Insulin Promotes Glycogen Storage and Cell Proliferation in Primary Human Astrocytes

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Abstract

Introduction: In the human brain, there are at least as many astrocytes as neurons. Astrocytes are known to modulate neuronal function in several ways. Thus, they may also contribute to cerebral insulin actions. Therefore, we examined whether primary human astrocytes are insulin-responsive and whether their metabolic functions are affected by the hormone.

Methods: Commercially available Normal Human Astrocytes were grown in the recommended medium. Major players in the insulin signaling pathway were detected by real-time RT-PCR and Western blotting. Phosphorylation events were detected by phospho-specific antibodies. Glucose uptake and glycogen synthesis were assessed using radio-labeled glucose. Glycogen content was assessed by histochemistry. Lactate levels were measured enzymatically. Cell proliferation was assessed by WST-1 assay.

Results: We detected expression of key proteins for insulin signaling, such as insulin receptor β-subunit, insulin receptor substrat-1, Akt/protein kinase B and glycogen synthase kinase 3, in human astrocytes. Akt was phosphorylated and PI-3 kinase activity increased following insulin stimulation in a dose-dependent manner. Neither increased glucose uptake nor lactate secretion after insulin stimulation could be evidenced in this cell type. However, we found increased insulin-dependent glucose incorporation into glycogen. Furthermore, cell numbers increased dose-dependently upon insulin treatment.

Discussion: This study demonstrated that human astrocytes are insulin-responsive at the molecular level. We identified glycogen synthesis and cell proliferation as biological responses of insulin signaling in these brain cells. Hence, this cell type may contribute to the effects of insulin in the human brain.

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Introduction

It was known for many years, that the insulin receptor is widely expressed throughout the central nervous system (CNS) [1]. Despite that, the brain was still considered to be a mostly insulin-independent organ, since glucose uptake is not significantly stimulated by insulin [2]. However, in the last years, evidence for an important role of this hormone in various brain functions emerged [3,4]. Among others, central insulin actions were found to be involved in the regulation of body weight and food intake [4], in the processing of food-related stimuli [5] as well as in memory [6]. Many of these studies were done in vitro without knowing the responsible cell type for insulin’s actions; most of the in vitro work focused on specific neuron subpopulations.

However, besides neurons, there are many other cell types within the brain that may potentially contribute to the function of the whole organ [7]. Indeed, there are at least as many glial cells as neurons [8]. Among these, astrocytes are very interesting from a metabolic point of view: they take up glucose and store energy as glycogen. Even if the astrocytes’ glycogen content is low compared to ‘classical’ glycogen storage organs such as liver and skeletal muscle, it is of great importance for neuronal function [9].

Further on, astrocytes release lactate, which may be taken up by neurons as an energy source in times of need, i.e. during neuronal activity or in hypoglycemia [9–11]. However, there is still debate about the significance of this lactate shuffling from astrocytes towards neurons in vivo [11–13].

Astrocytes are also part of the blood-brain barrier [14] and exert a pivotal role in the regulation of cerebral blood flow and thus contribute to the regulation of neurons’ supply of nutrients and oxygen [15]. Furthermore, these cells take up and release various neurotransmitters, thereby modulating and terminating the action of...
transmitters secreted from neurons or communicating with other cells [16].

More globally, astrocytes were recently shown to be involved in a brain function that is also influenced by insulin: memory formation [17].

On a cellular level, astrocyte-enriched cultures from rodents were shown to form glycogen after stimulation with very high concentrations of insulin [18–20]. Literature on the issue whether insulin additionally stimulates glucose uptake into astrocytes is inconsistent [20,21]. Furthermore, increased cell growth in response to high insulin levels was demonstrated in rodent astrocyte-enriched cultures [19]. Recently, a possible role of the insulin signaling cascade for the regulation of glutamate transporter 1, an important molecule for glutamate uptake into astrocytes, has been demonstrated [22].

However, most of these functions were detected in rodent astrocytes. Recently, fundamental differences between rodent and human astrocytes were reported (e.g. in size, structural complexity and diversity, kinetics of activation) [23]. Oberheim et al. suggested that these differences between astrocytes from rodent vs. human origin may even explain some of the general differences between mice and men [23]. Thus, findings in rodent astrocytes can not generally be transferred to humans.

The aim of this study was, hence, to analyze in human primary astrocytes whether insulin signaling occurs, and if so, whether metabolic functions are influenced by the hormone.

Results

First, we examined the mRNA expression of major insulin signaling molecules in human astrocytes in comparison to human myotubes and human adipocytes: Astrocytes express insulin receptor in similar amounts as myotubes (figure 1 A). Roughly, two thirds of astrocytes’ insulin receptors are isoform A and around one third is isoform B (figure 2 A). Expression levels of insulin receptor substrate (IRS)-1 as well as IRS-2 were significantly higher in astrocytes than in the other cell types (figures 1 B and C). Glucose transporter (GLUT) 1 mRNA was found in significantly greater amounts in astrocytes than in the other cell types (figures 1 D and E). Glucose transporter (GLUT) 1 mRNA was found in significantly greater amounts in astrocytes than in the other cell types (figures 1 F).

Figure 1. mRNA expression in human astrocytes (black bars) in comparison to human myotubes (light grey bars) and human adipocytes (dark grey bars). (A) Insulin receptor, (B) IRS-1, (C) IRS-2, (D) GLUT1, (E) GLUT3, (F) GLUT4. mRNA expression was normalized for mRNA of the housekeeping gene Rps13. Bars represent means + SEM. N = 3. There were significant differences between the groups in all mRNA expressions analyzed (ANOVA, all p<0.0254) except for GLUT3 mRNA (ANOVA, p = 0.2). * indicates significant difference from astrocytes (Tukey Kramer post hoc test p<0.05).

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other analyzed cells (figure 1 D), while GLUT3 expression was in a comparable range in all three cell types (figure 1 E). Nominally, more GLUT2 mRNA than in adipocytes or myotubes was present in astrocytes, but around 70-fold less than that found in HepG2 cells (figure 2 B). GLUT4 was barely detectable in astrocytes and myotubes compared to adipocytes (figure 1 F).

At the protein level, we detected insulin receptor’s β-subunit, IRS-1, Akt, and glycogen synthase kinase (GSK) 3 β in astrocytes (figure 3 A).

To investigate, if the insulin-signaling cascade is functional and can thus be activated in human astrocytes, we performed a PI-3 kinase assay. Following insulin stimulation, we found increased phosphorylation of L-β-phosphatidylinositol towards phosphatidylinositol-3'-phosphate indicating increased PI-3 kinase activity. The insulin effect was dose-dependent and could be detected at concentrations as low as 1 nM (figure 3 C). Accordingly, Akt phosphorylation on serine 473 was increased by insulin stimulation, starting at concentration of 1 nM (figure 3 B). Increase in Akt phosphorylation and PI-3 kinase activity reached statistical significance at 50 nM (figure 3 B and C).

Further on, we studied, whether metabolic functions of human astrocytes are influenced by insulin: glucose uptake into cells was not affected by treatment with either 50 or 100 nM of insulin for 15 minutes (ANOVA p = 0.7, N = 5). Lactate content of the supernatants was neither influenced by stimulation with 50 nM insulin for 8 hours nor by variation of glucose concentration (figure 4).

However, when analyzing incorporation of labeled glucose into glycogen, we detected a significant increase following insulin stimulation. To test if this occurs via the classical PI-3 kinase/Akt pathway, we added the PI-3 kinase inhibitor LY294002 and found a significant decrease of glucose incorporation into glycogen of around 30% under basal conditions, and insulin-stimulated glycogen synthesis was abolished when LY294002 was added (figure 5 A). Thus, basal glycogen synthesis as well as the insulin effect on glycogen synthesis are PI-3 kinase-dependent.

We furthermore stained NHA cells for glycogen content before and after stimulation with 50 nM insulin for 3 h under two different glucose concentrations, 1 g/l and 4.5 g/l. Whilst barely any of the unstimulated cells contained detectable amounts of glycogen, some of the insulin-stimulated cells stained positive for glycogen (figure 5 B). There were no detectable differences between the glucose concentrations, neither in the stimulated nor in the unstimulated cells (figure 5 B).

Finally, we investigated cell proliferation following insulin treatment for three days by WST-1 assay. With increasing insulin dose, there was an increase in dye formation that reached statistical significance at a concentration of 50 nM (figure 6), indicating cell proliferation. The morphology of the NHA cells did not appear different between the insulin concentrations.

Discussion

In the present study, we could clearly demonstrate the presence of major insulin signal transduction molecules in human primary astrocytes. In addition, the insulin signaling cascade was shown to be functionally active in these cells. We found insulin to stimulate glycogen formation and cell proliferation, while glucose uptake and lactate secretion were unaffected by the hormone.

Insulin receptor was present in comparable amounts as in the other two tested insulin-sensitive cell types. Around two-thirds of the insulin receptors in astrocytes were the receptor’s isoform A. The expression of this isoform A in the human brain is well known [24]. In contrast to isoform B, this isoform has, besides its binding capacity for insulin, a high affinity for insulin-like growth factor 2 (IGF-2) [24]. Substantial amounts of IGF-2 are present in various regions of the human brain [25] – effects on astrocytes have not been studied in detail, yet. Furthermore, isoform A was found to confer mitogenic responses [26]. In agreement, insulin induced proliferation of astrocytes in our experiments.

Downstream of the insulin receptor, we detected both, IRS-1 and IRS-2 in human astrocytes. Both of them are important for insulin signaling within the brain: Genetic variation within the IRS-1 locus was shown to determine insulin responsiveness of the human brain [27] and partially deregulated IRS-2 signaling causes hyperphagia and obesity in animals [28].
For the glucose transporters, human astrocytes showed high GLUT1 and GLUT3 expressions, while only very little GLUT2 and almost no GLUT4 was detected. The first-mentioned two transporters are insulin-independent, while GLUT4 is regulated by insulin [29]. The expression pattern, hence, explains why we could not detect any effect of insulin stimulation on astrocytes’ glucose uptake.

Even if expressed nearly ubiquitous [29], GLUT1 is believed to be responsible for glucose transport across the blood-brain-barrier [30], a structure to which astrocytes contribute [14,15]. The importance of this transporter within the brain is underlined by rare genetic defects within this gene causing cerebral damages [31].

While in cultured rat astrocytes GLUT3 expression was only detectable after pretreatment with endotoxin or hypoxia [32], we detected GLUT3 in primary human astrocytes even under basal conditions. Since this transporter has a high glucose affinity [29], it might possibly serve as the major glucose transporter in human astrocytes.

In this study, we demonstrated the insulin signaling cascade to be functional in terms of increased PI-3 kinase activity and Akt Serine 473 phosphorylation in these cells. Increments in PI-3 kinase activity were already detected at an insulin concentration as low as 1 nM, concentrations that are commonly exceeded in the blood of healthy humans after food intake. Even if insulin concentrations in the cerebrospinal fluid are markedly lower than those in the plasma [33], astrocytes might be exposed to comparable concentrations in vivo due to their close contact with blood vessels [14,15].

After characterizing human astrocytes as an insulin-responsive cell type, we investigated whether these cells’ metabolic functions are influenced by the hormone: similar to other glycogen-storing cell types like hepatocytes or muscle cells, we found increased glycogen storage in astrocytes following insulin stimulation. This energy storage is important to support neurons with energy [10], since neurons can not store glycogen for themselves. But neuronal activity triggers the mobilization of astrocytes’ glycogen [34], probably via the release of neurotransmitters [35]. This energy
possibly contribute to insulin-mediated changes within the brain. Thereby insulin-stimulated glycogen storage in astrocytes could be permissive for stimulation of neuronal activation. Action increase the rapidly available amount of energy and thus support is necessary especially during intense neuronal activity [9–11,36]. Thus, larger glycogen stores in astrocytes due to insulin has not been studied yet. Our results point towards this possibility. Thus, this cell type might contribute to the effects of insulin in the human brain.

Methods

Cell culture

Normal Human Astrocytes (NHA) derived from fetal human brain are commercially available as cryopreserved, primary-derived cultures (Lonza, Basel, Switzerland). These cells are guaranteed to stain positive for GFAP (Giall Fibrillary Acid Protein), one marker for astrocytes. NHA were grown in AGM medium (Lonza) containing 3% fetal bovine serum, 4.5% glucose, and reagents from BulletKits (Lonza) in a humidified incubator at 37°C and 5% CO2. Prior to each experiment, cells were washed twice with PBS (Lonza) and starved in DMEM (1 g/l glucose, Lonza) +0.5% FCS for 48 h. The FCS used for our experiments contains 34.9 ng/ml IGF-1. Since our starvation medium contains 0.5% FCS this results in a concentration of 0.27 ng/ml (0.04 nmol/l) in the starvation medium. For insulin stimulation, human recombinant insulin was used (Novo Nordisk, Bagsward, Denmark).

Human myotubes and adipocytes were grown from primary precursor cells and differentiated in vitro as described earlier [40,41]. Human HepG2 hepatoma cells were grown in MEM medium containing 10% FCS and 2 mM Glutamine until 80% confluence.

Western blot

The following antibodies were used: Insulin receptor β-subunit polyclonal antibodies from own production (detect KKN GRI LTI, PRS NPS); IRS-1 antibodies (Millipore, Billerica, MA, USA); Pan-Akt antibodies (R&D Systems, Minneapolis, MN, USA); GSK3β antibodies (Cell Signaling, Danvers, MA, USA); Phospho-Akt (Serine 473, Cell Signaling, Danvers, MA, USA).

Glycogen was detected using the periodic acid-Schiff (PAS) reaction. Mayer’s hemalum solution was used as a counterstain.

Glucose uptake

Cells were incubated in Krebs-Ringer-HEPES buffer for 3 hours. Afterwards, they were stimulated with the indicated insulin concentrations for 15 minutes. Following stimulation, a mixture of ²-deoxy-[¹³C] glucose (0.4 µCi/well) and 0.1 M non-labeled ²-deoxyglucose was added for three minutes. The reaction was stopped by adding cold deoxyglucose.
**Figure 5. Effects of insulin stimulation on glycogen synthesis (A) and glycogen content (B) of human astrocytes.**

**A**

The indicated cells were preincubated with the PI-3 kinase inhibitor LY294002 for 1 hour. Cells were stimulated with the indicated concentrations of insulin for 3 hours. Glycogen synthesis in the absence of insulin and LY294002 was set as 100%. Bars represent means of at least five independent experiments $\pm$ SEM. There were significant differences between the groups (ANOVA, $p<0.0001$). * indicates significant difference (Tukey Kramer post hoc test $p<0.05$).

**B**

Prior to experiment, NHAs were either starved in medium containing 1 g/l glucose (left lane) or kept in medium with 4.5 g/l (right lane). Medium was then replaced by new medium with the same glucose concentrations without (upper panels) or with 50 nM insulin (lower panels). After three hours of stimulation, cells were stained for glycogen (pink). Shown are representative examples of at least three independent experiments.

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layer chromatography. 32P-labeled phospholipids were detected by chloroform/methanol (1:1) twice. Products were separated by thin autoradiography.

Cells were washed and harvested by trypsinisation. Cells were lysed with RLT and homogenized using QIAshredder (Qiagen, Hilden, Germany). Total-RNA was isolated using RNaseasy columns (Qiagen), treated with RNase-free DNase I and transcribed to cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany). PCRs (in duplicates) were performed on a LightCycler 480 (Roche Diagnostics) using Probes Master and fluorescent probes from the Universal Probe Library (Roche Diagnostics). The following real-time PCR protocol was used: denaturation program (95°C for 5 minutes), an amplification and quantification program repeated 45 times (95°C for 10 seconds, 60°C for 30 seconds, 72°C for 1 second [fluorescence acquisition], and finally a cooling down program to 4°C. Primers were designed using the Roche Probe Design 2 software (Roche Diagnostics) and purchased from TIB MOLBIOL (Berlin, Germany).

Insulin receptor isoform A was amplified using the following primers: forward TT TTT CGT CCC CAG AAA AAC CTC T, reverse CCACGCTCAGATTTCCCAAC. Insulin receptor isoform B was amplified using primers: forward TTT TGG TGC CCA GGC CAT, reverse CCA CCG TGC CAT TGC CAA C. Both reactions used 5′-6-FAM phosphoramidite-TGG CCA AGG GAG CTT CGT T-BBQ (4,4-Bis-[2-butyloctyloxy]-p-quaterphenyl) as a probe.

The other reactions used standard Roche probes and the following primers: Insulin receptor substrate [IRS]-1 forward GCC TAT GCC AGC ATC AGT TT, reverse TTG CTG AGG TAA TTT AGG TCT T; IRS-2 forward TGA CCT CTT GTC CCA CCA CTT, reverse CAT CCT GGT GAT AAA GCC AGA; insulin receptor forward GGT GGA TTA TTG CCT CAA AGG, reverse TGA GAA TCT TCA GAC TGG AAT GG; glucose transporter (GLUT) 1 forward GGT TGT GCC ATA CTC AGG, reverse CAG ATA GGA CAT CCA GGG TAG C; GLUT2 forward TGG TTT TGA CTT CTC, reverse CAT TCC AAC TAG AGA GAG GAA AGC T; GLUT3 forward GCC CCT AAA GTC CCA GAT TT, reverse TCT ATC ATC TCC TGG ATG TCT TGG; GLUT4 forward CTT GTC CAT CCT GAT CAG TG, reverse CGT AGC TGA TGG CTG CAA GT, RPS13 forward CAC TTT GGT GTT GAA GGT GA, reverse AGA CCA TGT GAA CTT AGG A. All RNA data is presented relative to the housekeeping gene RPS13 using the ΔΔCt method.

Glycogen synthesis

Cells were washed and harvested by trypsinisation. Cells were lysed with RLT and homogenized using QIAshredder (Qiagen, Hilden, Germany). Total-RNA was isolated using RNaseasy columns (Qiagen), treated with RNase-free DNase I and transcribed to cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany). PCRs (in duplicates) were performed on a LightCycler 480 (Roche Diagnostics) using Probes Master and fluorescent probes from the Universal Probe Library (Roche Diagnostics). The following real-time PCR protocol was used: denaturation program (95°C for 5 minutes), an amplification and quantification program repeated 45 times (95°C for 10 seconds, 60°C for 30 seconds, 72°C for 1 second [fluorescence acquisition], and finally a cooling down program to 4°C. Primers were designed using the Roche Probe Design 2 software (Roche Diagnostics) and purchased from TIB MOLBIOL (Berlin, Germany).

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Cell proliferation assay

The water soluble tetrazolium (WST)-1 assay was used to estimate astrocytes cell numbers according to the manufacturer’s instructions (Roche Molecular Biochemicals, Mannheim, Germany). The amount of formazan dye formed directly correlates to the number of metabolically active, viable cells. Equal amounts of NHA were seeded in each well of a 96-well plate. After starvation for at least 24 hours, cells were treated with insulin for 3 days.

Statistical analyses

For all statistical analyses, the software package JMP 8.0.2 (SAS Institute, Cary, NC, USA) was used. Two-group comparisons were performed using Student’s t-test. Differences between multiple groups were tested by ANOVA. Tukey-Kramer test was used as a post-hoc test. Results with values of p<0.05 were considered statistically significant.
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