Intermittent high glucose concentrations reduce neuronal precursor survival by altering the IGF system: the involvement of the neuroprotective factor DHCR24 (Seladin-1)

S Giannini¹, S Benvenuti², P Luciani², C Manuelli³, I Cellai², C Deledda², A Pezzatini¹, G B Vannelli⁴, E Maneschi², C M Rotella¹, M Serio² and A Peri²

¹Diabetes and Metabolic Diseases Unit and ²Endocrine Unit, Department of Clinical Physiopathology, Center for Research, Transfer and High Education on Chronic, Inflammatory, Degenerative and Neoplastic Disorders for the Development of Novel Therapies (DENOTe), ³Institute of Dermatology and Venereology and ⁴Department of Anatomy, Histology and Forensic Medicine, University of Florence, Viale Pieraccini, 6, 50139 Florence, Italy
(Correspondence should be addressed to A Peri; Email: a.peri@dfc.unifi.it)

Abstract

The exposure of neurons to high glucose concentrations is considered a determinant of diabetic neuropathy, whereas members of the IGF system are neurotropic factors. Here, we investigated the effects of constant and intermittent high glucose concentrations on IGF1 and IGF-binding proteins (IGFBPs) in human neuroblast long-term cell cultures fetal neuroepithelial cells (FNC). These cells express the IGF1 receptor, and express and release in the culture medium IGFBP2, IGFBP4, and IGF1. The release of IGF1 was significantly increased by 17 β -estradiol (10 nM). IGF1 (100 nM) treatment determined a significant increase of IGFBP2 and a decrease of IGFBP4 release. In addition, IGF1 (1–100 nM) stimulated FNC cell proliferation in a dose-dependent manner. We hypothesized that this effect may be, at least partially, due to IGF1-induced up-regulation of the expression of the Alzheimer's disease related gene *SELADIN-1*

(now known as *DHCR24*), which acts as a pro-survival factor for neuronal cells. Conversely, the exposure to intermittent (20/10 mM), but not stable (20 mM), high glucose concentrations decreased the release of IGF1 and IGFBP2 in the culture medium and inhibited FNC growth by inducing apoptosis. The latter was prevented by the addition of IGF1 to the culture medium. Furthermore, high glucose concentrations reduced the expression of *DHCR24*. In conclusion, our results indicate for the first time that intermittent high glucose concentrations, similar to those observed in poorly controlled diabetic patients, may contribute to the development of diabetic neuropathy by interfering with the tropic effects exerted by the IGF system, and suggest the involvement of the neuroprotective factor *DHCR24*.

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Introduction

Neuropathy can be a highly debilitating clinical condition for diabetic individuals. Although several mechanisms are involved in this complication, most pathogenic theories generally accept that damage to nerves is a direct or indirect effect of glucose levels (Tomlinson & Gardiner 2008). In fact, altered biological products such as advanced glycation end products formation, cellular accumulation of polyols, decreased myo-inositol content, impaired Schwann cell function, and microangiopathy with ischemia have been proposed to cause neuropathy (Vincent *et al.* 2004, Ho *et al.* 2006). *In vivo* experimental diabetes clearly determined an impairment of axonal regeneration, and neuron loss at various stages of degeneration has been reported in the presence of chronic high glucose (Yagihashi *et al.* 1990, Toth *et al.* 2004), whereas *in vitro* studies showed that intermittent high glucose is more toxic than constant high glucose concentrations for human cells (Picconi *et al.* 2006).

The insulin-like growth factor (IGF) system consists of IGF1, IGF2, IGF1 receptor (IGF1R), IGF2R, and some protein carriers named IGF-binding proteins (IGFBPs 1–6). Most of the IGFs in biological fluids and *in vitro* cell cultures form complexes with IGFBPs that bind to IGFs with affinities 10- to 100-fold greater than the IGF1R, thus modulating the bioavailability of IGFs (Mohan & Baylink 2002). Several studies have documented the presence of IGFs in the central nervous system (CNS) and, although the precise roles of these growth factors remain to be elucidated, there is evidence that this system plays an important role in neuronal development, metabolism, survival, and regeneration (Matthews & Feldman 1996, Russo *et al.* 2004, 2005). On the contrary, little is known about the types and the regulation of IGFBPs in human neuronal cells. Under basal conditions, human neuroblastoma cells have been demonstrated to secrete a large amount of IGFBP2, a lower amount of IGFBP4, and traces of IGFBP6 (Babajko *et al.* 1997). IGFBP2 is the most representative IGFBP in the cerebrospinal fluid

suggesting an important role in the CNS (Roghani *et al.* 1993). IGF1 and other growth factors have been demonstrated to differently regulate the levels of IGFBPs in the CNS depending on the type of neuronal or glial cells studied (Pons & Torres-Aleman 1992, Kummer *et al.* 1996).

SELADIN-1 (for SElective Alzheimer's disease INDicator-1 that is now known as *DHCR24*) is a recently described gene found to be down-regulated in brain regions affected by Alzheimer's disease (Greeve *et al.* 2001). A neuroprotective role has been described for this gene. In fact, when *DHCR24* was overexpressed in neuroglioma H4 cells it counteracted the toxicity of β -amyloid deposits and oxidative stress (Greeve *et al.* 2001). The biological effects of *DHCR24* appear to be due, at least in part, to its anti-apoptotic activity related to the inhibitory effect on the activation of CASP3. We have demonstrated previously that 17 β -estradiol increases cell proliferation, whereas it counteracts β -amyloid-induced apoptosis in human fetal neuroepithelial cells (FNC) and up-regulates the expression of the *DHCR24* gene. These findings suggested that this gene may be a mediator of the pro-survival effects of estrogen in the brain (Benvenuti *et al.* 2005). This hypothesis was supported by the very recent finding that *DHCR24* silencing abolishes the protective effects of estrogen in FNC cells (Luciani *et al.* 2008). These cells are GNRH1-secreting neuroblast long-term cell cultures, previously isolated from human fetal olfactory epithelium (Vannelli *et al.* 1995). FNC show unique features, because they synthesize both neuronal proteins and olfactory markers and respond to odorant stimuli, suggesting their origin from the stem cell compartment that generates mature olfactory receptor neurons. Interestingly, there is evidence of a tight link between estrogen and the IGF system in the CNS in terms of neuronal cell differentiation, survival, and regeneration (Mendez *et al.* 2003, 2006).

The aim of this study was to clarify the involvement of the IGF system in glucose-related neuropathy. To this purpose, we used FNC to i) characterize the IGF system in this neuronal cell model; ii) determine whether IGF1 has pro-survival effects in these cells; iii) assess whether the exposure to constant and intermittent high glucose concentrations, similar to those observed in poorly controlled diabetics, affects the integrity of the IGF system; and iv) determine whether IGF1 and high glucose concentrations affect the expression of the *DHCR24* gene.

Materials and Methods

Cell cultures

The primary neuroblast long-term cell culture FNC was established, cloned, and propagated *in vitro* from human fetal olfactory neuroepithelium (Vannelli *et al.* 1995). FNC cells synthesize both neuronal proteins and olfactory markers and respond to odorant stimuli, suggesting that their origin from the stem cell compartment that generates mature

olfactory receptor neurons. In addition, they display neuroendocrine features (Maggi *et al.* 2000). FNC cell cultures were propagated at 37 °C in 5% CO₂ in Coon's modified Ham's F-12 medium (Sigma Chemicals Co.), with 1·802 g/l glucose, 10% fetal bovine serum (HyClone, Logan, UT, USA), and antibiotics (penicillin, 100 IU/ml; streptomycin 100 μ g/ml). The B4 clone, showing the highest levels of expression of neuronal and olfactory markers (Vannelli *et al.* 1995), was used in this study.

Determination of IGF1R mRNA and protein levels

Total RNA was extracted from cells using RNeasy kit (Qiagen) and treated with DNase I (Qiagen) to eliminate possible genomic DNA contamination. cDNA of each sample was synthesized from 1 μ g total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA USA), following the manufacturer's protocol. Real-time RT-PCR was performed using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. All PCR amplifications were performed on MicroAmp optical 96-well reaction plate with Taqman Universal Master Mix (Applied Biosystems) and using Assay on Demand (Applied Biosystems). The PCR was performed using the template cDNA with specific primers for human IGF1 receptor (Forward: 5'-TTA AAA TGG CCA GAA CCT GAG-3'; Reverse: 5'-ATT ATA ACC AAG CCT CCC AC-3'). Each assay was carried out in duplicate and included a no-template sample as negative control. RT-negative samples were used to demonstrate that the signals obtained were RT dependent. Relative expression of mRNA levels were determined by comparing experimental determinations to a standard curve generated using serial dilutions of cDNAs obtained from human leukocytes (Liotta *et al.* 2007).

For immunoblot analysis of IGF1R, cell lysates were prepared as described previously (Cho *et al.* 2003). Total cell lysates were resolved on 4–20% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The blots were blocked for 1 h in TBS-T (20 mmol/l Tris-HCl, pH 7·5, 150 mmol/l NaCl, 1 g/l Tween 20) containing 50 g/l non-fat dry milk powder and incubated for 1 h with anti-IGF-IR β (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody. The blots were then incubated with anti-mouse or anti-rabbit HRP-conjugated antibody. Signals were detected by chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA).

Collection of conditioned medium (CM), identification, and immunocharacterization of IGFBPs

Cells were seeded in 25 cm² tissue culture flasks at a density of 35×10³ cells/cm² in regular medium. After 24 h, the cells were washed with PBS and cultured in 1·5 ml serum-free culture medium for 48 h (basal condition) or in serum-free culture medium for 24 h, and then in serum-free culture

medium containing 100 nM IGF1 (Sigma Chemicals Co.) for an additional 24 h. CM was then collected and stored at -80°C , as described previously (Giannini *et al.* 1994). After the collection of CM, cell numbers were determined and the volume for analysis adjusted accordingly. IGFBPs in the CM were examined by western ligand blotting, carried out according to the method of Hossenlop *et al.* (1986). Briefly, 100 μl CM, together with pre-stained molecular weight marker proteins (Bio-Rad) underwent 12% SDS-PAGE in non-reducing conditions and subsequent transfer onto nitrocellulose membranes (Sartorius AG, Göttingen, Germany). The membranes were then incubated with ^{125}I [IGF2] (Amersham). Protein band intensity was analyzed by scanning densitometry of the original autoradiographic film, after 3 days of exposure, with a Flowvision densitometer (Lynx, San Mateo, CA, USA). To quantify the intensity of radioactivity of various IGFBPs, several autoradiographs developed after different times of exposure of the same blot were measured, and data within a linear range were used.

For immunoblotting, CM was subjected to SDS-PAGE under non-reducing conditions and then electrophoretically transferred onto a nitrocellulose membrane as described above for the western ligand blotting. Polyclonal antibodies against IGFBP1 to IGFBP6 were purchased from UBI (Lake Placid, NY, USA) and goat antibody immunoglobulin G conjugated with alkaline phosphatase was obtained from Sigma Chemicals Co. The signals were detected according to the manufacturer's instructions.

For glycosylation studies, a 10-fold concentrated lyophilized FNC serum-free CM was analyzed as follows: a 2 mg/ml solution of FNC CM were prepared and 5 μl 7.5% NP-40 and then 8 μl *N*-glycanase enzyme (Genzyme Co., Cambridge, MA, USA) were added to 10 μl of the above obtained solution. After the addition of 7 μl distilled water, the solution was incubated for 12 and 24 h at 37°C . The reaction was finally stopped by the addition of a proper amount of PAGE loading buffer and the samples were analyzed by ligand blot after SDS-PAGE (Giannini *et al.* 1999).

IGF1 determination by ELISA

Secreted IGF1 was determined using acid ethanol extraction (Daughaday *et al.* 1980) and the Quantikine human IGF1 kit (R&D system, Minneapolis, MN, USA). An aliquot of 600 μl of the above described CM, exposed to 17β -estradiol, high glucose, or intermittent high glucose concentrations, was lyophilized to a dry pellet. An aliquot of 20 μl water was added to resuspend the pellet. An aliquot of 80 μl of 12.5% 2 M formic acid/87.5% ethanol was added and incubated for 30 min at room temperature to avoid the IGFBP complexes and to free IGF1. The solution was centrifuged and the supernatant was neutralized by adding 0.4 volume of 0.855 M Tris base. Seven volumes of 1 mg/ml BSA with 500 ng/ml IGF2 in 0.1 M Tris (pH 7.4) were added; 50 μl of the prepared sample were used in each well of the Quantikine kit following the manual instructions for each experimental condition.

Cell growth

For cell growth experiments, 5×10^4 cells were seeded onto 6-well plates in growth medium. After 24 h, the growth medium was removed, the cells were incubated in the same medium with different stimuli: IGF1 (Sigma Chemicals Co.), recombinant human IGFBP2 (UBI), and αIR3 antibody (kind gift from Prof Mario Maggi, University of Florence, Italy). During the prolonged (5 days) glucose cell culture experimental protocol, three groups of FNC were considered, each receiving the following fresh medium every 24 h: 1) continuous normal glucose medium (10 mM); 2) continuous high glucose medium (20 mM); and 3) intermittent high glucose media (20/10 mM) alternating every 24 h. Osmotic control was assured by incubating FNC with equimolar concentrations of mannitol, both continuously and in an alternating manner. The cells were then trypsinized and counted using a hemocytometer. For each experimental point, the mean value from at least five different fields for each well was considered. Each determination was repeated in duplicate. The results of the experiments were expressed as growth percentage ($\pm$ S.E.M.) versus the corresponding control.

TUNEL analysis for the determination of apoptotic cells

Apoptosis was analyzed by TUNEL analysis, using a commercially available detection kit (FragEL DNA Fragmentation Detection Kit, Oncogene Research Products, Boston, MA, USA). FNC cells were cultured in chamber slides for 5 days. Fresh medium was changed every 24 h and four groups were considered: 1) continuous normal glucose medium (10 mM); 2) intermittent high glucose media (20/10 mM) alternating every 24 h; 3) IGF1 (10 nM); and 4) IGF1 (10 nM) and intermittent 20/10 mM glucose. Three experiments were performed. Apoptotic cells/field were counted in twenty fields ($20\times$) and the results were expressed as percentage of apoptotic cells/field.

Quantitative real-time RT-PCR for DHCR24 transcript

The absolute quantification of *DHCR24* mRNA was performed by real-time RT-PCR, based on TaqMan technologies, as described previously (Luciani *et al.* 2004). Total RNA to be subjected to reverse transcription was extracted from FNC in basal condition and after IGF1 or high glucose concentrations exposure. The results were referred to microgram of total RNA. The experiments ($n=3$) were run in triplicates.

Statistical analysis

Data were expressed as mean $\pm$ S.E.M. Statistical differences were analyzed using one-way analysis of variance. The significant level was taken at $P<0.05$. All statistical tests were performed with the program, Statistical Package for Social Sciences (SPSS version 12.0; SPSS, Chicago, IL, USA).

Results

Characterization of the IGF system in FNC

No previous study characterized the IGF system in the cell model we used, i.e. FNC. Initially, the presence of *IGF1R* was investigated. The amount of mRNA was determined by real-time RT-PCR. *IGF1R* transcript was detectable in FNC and the level of expression was about four-fold higher than in a human cell line from glomerular endothelial cells (GENC), previously obtained in our laboratory and used as the positive control (Giannini *et al.* 1999; Fig. 1A). Western immunoblot analysis of the *IGF1R* in cell lysates of FNC confirmed the presence of a band of about 95 kDa, corresponding to the mature form of the *IGF1R* and a faint band of 200 kDa that has been reported to correspond to a precursor form of this receptor (Kim *et al.* 2005; Fig. 1B).

Figure 1 (A) Expression of *IGF1R* mRNA in FNC, FNC exposed to 17 β -estradiol (E₂; 10 nM), and GENC used as the positive control, as assessed by quantitative RT-PCR. Relative quantity of IGF1R indicates that mRNA levels were determined by comparing experimental determinations to a standard curve generated using serial dilutions of cDNAs obtained from human leukocytes (Liotta *et al.* 2008). **P* < 0.05 (B) Expression of IGF1R precursor protein and mature IGF1R protein in FNC and GENC. The western blot shown in the figure is representative of three independent experiments.

With regard to IGFBPs, after 48 h in serum-free medium, western ligand blot analysis revealed the presence of two major IGFBP species of 34 and 24 kDa and a faint 32–30 kDa doublet in FNC (Fig. 2A). The 34 kDa band with the migration pattern typical of IGFBP2 was recognized by the anti-IGFBP2 antibody (Fig. 2B). This antibody did not recognize other proteins with a lower molecular weight. The 24 kDa band, with migration characteristics of IGFBP4, was identified as such by the specific antibody (Fig. 2B). However, this antibody also cross-reacted with the 32–30 kDa doublet, which could correspond to the glycosylated form of IGFBP4 (Fig. 2B; Babajko *et al.* 1997), as confirmed by *N*-glycanase treatment of the CM, which determined the disappearance of

Figure 2 IGFBPs characterization in FNC. (A) ¹²⁵I[IGF2] ligand blot of FNC CM without (CTRL) or with *N*-glycanase (*N*-Glyc) treatment for 12 and 24 h. The molecular weight of the detected signals is reported on the right-hand side of the figure. (B) Immunoblotting for the characterization of the IGFBPs released by FNC. Immunoreactive signals specific for IGFBP2 and IGFBP4, and the 32–30 kDa doublet are shown.

this band in the western ligand blot analysis (Fig. 2A). No signals, instead, were obtained with anti-IGFBP1, -3, -5, or -6 antibodies (data not shown). In the presence of 100 nM IGF1, western ligand blot analysis of the IGFBPs released in the CM showed an increased intensity of the 34 kDa signal (IGFBP2) band with a concomitant dramatic reduction of the 24 kDa signal (IGFBP4; Fig. 3). In addition, the exposure to IGF1 markedly increased the signal corresponding to the 32–30 kDa doublet. The densitometry of the signals showed in Fig. 3 is reported in Table 1. Immunoblot analysis, using the anti IGFBP4 antibody, confirmed the results of western ligand blot analysis, with regard to the variations of the 24 and 32–30 kDa signals upon IGF1 treatment (data not shown).

Furthermore, the production of IGF1 by FNC cells was assessed. Detectable levels of immunoreactive IGF1 were found in the culture medium (Fig. 4). According to previous evidence that a cross-talk between the IGF system and estrogen in the brain occurs (Mendez *et al.* 2003, 2006), the effect of 17 β -estradiol on IGF1 production was determined. The exposure of FNC to 17 β -estradiol (10 nM) significantly increased the release of IGF1 in the culture medium (Fig. 4). Conversely, estrogen exposure did not significantly alter the amount of expression of IGF1R (Fig. 1A).

Effect of IGF1 on FNC cell proliferation

The effect of increasing concentrations of IGF1 on FNC cell proliferation was investigated in FNC cells maintained in CM. The results of these experiments are shown in Fig. 5. IGF1 significantly increased the cell number in a dose-dependent manner with a maximum effect at 10 nM (2.1-fold

Table 1 Densitometric quantification of western ligand blot showing the effect of insulin-like growth factor 1 (IGF1) on IGF-binding proteins (IGFBPs) modulation in fetal neuroepithelial cells (FNC; Fig. 3)

	Control	IGF1
IGFBP4	200.3 $\pm$ 37.8	83 $\pm$ 11*
32–30 kDa doublet	10.6 $\pm$ 2.9	689.3 $\pm$ 68.7†
IGFBP2	41.6 $\pm$ 5.5	496.3 $\pm$ 55.6†

Data are expressed as mean values $\pm$ S.E.M. of three different experiments. * P <0.05; † P <0.01 versus control.

versus untreated cells). The addition of the monoclonal antibody α IR3, which specifically inhibits IGF1 binding to its receptor, blunted the effect of IGF1 (10 nM). By contrast, the co-presence of an equimolar concentration (10 nM) of IGF1 and recombinant IGFBP2 maintained the stimulatory effect of IGF1 on cell growth (2.05-fold versus untreated FNC cells).

Effect of glucose exposure on FNC cell proliferation and on IGF1/IGFBPs release

The exposure to a constant high (20 mM) glucose concentration did not modify the growth profile of FNC compared with control untreated cells. Instead, when the cells were alternatively exposed to intermittent high glucose concentrations (20/10 mM), the number of cells at the end of the treatment was significantly lower compared with untreated cells (Fig. 6). The inhibitory effect of 20/10 mM glucose on cell growth was effectively counteracted by IGF1 treatment (10 nM). Osmotic control with mannitol did not significantly affect the cell number.

The effect of the 20 and 20/10 mM glucose exposure on IGFBPs secretion was also checked (Fig. 7). The amount of IGFBP4 in the CM was not modified by glucose exposure,

Figure 3 125 I[IGF2] ligand blot of FNC CM harvested after 48-h serum-free culture in the presence or not (CTRL) of 100 nM IGF1.

Figure 4 Amount of IGF1 released by FNC in the culture medium in basal conditions (control) and after treatment with 17 β -estradiol (E₂; 10 nM). The results are reported as mean values $\pm$ S.E.M. of three different experiments. * P <0.05.

Figure 5 Effect of IGF1 on FNC cell growth. The results are reported as mean values $\pm$ S.E.M. of three different experiments and are reported as percentage of cell number versus untreated cells (control considered as 100%). * $P < 0.05$.

whereas the intensity of the IGFBP2 and of the 32–30 kDa doublet signals was significantly reduced upon 20/10 mM glucose exposure, as determined by densitometric analysis, reported in Table 2. Osmotic control with mannitol did not affect the IGFBPs levels.

Figure 6 Effect of stable high (20 mM) and oscillating high glucose (Gluc; 20/10 mM) concentrations on FNC cell growth. The effect of the exposure to IGF1 (10 nM) in addition to glucose 20/10 mM is also shown. Equimolar concentrations of mannitol (mann) were used as osmotic control. The results are reported as mean values $\pm$ S.E.M. of three different experiments and are reported as percentage of cell number versus untreated cells (control, considered as 100%). * $P < 0.05$ versus control; ** $P < 0.05$ versus glucose 20/10 mM.

Figure 7 125 I[IGF2] Western ligand blot of FNC CM in basal conditions (CTRL) or after exposure to alternating high (20/10 mM) and constant high (20 mM) glucose (Gluc) concentrations. Equimolar concentrations of mannitol (Mann) were used as osmotic control. The molecular weight of the detected signals is reported on the left-hand side of the figure.

Interestingly, the amount of IGF1 released in the CM was also affected by the exposure to glucose; in particular, a significant reduction was obtained upon exposure to glucose 20/10 mM (Fig. 8).

Effect of IGF1 and glucose on FNC cell apoptosis

In order to determine whether the inhibitory effect of intermittent high glucose concentrations on FNC cell growth was influenced by altered cell survival, apoptosis was evaluated by TUNEL analysis. The exposure to 20/10 mM glucose significantly increased the number of apoptotic cells. As already observed in cell proliferation experiments, IGF1 (10 nM) was able to counteract the pro-apoptotic effect of intermittent high glucose concentrations (Fig. 9).

Effect of IGF1 and glucose on DHCR24 expression in FNC

To examine whether the IGF system affects the *DHCR24* gene expression in FNC, quantitative RT-PCR was performed. In these experiments, IGF1 (10 nM) and IGFBP2 (10 nM), alone or in combination, were used. IGF1 increased the amount of *DHCR24* mRNA by ~ 2.4 -fold compared with untreated FNC cells. Conversely, the treatment with IGFBP2 had no significant effect on *DHCR24* mRNA (Fig. 10). The simultaneous exposure to IGF1 and IGFBP2 did not modify the effect observed in the presence of IGF1 alone. At variance with the stimulatory effect of IGF1, both glucose 20/10 and 20 mM were able to determine a statistically significant reduction of the amount of *DHCR24* expression (Fig. 10).

Table 2 Densitometric quantification of western ligand blot showing the effect of alternating high (20/10 mM) or constant high (20 mM) glucose (Gluc) concentrations, or equimolar concentrations of mannitol (Mann), on insulin-like growth factor-binding proteins (IGFBPs) release. Data are expressed as mean values $\pm$ S.E.M. obtained from triplicate determinations (Fig. 7)

	Control	Mann 20/10	Mann 20	Gluc 20/10	Gluc 20
IGFBP4	180 $\pm$ 30.6	168 $\pm$ 19.6	188 $\pm$ 23.1	147 $\pm$ 18.5	17.8 $\pm$ 26.6
32–30 kDa doublet	16 $\pm$ 2.9	12 $\pm$ 2.3	23 $\pm$ 4	3 $\pm$ 1.2*	15 $\pm$ 4.6
IGFBP2	89 $\pm$ 14.4	96 $\pm$ 13.3	94 $\pm$ 17.9	12 $\pm$ 1.7*	59 $\pm$ 6.9

* $P < 0.05$ versus control.

Conversely, the exposure to mannitol did not modify the amount of mRNA (data not shown).

Discussion

The pathogenesis of diabetic neuropathy is complex and involves different molecular mechanisms. IGF1, together with its related IGFBPs, is well recognized as a potent physiological neurotrophic factor because of its ability to promote the proliferation, survival, migration, and differentiation of immature glial and neuronal cells (Russo *et al.* 2005). The role of the IGFBPs in these processes remains unclear, but it is likely to involve the transport and targeting of IGF1 to its receptor (Russo *et al.* 2005). IGF1 has a well-demonstrated protective effect on neuronal cells even when exposed to many biological insults (Matthews *et al.* 1997, Russo *et al.* 2004, Willaime-Morawek *et al.* 2005). Nevertheless, a few data on the protective effect of IGF1 in human neuronal cells in the presence of high glucose concentrations are available (Gustafsson *et al.* 2004) and, to our knowledge, no study has assessed the modification of IGF1 or IGFBPs levels in neuronal cells during rapid changes of glucose concentration, as it might occur in poorly controlled diabetes. To clarify this issue, in the present study, we used a unique human cell model, i.e. FNC.

First, we characterized the IGF system in these cells and we found that FNC express the IGF1 receptor, which is known to be variably expressed in the developing brain, with a higher level in the early stages of embryogenesis (Gammeltoft *et al.* 1985). With regard to IGFBPs, the presence of low molecular weight IGFBPs (24–34 kDa) in the CM of neuronal cells has been described (D'Ercole *et al.* 1996). The most abundant immunocharacterized IGFBPs in these cells are IGFBP2, IGFBP4, and IGFBP5 (Han *et al.* 1996). For the other IGFBPs, the demonstration of their presence is limited, variable, and when expressed their levels are low (Naeve *et al.* 2000). Under particular experimental conditions, the induction of IGFBP6 that is not normally expressed in the CNS has been reported (Zhou *et al.* 2001). In the present study, we identified in the CM of FNC cells two major bands of 24 and 34 kDa immunocharacterized as IGFBP4 and IGFBP2 respectively. These data confirm a previous report on neuroblastoma cells (SK-N-SH) that, under basal conditions, secrete these two IGFBPs (Menouny *et al.* 1997). However, in agreement with another human neuroblastoma cell line (SH-SY5Y), in the CM of FNC we detected an additional weak signal migrating as a doublet around 32–30 kDa, which we characterized as the *N*-glycosylated form of IGFBP4 (Babajko *et al.* 1997). IGF1 treatment increased the amount of immunoreactive IGFBP2 in the CM, whereas it decreased

Figure 8 Amount of IGF1 released by FNC in the culture medium in basal conditions (control) and after treatment with alternating high (20/10 mM) or constant high (20 mM) glucose (Gluc) concentrations. The results are reported as mean values $\pm$ S.E.M. of three different experiments. * $P < 0.05$ versus control.

Figure 9 Amount of apoptotic cells, as detected by TUNEL analysis, in basal conditions (control) and after exposure to glucose (Gluc) 20/10 mM, IGF1 (10 nM), or Gluc 20/10 mM + IGF1 (10 nM). * $P < 0.05$ versus control; ** $P < 0.05$ versus glucose 20/10 mM.

Figure 10 Quantification of *DHCR24* mRNA by real-time RT-PCR in untreated FNC (control), or upon exposure to 10 nM IGF1, 10 nM IGFBP2 (BP2), 10 nM IGF1 + 10 nM IGFBP2, alternating high (20/10 mM) or constant high (20 mM) glucose (Gluc) concentrations. * $P < 0.05$.

the amount of IGFBP4. The altered ratio between these two IGFBPs may account, at least in part, for the observed stimulatory effect of IGF1 on FNC cell proliferation, which confirmed the large number of *in vitro* studies demonstrating that IGF1 promotes cell proliferation in neuronal cells (Russo *et al.* 2005). In fact, IGFBP2 is produced in various regions of the CNS, it is one of the major IGFBPs in the cerebrospinal fluid, and its mRNA is detectable in abundance in some brain regions with higher dynamic remodeling such as the olfactory bulb (D'Ercole *et al.* 1996). IGFBP2 is associated with membrane proteoglycans in neuronal cells where the complex IGF1/IGFBP2 stimulates neurogenesis by a mechanism facilitating the binding of IGF1 to its receptor (Brooker *et al.* 2000). On the contrary, IGFBP4 is generally accepted as a potent inhibitor of IGF1 biological action in most of the *in vitro* experimental models used (Mazebourg *et al.* 2004) and its mRNA has been reported also in fetal neuronal cells (Chernausk *et al.* 1993). The reason for the increased intensity of the 32–30 kDa doublet, identified as the *N*-glycosylated form of IGFBP4, after the addition of IGF1 remains unclear. In fact, the glycosylation of IGFBP4 does not affect its binding to IGFs and the physiological role of this phenomenon has not been clarified, so far (Zhou *et al.* 2003). Anyway, the same effect has been previously described in SH-SY5Y neuroblastoma cells (Babajko *et al.* 1997), suggesting that this differential modulation of IGFBPs by IGF1 could be the result of an autocrine regulatory loop aimed to control cell proliferation.

Furthermore, we found that FNC themselves are able to release immunoreactive IGF1, which is significantly increased by the exposure to 17 β -estradiol (10 nM). The latter finding is in agreement with the reported cross-talk between the IGF system and estrogen in determining neuronal differentiation, survival, adult neurogenesis, and synaptic plasticity (Mendez *et al.* 2006).

Then, we investigated the effect of high glucose concentrations, which cause many of the pathological consequences of diabetes mellitus, on cell growth and the integrity of the IGF system in FNC cells. Much of the damage induced by glucose is suggested to be a consequence of both high glucose levels as well as glucose fluctuations (Ceriello 1998), which are strong predictors of diabetic microangiopathy, including neuropathy (The Diabetes Control and Complications Trial Research Group 1995). Therefore, FNC were exposed to chronic and intermittent high glucose concentrations, a condition that partially mimics what happens *in vivo* in diabetic patients. We found that intermittent high glucose inhibited FNC growth whereas stable high glucose did not. Furthermore, apoptosis evaluation indicated that intermittent high glucose induced apoptotic cell death. The stimulatory effects of 20/10 mM glucose on apoptosis were effectively counteracted by IGF1. It is generally accepted that chronic high glucose impairs the rate of neuronal cells growth, as confirmed also by a recent study (Pal *et al.* 2007); however, some other reports indicated that high glucose is not a potent inducer of neuronal cell death and that neurite regeneration is enhanced by high glucose (Sango *et al.* 2002). Nevertheless, our data are in agreement with observations from different experimental models, such as fibroblasts, mesangial, tubulointerstitial, and endothelial cells, indicating that glucose variations are more detrimental for cells than stable high glucose (Piconi *et al.* 2006). Some of these effects probably occur as a result of changes in the secretion or modified local bioavailability of autocrine or paracrine growth factors (Sango *et al.* 2006). To our knowledge, this is the first demonstration of a negative effect on cell survival in neuronal precursors upon exposure to intermittent high glucose. A possible explanation for this observation may be found in the marked reduction in the release of IGFBP2, but not of IGFBP4, induced by this experimental condition. We reported previously similar results in human retinal endothelial cells (Giannini *et al.* 2001). IGFBP2, in contrast to IGFBP4, presents sites of association on the matrix/cell surfaces (Bach & Rechler 1994); thus, it might be hypothesized that one of the mechanisms regulating the levels of IGFBP2 could be a biochemical modification of protein matrix/cell surface or of the components involving the IGFBP heparin-binding sequence. Because of the opposite effects of IGFBP2 and IGFBP4 on the bioavailability of IGF1, it is conceivable that the altered ratio among these two proteins was the determinant of the reduction of cell growth, that we observed upon exposure to intermittent high glucose concentrations. This hypothesis is in agreement also with the reduced amount of IGF1 detected in the culture medium of FNC after treatment with intermittent high glucose. Furthermore, our findings are in keeping with the description of reduced levels of IGF1 and IGFBP2 in the cerebellum of poorly controlled diabetic rats (Busiguina *et al.* 1996), together with an altered balance between the level of IGF1 in the cerebrospinal fluid and the blood (Armstrong *et al.* 2000).

Finally, in our study, we addressed the effect of IGF1 and high glucose concentrations on the expression of the *DHCR24* gene. The expression of this neuroprotective factor had been detected previously in FNC and found to be up-regulated by estrogen (Benvenuti *et al.* 2005). Interestingly, 17 β -estradiol was also able to induce cell proliferation and counteract β -amyloid-induced apoptosis in FNC. Thus, it was hypothesized that *DHCR24* might be a mediator of these effects (Benvenuti *et al.* 2005). More recent studies in FNC cells exposed to estrogen after *DHCR24* gene silencing confirmed this hypothesis (Luciani *et al.* 2008). Here, we found that IGF1 significantly up-regulated the amount of *DHCR24* mRNA, whereas high glucose reduced it, thus indicating this factor as a possible mediator of the tropic effects of IGF1 in neuronal cells. This hypothesis, although preliminary, appears to be corroborated by the common intracellular pathways activated by ER- and IGF1R-mediated signaling, involving for instance the MAPK and the PI3K pathways (Mendez *et al.* 2003, Jover-Mengual *et al.* 2007).

In conclusion, our results indicate for the first time that intermittent high glucose concentrations, similar to those observed in poorly controlled diabetic patients, may contribute to the development of diabetic neuropathy by interfering with the tropic effects exerted by the IGF system, and suggest the involvement of the pro-survival factor *DHCR24*. Nevertheless, additional studies, performed for instance in cells subjected to *DHCR24* gene silencing, are needed to clarify the exact role of *DHCR24* in mediating the effects of IGF1 and high glucose in neuronal cells.

Declaration of Interest

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

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