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Exendin-4 Protects Neural Progenitor Cells from Glucolipoapoptosis

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Abstract

Type 2 diabetic and obese patients are under high risk to prematurely develop neurological complications such as stroke and Alzheimer's disease. Interestingly, type 2-diabetes impairs adult neurogenesis in rodent animal models and this impairment has been suggested to play a role in the brain complications of this disease. Recent work from us and others showed that the treatment with the Glucagon-Like Peptide 1 Receptor (GLP-1R) agonist Exendin-4 stimulates adult neurogenesis in rodents. Based on these findings we have raised the hypothesis that Exendin-4 may counteract the detrimental effects induced by diabetes in neural stem/progenitor cells.

The aim of this study was to investigate whether Exendin-4 protect neural progenitor cells from glucolipotoxicity and to analyse if the regulation of apoptosis may be involved in the Exendin-4 protecting effect.

Murine neural progenitor cells were exposed to high palmitate and glucose, which characterize diabetic glucolipotoxicity, in presence/absence of Exendin-4. To determine whether neural progenitor cells proliferation was impacted by the Exendin-4 treatment, [^{3}H] thymidine incorporation experiments were also performed. The expression of apoptosis key players, such as cleaved-caspase 3 and Bcl-2, were evaluated by western blotting.

We show that Exendin-4 counteracts the impaired neural progenitor cell viability induced by glucolipotoxicity. Cell proliferation was not influenced by the Exendin-4 treatment. The protective effect induced by Exendin-4 correlated with decreased apoptosis. In addition, the Exendin-4 protective effect was completely abolished by using the GLP-1R antagonist Ex-9-39, indicating that the protective effect by Exendin-4 was GLP-1R-mediated.

In conclusion, we show a direct survival effect of GLP-1R activation on neural progenitor cells challenged by diabetic-like conditions. The results support a potential therapeutic role of GLP-1R agonists, based on neurogenesis stimulation, for the treatment of the neurological complications in Type 2-diabetes and obesity.

Keywords: C17.2 cells; Exendin-4; GLP-1R; Palmitate; Neural progenitor cells

Introduction

During the last decade, several studies have inferred an association between diabetes/obesity and neurodegenerative disorders [1]. Particularly, a link between Type 2 Diabetes (T2D) and Alzheimer's disease (AD) has been shown [2]. In addition, T2D patients are at higher risk to develop stroke and show decreased recovery and increased mortality [3].

In the adult mammalian brain, new neurons can be generated from a proliferating population of adult Neural Stem Cells (NSCs) occurring in two specific brain regions, namely the subgranular zone of hippocampus and the subventricular zone of the lateral ventricle wall (SGZ and SVZ, respectively). Neuroblasts from the SVZ migrate to the olfactory bulb to differentiate into mature interneurons, while in the SGZ new neurons are generated in the granular cell layer of the hippocampus. This process is known as adult neurogenesis [4]. Adult neurogenesis can be regulated by different factors and in response to different diseases such as stroke, AD, PD and Huntington's disease (HD) in the human suggesting that this process could play a role in the development and/or response to neurodegeneration as well as represent a potential target for therapeutic intervention [5-7]. Interestingly, our recent work has shown that glucolipotoxicity mediated by high glucose and fatty acids, namely palmitate, decreases NSC viability [8]. Furthermore, pre-clinical *in vivo* studies have shown that adult neurogenesis is impaired in diabetes [9].

Glucagon-Like Peptide-1 (GLP-1) is a brain-gut insulinotropic peptide that plays an important role in the regulation of glucose homeostasis [10]. GLP-1 receptor (GLP-1R) agonists are current treatments for T2D based on their properties to stimulate glucose-dependent insulin secretion [11]. Exenatide (synthetic Exendin-4 (Ex-4)) is a stable GLP-1 analogue that is resistant to degradation and is approved in Europe and US for the clinical treatment of T2D [11]. Beside its well-known anti-hyperglycemic effects it has been showed that Ex-4 can cross the blood-brain barrier and studies have shown that exogenous Ex-4/GLP-1 also act as neuroprotectants in models of AD, PD and stroke [12,13]. Moreover, recent work has shown that Ex-4 can stimulate NSC proliferation and neurogenesis in both mice and rats [14-17]. In addition, a recent study has shown neuroprotective actions of Ex-4 in differentiated human Neural Progenitor Cells (NPCs) [18]. Finally, we recently showed that treatment with Ex-4 in diabetic rats reduces brain damage after stroke and increases stroke-induced NSCs proliferation [19].

While effects mediated by GLP-1R activation on NSCs/NPCs have been recently shown, it has not been studied whether GLP-1R activation can directly stimulate the survival of these cells in response to diabetes.

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The aim of this study was to determine whether Ex-4 protects NPC-derived C17.2 cells against a diabetic glucolipotoxic *milieu in vitro* [20]. Furthermore, we studied apoptosis as a potential mechanism behind such protective effect.

Material and Methods

Cell culture

C17.2 NPCs were originally isolated from neonatal mouse cerebellum and immortalized [20]. NPCs were maintained in plastic culture flask in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 5% horse serum, 2 mM glutamine, 100 U/mL penicillin, 100 µg/ml and streptomycin sulfate in 5% CO_2 and 95% humidity at 37°C.

Hyperglycemic and fatty acid-enriched medium

To mimic a diabetic *milieu* (hyperglycemic and hyperlipidemic) *in vitro*, DMEM (glucose 19 mM) and sodium palmitate (Sigma-Aldrich, St Louis, MO, USA) at the concentration of 0.3-0.4 mM were used as previously described by our group [8,21]. To obtain the desired palmitate concentration, NPC medium was supplemented with 0.25% bovine serum albumin (BSA, fatty acid free) (Roche Diagnostics, Mannheim, Germany) before adding the palmitate (from a 100 mM palmitate stock solution in 12.5% EtOH). Control cells were given vehicle with equal amounts of ethanol as the palmitate exposed cells.

Cell viability

Previous reports have indicated that intracellular ATP levels correlate to cell numbers [22]. To measure NPC viability, NPCs were seeded into 96 or 12-well plates (Corning B.V. Life Sciences, Amsterdam, Netherlands) at the final concentration of 3000 cells/well or 20,000 cells respectively. Ex-4 (1 nM, 10 nM, 100nM) (Sigma-Aldrich, St Louis, MO, USA), was added to NPCs for 15 min prior to palmitate exposure at concentrations shown in the results section. The treatments were incubated at 37°C (5% CO_2, 98% humidity), for 24 hours. Ex-9-39 (100 nM) (Polypeptide Laboratories, Torrance, USA), a specific and competitive antagonist for Ex-4, was administered to the cells 5-10 min before Ex-4. After 24 hours intracellular ATP levels were measured using the Cellular ATP Kit HTS according to the manufacturer's instructions (BioThema, Stockholm, Sweden). Trypan blue exclusion by cellular counting was also employed to validate the ATP-based results. In these experiments, the effect of each treatment at a certain concentration was determined between 4-7 samples in 3-10 different sets of experiments.

[^{3}H] Thymidine incorporation

To determine [^{3}H] thymidine incorporation into DNA, NPCs were plated in 12-wells plates at the final concentration of 30,000 cells/well. Palmitate alone or palmitate plus Ex-4 (10 nM) were added and cells were incubated for 24 hours. [^{3}H] thymidine (1 µCi/ml; Amersham Biosciences, Piscataway, NJ) was present from after plating until the cells were harvested. Cells were harvested and radioactivity was measured using a microplate scintillation and luminescence counter (Wallac MicroBeta Trilux; PerkinElmer). In these experiments, the effect of each treatment was determined in duplicates in 4 different set of experiments.

RT-PCR

To assess GLP-1R expression in NPCs, the total RNA was extracted using Aurum total RNA-mini kit (Bio-Rad Laboratories, Stockholm, Sweden) and the RNA was treated with DNase I (Bio-Rad Laboratories, Stockholm, Sweden) to eliminate possible DNA contamination, according to the manufacturer's protocol. Total mRNA was reversely transcribed into cDNA by using an iScript™cDNA Synthesis Kit (Bio-Rad Laboratories, Stockholm, Sweden). The expression levels of mRNAs were measured by SYBR green based quantitative RT-PCR (iQ™ SYBR® Green Supermix; Fermentas, St. Leon-Rot, Germany) using mouse-specific primer pairs for GLP-1R (Supplementary Table 1 for primer sequence) (Invitrogen, Stockholm, Sweden). β-actin was used as an internal standard. Reactions were resolved on a 2% agarose gel containing ethidium bromide and the bands were visualized under UV light to verify correct sizes of the amplification products.

Western blotting

NPCs were plated in 10 cm Petri dish (see under cell cultures) at the final concentration of 250,000 cells with/without Ex-4 (10 nM) added 15 min prior to palmitate exposure at concentrations shown in the results section. Treatments were incubated at 37°C (5% CO_2, 98% humidity) for 24 hours. NPCs were harvested, washed twice with PBS and homogenized on ice. Samples were clarified by centrifugation. The supernatants were transferred to new tubes and the total protein concentration was determined by Lowry protein assay (Bio-Rad Laboratories, Stockholm, Sweden). Samples were then mixed with reducing SDS-PAGE sample buffer and boiled for 10 min before performing SDS-PAGE. After electrophoresis, proteins were transferred onto Polyvinylidene Fluoride (PVDF) membranes (Bio-Rad Laboratories, Stockholm, Sweden). As a positive control for GLP-1R lysated from the beta cell line MIN-6 were used as control. Immunoblot analyses were performed with antibodies against GLP-1R (1:1000) (Abcam, Cambridge, MA, USA), cleaved form of Caspase-3 (1:1,000) (Cell Signaling Technology, Danvers, MA, USA) and Bcl-2 (1:200) (Abcam, Cambridge, MA, USA) [23]. Immuno-reactive bands were developed using ECL (GE Healthcare, Stockholm, Sweden), imaged with a GelDoc system and quantified with Quantity One software (Bio-Rad Laboratories, Stockholm, Sweden). After imaging, to verify equal protein loading, the PDVF membranes were stained with Coomassie blue (Bio-Rad Laboratories, Stockholm, and Sweden). In these experiments, the effect of each treatment a certain concentration was determined in duplicates in 5 different set of experiments.

Statistical analysis

Data are presented as mean ± SEM; multiple comparisons were made by one-way ANOVA followed by *post hoc* Fisher LSD test or Kruskal-Wallis if data was not normally distributed. All statistical analyses were performed using Sigma Plot software v. 11. $P<0.05$ was considered statistically significant.

Results

The results in Figure 1 show mRNA expression of GLP-1R in NPCs by RT-PCR. Moreover, GLP-1R protein expression was detected in C17.2 cells by Western blot analysis (data not shown).

To determine whether the activation of GLP-1R by Ex-4 could exert a protective effect against a diabetic *milieu,* NPCs were shortly pre-treated with Ex-4 (1-100 nM) before exposure to high glucose and palmitate for 24 hours. Exposure to these diabetic-like conditions resulted in reduced viability of the NPCs as reflected by a significant decrease of intracellular ATP levels (Figure 2A). However, Ex-4 pre-treatment 10nM significantly counteracted the decreased NPC viability induced by diabetic-like conditions (Figure 2A). The results were also confirmed by reduced cell counts of Trypan blue stained cells (data not

shown). To determine whether the enhanced NPCs viability by Ex-4 was due to increased proliferation, [^{3}H] thymidine incorporation was assessed. Hyperglycemic and hyperlipidemic challenge resulted in a large (approximately 60%) reduction of [^{3}H] thymidine incorporation in the NPCs (Figure 2B). However, the co-incubation with Ex-4 (10 nM) did not impact [^{3}H] thymidine incorporation (Figure 2B).

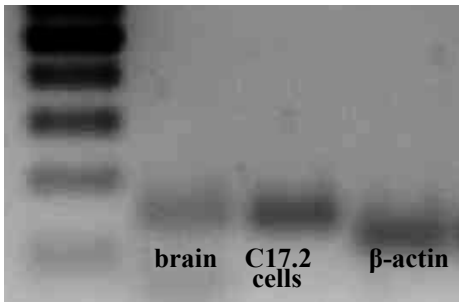

Figure 1: GLP-1R is expressed by NPCs *in vitro*. RT-PCR experiments were performed using primer pairs specific for GLP-1R gene (Table 1 for primer sequence). Arrows indicate bands corresponding to the correct size of each product of RT-PCR amplification. Control reactions of GLP-1R with brain lysates and β-actin primers were carried out.

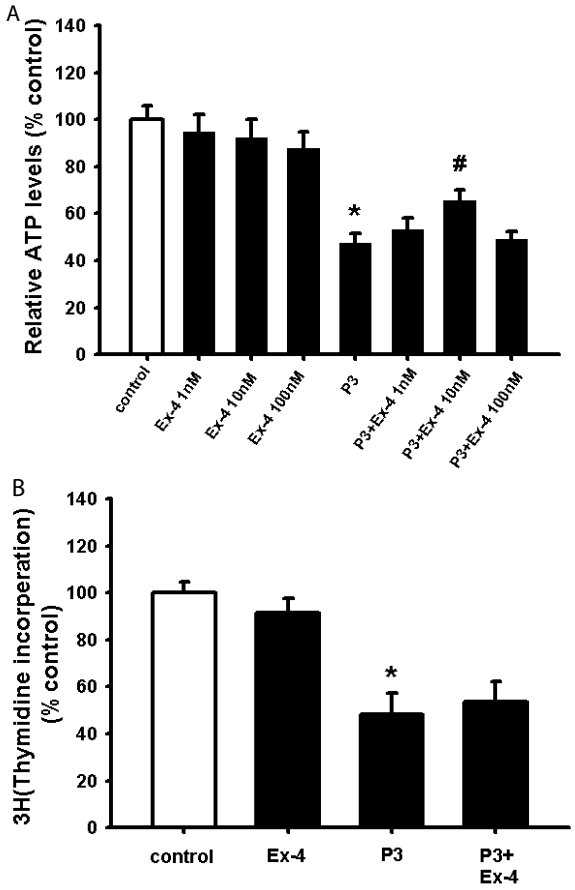

Figure 2: Ex-4 increases NPC viability in response to diabetic-like conditions. NPCs were plated in the presence or absence of high glucose and palmitate (19 mM and 0.3 mM respectively) for 24 hours. prior to palmitate addition cells were shortly pre-incubated with Ex-4 (1 nM, 10 nM or 100 nM). **(A)** Intracellular ATP levels and **(B)** [^{3}H] thymidine incorporation was assessed after 24 hours. Values are shown as mean ± SEM (A, n=30 B, n=8). One way ANOVA followed by Kruskal-Wallis *post hoc* test was used. Differences were considered significant at P<0.05. * denotes P<0.05 compared with control. # denotes P<0.05 compared with P3.

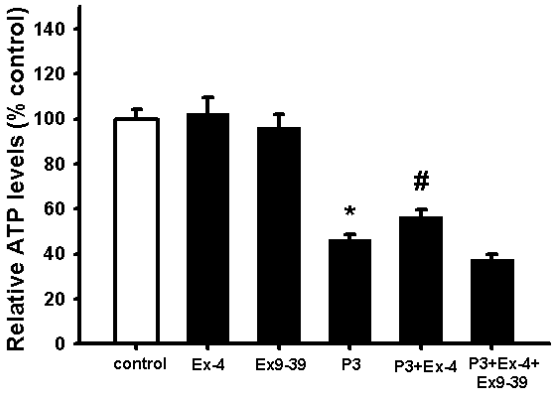

Figure 3: Ex-4 protects NPCs from glucolipotoxicity *via* a GLP-1R dependent pathway. NPCs were plated in the presence or absence of high glucose and palmitate (19 mM and 0.3 mM respectively). Prior to palmitate addition, cells were incubated with Ex-9-39 (100 nM) for 10 min before to Ex-4 (10 nM) exposure. Intracellular ATP levels were assessed after 24 hours. Values are shown as mean ± SEM (n=25-50). One way ANOVA followed by Kruskal-Wallis *post hoc* test was used. Differences were considered significant at P<0.05. *denotes P<0.05 compared with control, # denotes P<0.05 compared with P3.

In order to determine whether the effects of Ex-4 were conveyed through a GLP-1R dependent pathway, NPCs were pre-treated with the specific GLP-1R antagonist (Exendin 9-39 (Ex-9-39)) before exposure to Ex-4 and diabetes-like conditions [24]. The results show that the pre-treatment with Ex-9-39 led to loss of Ex-4-mediated protection against glucolipotoxicity (Figure 3).

Previous results from our group have shown that a diabetic *milieu* induces apoptosis in murine primary NSCs [8,21]. To determine whether the protective effect of Ex-4 correlated with decreased apoptosis in response to a diabetic *milieu*, the expression of the anti-apoptotic protein Bcl-2 and the active form of the protease Caspase-3 were assessed by Western blot [25]. The results show that hyperglycemia and palmitate induced a profound (approximately 4-folds) increment of the cleaved form of caspase-3 (Figure 4A). However, co-incubation with Ex-4 (10 nM) counteracted completely the enhanced levels of cleaved caspase-3 evoked by the diabetic *milieu* (Figure 4A), reflecting an attenuated apoptosis. Furthermore, glucolipotoxicity induced a significant reduction (approximately 50%) of anti-apoptotic Bcl-2 levels (Figure 4B). Conversely, co-incubation with Ex-4 (10 nM) entirely counteracted the decrease of Bcl-2 induced by the diabetic *milieu* (Figure 4B).

Discussion

Recent work from our and laboratories has showed that GLP-1R activation leads to increased NSC/NPC proliferation/survival and neurogenesis *in vitro* and *in vivo* (see Intro). However, the potential direct protective effect of GLP-1R activation in these cells against a diabetic glucolipotoxic environment has not been previously investigated.

The main findings of this study are as follows: exposure of NPCs to a glucolipotoxic diabetic *milieu* induces cell death. The results also indicate that glucolipotoxicity induces apoptosis as reflected by increased protein levels of cleaved-caspase 3, and reduced levels of Bcl-2. Ex-4 confers cellular protection against glucolipotoxicity in

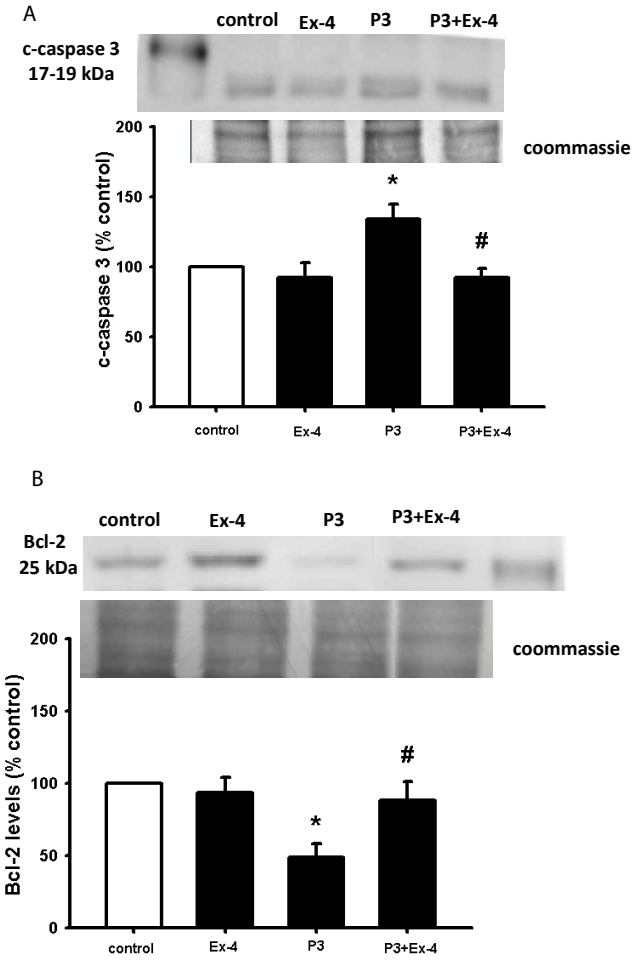

Figure 4: GLP-1R activation by Ex-4 counteracts glucolipoapotosis in NPCs *in vitro*. NPCs were plated in the presence or absence of high glucose and palmitate (19 mM and 0.3 mM respectively). Prior to palmitate addition cells were incubated with Ex-4 (10 nM) for 10 min. After 24 hours incubation cells were harvested for Western blot experiments. To obtain quantitative measurements **(A)** cleaved caspase 3 **(B)** Bcl-2 protein levels were normalized against Coomassie blue. Data are shown as mean ± SEM (A, n=10; B, n=10). One way ANOVA followed by Kruskal-Wallis *post hoc* test was used. Differences were considered significant at P<0.05. *denotes P<0.05 compared with control, # denotes P<0.05 compared with P3.

correlation with decreased apoptosis. Increased NPCs survival in response to Ex-4 does not occur by increased proliferation since [^{3}H] thymidine incorporation was unchanged.

Effects mediated by GLP-1 in the cardiovascular system have been observed in *glp-1r* $^{-/-}$ knockout mice due to the formation of the GLP-1 metabolite GLP-1 (9-36) following Dipeptidyl Peptidase-4 (DPP-4) degradation [26]. There also are reports of physiological actions on liver, skeletal muscle and fat cells by GLP-1; tissues lacking expression of GLP-1R. One explanation for this complexity might be due to a not yet known second GLP-1R [27]. Our results show that the protective effect of Ex-4 in NPCs occurs specifically via GLP-1R since the specific GLP-1R antagonist Ex-9-39 counteracted entirely the Ex-4-mediated increased NPC viability.

Diabetes/obesity, with concomitant hyperglycemia and hyperlipidemia, is a disease negatively affecting the CNS (see Intro). Insulin is a common treatment in T2D patients. However, although beneficial neuroprotective effects induced by insulin have been reported, insulin can induce a substantial risk for inducing hypoglycaemia. In the SELESTIAL trial, sought to determine the effects of glucose Potassium Insulin Infusion (GKI) on infarct growth in ischemic stroke, a high incidence of insulin-induced hypoglycaemia (76%) was reported [2,3,28]. Moreover GKI was associated with greater infarct growth in patients with persistent arterial occlusion [29]. Further, recurrent hypoglycaemic events have been associated to dementia and AD and obviously insulin is not the best drug aiming to treat CNS disorders in T2D [2]. Interestingly, GLP-1R agonists are associated with a minimal risk of hypoglycaemia since their metabolic effects are glucose-dependent [11]. In addition, there is a rapid growing body of evidence showing that GLP-1R agonists exert neuroprotective effects, irrespective of glycemia regulation [19]. Adult neurogenesis is impaired in T2D, both in hippocampus and SVZ [30,31]. Interestingly, hippocampal neurogenesis impairment has been suggested to be at the basis of cognitive dysfunction in T2D patients [28]. Furthermore, it has been recently shown that neuroblasts in the SVZ regenerate the human striatum along the whole adult life [32]. Altogether, these results support the therapeutic potential of the stimulation of adult neurogenesis for the treatment not only of AD/dementia but also of stroke and other striatal diseases like HD. In line with this hypothesis, our finding that NPCs are directly protected by Ex-4 against glucolipoapoptosis strengthens the idea that elderly T2D patients at high risk to develop dementia and stroke could be kept on a therapy based on GLP-1R activation. A GLP-1R activation therapy could be beneficial primarily against their diabetes (i.e., anti-hyperglycemic) while at the same time stimulating adult neurogenesis, with minimal risks of inducing hypoglycemia.

In conclusion, we report a clear neuroprotective effect of the GLP-1R analogue Ex-4 on NPCs exposed to a diabetic glucolipotoxic *milieu*. These results motivate further studies investigating the protective effects of GLP-1R agonists to counteract the increased neurodegeneration and dementia as well as the decreased recovery after stroke that T2D patients encounter.

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