001$ on comparing EGF- and EGF plus GLP-2-treated cells

but nonsignificant increase in gucy-α2 expression in the cytosolic fraction at both 1 and 24 h of incubation (Fig. 7). Likewise, the expression of gucy-α2 was strongly enhanced (275 %) in cells treated with insulin for 1 h and the addition of GLP-2 produced a dose-dependent decrease, which was 40 % lower in the presence of 1 μM GLP-2. However, in cells treated with insulin for 24 h, only a 30 % increase in gucy-α2 expression was obtained, while the addition of GLP-2 did not produce any significant effect.

Discussion

Many studies have attempted to study the downstream mechanism involved in GLP-2/GLP-2R interaction through both cAMP-dependent and -independent signal transduction pathways [8] and their involvement in different pathophysiological processes. However, studies on the effect of GLP-2 on cGMP production and on sGC regulation have not yet been reported. In this study, we report a direct and specific effect of GLP-2 on the cGMP signalling pathway in cultured astrocytes. These cells have GLP-2 receptors, and GLP-2 induces cell proliferation through cAMP-dependent [11] and -independent [12] mechanisms. Here, we tested the effects of GLP-2, insulin, and certain growth factors on NO-

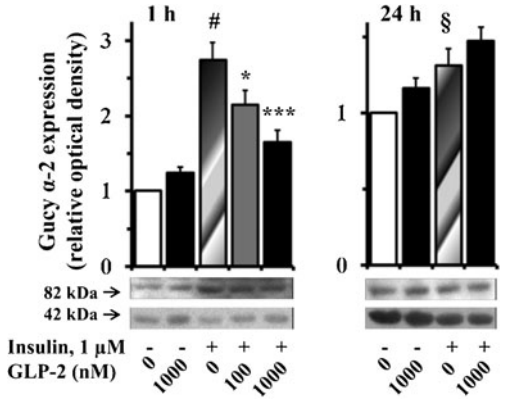

Fig. 7 Effect of GLP-2, insulin and insulin plus GLP-2 on the expression of the soluble guanylyl cyclase α2 subunit in cultured rat astrocytes. Astrocytes were incubated with 1 μM insulin in the absence and presence of the indicated concentrations of GLP-2. Figures show densitometric analyses from n=3 independent experiments carried out two times and representative band (82 kDa) from Western blot analysis, performed on the cytosolic fraction (100,000g), in which β-actine (42 kDa) was used as a loading control. $^{\#}p<0.001$ on comparing insulin treated and untreated cells. $^{*}p<0.1$, $^{***}p<0.001$ on comparing insulin and insulin plus GLP-2 treated cells

sensitive guanylyl cyclase activity as SNP-induced cGMP production. Under our experimental conditions, astrocytes displayed similar basal levels to those previously reported in cortical rat astrocytes [30]. We suggest that the dose-dependent reduction of intracellular cGMP induced by GLP-2 could be related to a decrease in the content of both the $\alpha 1$ and $\beta 1$ subunits (at 1 h) and at least of the $\beta 1$ subunit (24 h) in the cytosolic fraction, because the expression of $\alpha 2$ subunit was not affected at any time tested. Therefore, although both heterodimeric enzymes—$\alpha 1\beta 1$ and $\alpha 2\beta 1$—may be present in astrocytes, we believe that under our experimental conditions $\alpha 1\beta 1$ would be the major active form of sGC that is downregulated by GLP-2 in these cells. Although different spatial localizations of the sGC isoforms targeted to subcellular compartments may have specific functional roles [31], a 1:1 stoichiometry could be necessary for maintaining their biochemical and pharmacological properties. However, the localization of $\alpha 1$ and $\beta 1$ subunits and the distribution of maximal sGC activity do not seem to be correlated [32, 33]. In addition, the heterodimerization of sGC is a regulated process that changes during postnatal cerebral development [34] and the differing efficiencies in heterodimer formation may be an important reason for the lack of correlation between sGC protein expression and sGC activity in astrocytes after 24 h of incubation with GLP-2. An additional reason could be the formation of homodimeric complexes that exist in vivo and that have a different function from cGMP signalling [35]. Our results suggest that both the turnover and temporal subcellular localization of the subunits do not present a synchronic response to GLP-2, as indicated by the significant differences between the levels of expression of $\alpha 1$ and $\beta 1$ in the cytosolic and total fractions at the different times of incubation, possibly in accordance with different roles for sGC subunits in these cells [33].

Although it has been reported that IGF-I increases cGMP signaling pathway in cerebral granule cells [36], in cavernosal smooth muscle [37] and in aorta [38], neither IGF-I nor IGF-I plus GLP-2 affected the intracellular production of cGMP in astrocytes, and hence experiments addressing the effect of GLP-2 in the presence of IGF-I on the expression of the subunits of sGC were not performed. However, insulin enhanced cGMP production at 1 h, correlated with an increase in the expression of $\alpha 1$ and $\alpha 2$, but presumably also of $\beta 1$, in the cytosolic fraction, which could permit the formation of both $\alpha 1/\beta 1$ and $\alpha 2/\beta 1$ heterodimeric active forms of sGC in these cells. Moreover, the content of cGMP did not change in cells incubated with insulin for 24 h, which could be due to a lower presence of the $\beta 1$ subunit in the cytosolic fraction, but presumably also to an increase in the formation of homodimeric forms [35], such as $\alpha 1\alpha 1$ and $\alpha 2\alpha 2$, which do not participate in cGMP production. These results are in accordance with those reported in human and experimental animals cells and tissues [39–41], indicating that insulin enhances cGMP production via the induction of eNOS activity, as well as with those indicating that insulin increases the reduced levels of cGMP [42] and sGC expression [43] present during uncontrolled diabetes. In astrocytes, the co-incubation of GLP-2/insulin for 1 h induced a decrease in both cGMP production and the protein expression of the $\alpha 1$, $\beta 1$, and $\alpha 2$ isoforms, while co-incubation for 24 h did not change cGMP levels, presumably due to the unvarying amount of $\beta 1$ subunit in the cytosolic fraction. Considered together, our results strongly suggest that cGMP production and the expression of the isoforms of GC are closely correlated.

The effect of EGF on cGMP signaling has also attracted little attention. Thus, it has been reported that EGF stimulates the release of cGMP in rabbit ovarian cells [44] and in rat uterine horns [45]; likewise, EGF enhances guanylyl cyclase activity and DNA synthesis in the cecum, liver, and kidney of mice [46]. However, it has also been reported that ANP or SNP inhibit the EGF-stimulated cell proliferation in rabbit corneal epithelial cells [47]. We have previously reported [12] that EGF, GLP-2, and EGF plus GLP-2 induce cell proliferation in astrocytes; but our present results clearly indicate that this effect takes place through mechanisms that induce a down-regulation of the expression of both the $\alpha 1$ and $\beta 1$ subunits of the sGC and a decrease of cGMP production. Also, we think that GLP-2, insulin and EGF act in an independent manner on cGMP signaling, although further experiments should be made to confirm this issue.

To test whether GLP-2 directly activated GLP-2R, we used the GLP-1R antagonist exendin (9–39) for blocking the effects of GLP-2 in astrocytes, according with the results previously published by us [12] in which exendin (9–39) inhibited the effect of GLP-2 on astrocyte proliferation and phospho-ERK1/2 expression, or those of other authors [48] in which no differences were found by blocking the GLP-2 anorectic actions with the GLP-2R antagonist GLP-2 (3–33) or with the GLP-1R antagonist exendin (9–39). Here, we describe that under our experimental conditions the inhibitory effect of GLP-2 on both insulin-induced and EGF-reduced cGMP production is also reversed by exendin (9–39), which seem clearly to indicate that GLP-2 acts through its own receptor in these cells.

In this report, we present experimental evidence that GLP-2 reduces the intracellular cGMP content, inhibits the insulin-stimulated formation, and decreases the EGF-reduced cGMP production in cultured rat astrocytes. We propose that this effect would in part be due to a decrease in the expression of at least one of the three subunits ($\alpha 1$, $\beta 1$, and $\alpha 2$) that constitute the heterodimeric active form ($\alpha 1\beta 1$ and $\alpha 2\beta 1$) of soluble guanylyl cyclase. Whether GLP-2 has some effect on sGC activity or on other members of the cGMP signaling pathway should be further studied. In addition, there are many

ligands that enhance cGMP production and induce an inflammatory response, and hence study of the effect of GLP-2 on cGMP signaling pathway as well as study of the regulation of mRNA- and protein–GLP-2R expression under these experimental conditions should be of interest and should shed further light on our knowledge of the role of this neuropeptide in astrocytes.

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