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Protein targeting to glycogen is a master regulator of glycogen synthesis in astrocytes

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ABSTRACT

The storage and use of glycogen, the main energy reserve in the brain, is a metabolic feature of astrocytes. Glycogen synthesis is regulated by Protein Targeting to Glycogen (PTG), a member of specific glycogen-binding subunits of protein phosphatase-1 (PPP1). It positively regulates glycogen synthesis through dephosphorylation of both glycogen synthase (activation) and glycogen phosphorylase (inactivation). In cultured astrocytes, PTG mRNA levels were previously shown to be enhanced by the neurotransmitter noradrenaline. To achieve further insight into the role of PTG in the regulation of astrocytic glycogen, its levels of expression were manipulated in primary cultures of mouse cortical astrocytes using adenovirus-mediated overexpression of tagged-PTG or siRNA to downregulate its expression. Infection of astrocytes with adenovirus led to a strong increase in PTG expression and was associated with massive glycogen accumulation (>100 fold), demonstrating that increased PTG expression is sufficient to induce glycogen synthesis and accumulation. In contrast, siRNA-mediated downregulation of PTG resulted in a 2-fold decrease in glycogen levels. Interestingly, PTG downregulation strongly impaired long-term astrocytic glycogen synthesis induced by insulin or noradrenaline. Finally, these effects of PTG downregulation on glycogen metabolism could also be observed in cultured astrocytes isolated from PTG-KO mice. Collectively, these observations point to a major role of PTG in the regulation of glycogen synthesis in astrocytes and indicate that conditions leading to changes in PTG expression will directly impact glycogen levels in this cell type.

1. Introduction

Glycogen is the main energy reserve in the central nervous system (CNS) and is localized primarily in astrocytes (Magistretti et al., 1993a). At the physiological level, the role of glycogen as an energy reserve in energy deficiency has been well documented (see e.g. Magistretti and Allaman, 2015 and references therein). More recent evidence also points to an important role played by astrocytic glycogen in higher brain functions in mammals such as learning and memory (Boury-Jamot et al., 2016; Duran et al., 2013; Gibbs, 2016; Newman et al., 2011; Obel et al., 2012; Suzuki et al., 2011). In particular, an activity-dependent mobilization of glycogen associated with astrocytic lactate production and transfer to neurons is crucial for the establishment of long term memory in rodents in an inhibitory avoidance learning paradigm (Gao et al., 2016; Steinman et al., 2016; Suzuki et al., 2011). A direct correlate of such observations is that tight regulation of glycogen metabolism, and in particular the maintenance of a releasable glycogen pool, is crucial to sustain such physiological processes.

As in peripheral organs, cerebral glycogen mobilization and synthesis is under complex metabolic regulations involving both allosteric and covalent modifications and for which glycogen synthase (GS) and glycogen phosphorylase (GP) represent the two central elements (see e.g. Allaman and Magistretti, 2015; Obel et al., 2012; Roach et al., 2012). In particular, protein kinase-catalyzed phosphorylation inactivates GS and activates GP, resulting in

Abbreviations: PTG, Protein targeting to glycogen; GS, Glycogen synthase; GP, Glycogen phosphorylase; NA, Noradrenaline; Ins, Insulin.

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glycogenolysis and generation of glucose and glucose 6-phosphate whereas dephosphorylation of both enzymes leads to inactivation of GP and activation of GS allowing synthesis of glycogen from the precursor UDP-glucose. While phosphorylation of both GS and GP involves different set of kinases, protein phosphatase-1 (PPP1) is the enzyme responsible for dephosphorylation of both GS and GP. In order to be active, PPP1 must be bound to glycogen through PPP1 glycogen-binding subunits (Ceulemans et al., 2002; Korrodi-Gregorio et al., 2014). So far, at least seven distinct glycogen-binding subunit have been identified (PPP1R3A to G) which show specific cellular and tissue distributions (Ceulemans et al., 2002). The major ones include PPP1R3A (G_M) which is muscle-specific (Suzuki et al., 2001), PPP1R3B (G_L) which is the primary PPP1R3 expressed in the liver (Doherty et al., 1995), and the relatively widespread-ubiquitous isoforms PPP1R3C (PPP1R5 or PTG for protein targeting to glycogen) (Printen et al., 1997) and PPP1R3D (PPP1R6) (Armstrong et al., 1997). Interestingly, the gene encoding PTG is abundantly expressed in astrocytes of the CNS (Lovatt et al., 2007; Zhang et al., 2014), and several lines of evidence suggested that PTG plays an important role in the regulation of glycogen metabolism in this cell type (Allaman et al., 2000, 2003; Petit et al., 2013; Vilchez et al., 2007).

Astrocytic glycogen levels are under tight control by numerous glycogenolytic and glycogenic neuro-active substances including, noradrenaline (NA), VIP, insulin (Ins), insulin-growth factors I and II, glutamate, glucocorticoids, ATP or adenosine (see e.g. Brown, 2004; Magistretti et al., 1993a, 1993b; Obel et al., 2012). Regarding the maintenance of a releasable glycogen pool, NA and Ins represent interesting cases since both are known to promote long-term glycogen synthesis in astrocytes (Allaman et al., 2003; Dringen and Hamprecht, 1992; Hamai et al., 1999; Heni et al., 2011; Kum et al., 1992; Sorg and Magistretti, 1992). While NA and Ins glycogenic effects ultimately involve dephosphorylation and activation of GS (Allaman et al., 2004; Hamai et al., 1999), the activation cascades resulting in GS activation differ between the two agents (cAMP and PI3-kinase cascades for NA and Ins respectively). Interestingly, we have previously shown that NA, but not Ins, stimulates PTG mRNA expression concomitantly with glycogen resynthesis, suggesting PTG as the main mediator of glycogen resynthesis induced by NA.

In this study, we manipulated PTG expression in primary cultures, by up- or down-regulation, to determine its role in the regulation of glycogen content in astrocytes. Moreover, we aimed to address PTG involvement in the glycogenic action of NA and Ins.

2. Materials and methods

2.1. Reagents and antibodies

All chemicals and culture mediums, if not otherwise specified are from Sigma-Aldrich, Buchs SG, Switzerland.

2.2. Astrocytes cultures

Experiments were conducted in accordance with the Swiss Federal Guidelines for Animal Experimentation and were approved by the Cantonal Veterinary Office for Animal Experimentation (Vaud, Switzerland). Primary cultures of cerebral cortical astrocytes were prepared as previously described (Bélanger et al., 2011) from newborns (1-2 days-old) of OF1 mice (Charles River Laboratories, L'Arbresle, France) or heterozygous C57/Bl6j PTG KO mice (produced by A. A. DePaoli-Roach). For PTG KO astrocytic cultures, newborn pups from heterozygous breedings of C57/Bl6j PTG KO mice were genotyped by quantitative PCR. From each wild-type and knock-out homozygote mice, one littermate was selected per experiment and brain was dissected for astrocytic cultures. Cells were plated in 35 mm Ø dishes or glass coverslips and incubated in culture medium (Dulbecco's Modified Eagle Medium [DMEM] containing 25 mM glucose, Sigma-Aldrich, D7777) supplemented with 10% fetal calf serum, at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. The culture medium was renewed 3–5 days after seeding and subsequently twice a week. Experiments were performed on confluent 14 days-old cultures (DIV14) with the exception of immunofluorescence experiments which were performed on non-confluent (low density plated) cultures (DIV14) to facilitate cellular localization of Flag-PTG.

2.3. Small interfering RNA (siRNA) transfection

Specific PPP1R3C (PTG) downregulation in astrocytes was achieved using Stealth Select RNAi™ (siRNA, Ref. no. PPP1R3C/MSS284999; Invitrogen, Basel, Switzerland). Control groups (Mock siRNA) were transfected with siRNA negative controls (Hi GC complex #2, Invitrogen). For each 35 mm Ø dish, 100 pmoles siRNA was transfected following a previously described protocol (Bélanger et al., 2011). Each siRNA was applied to astrocytes for 4 days, followed by Western blot analysis, quantitative PCR and glycogen determination.

2.4. Adenovirus mediated Flag-PTG overexpression

To infect astrocytes, DIV9 cortical astrocytes (around 80% of confluency) were incubated in 1 mL serum free DMEM (Sigma-Aldrich, D5030) supplemented with (final concentrations) 5 mM glucose, 44 mM $NaHCO_3$, 0.06 g/L penicillin and 0.1 g/L streptomycin ($DMEM_5$). 1×10^6 Pfu of adenovirus containing Flag-tagged mouse PTG (Ad-Flag-m-PPP1R3C, ADV-269217 clone) or enhanced GFP (EGFP) (Ad-GFP used as control adenovirus, cat. n° 1060) both under the control of the CMV promoter, obtained by Vector BioLabs (Philadelphia, USA), were added directly in the dishes and incubated overnight. The next day, $DMEM_5$ medium was replaced by fresh culture medium. Experiments using adenovirus were conducted in biosafety level 2 laboratory and performed 5 days after infection. When indicated siRNAs were applied 24 hours after adenoviruses infection following the above-described protocol.

2.5. Cell treatment with noradrenaline and insulin

During all incubations, primary cultures of astrocytes were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. 16 h before treatment, the culture medium was removed and astrocytes were incubated in serum free $DMEM_5$. Without changing the medium, the cells were then exposed to noradrenaline (NA) or Insulin (Ins) for 6 h followed by glycogen determination. It was previously demonstrated that under these experimental conditions glucose concentration is not a limiting factor for glycogen synthesis induced by glycogenic agents (Allaman et al., 2003, 2004).

2.6. Quantitative PCR (qPCR)

Gene expression analysis: RNA isolation, cDNA synthesis and qPCR amplification were performed as previously described (Bélanger et al., 2011). Forward and reverse sequences were as follows: β-*Actin*: 5′-GCTTCTTTGCAGCTCCTTCGT-3′ and 5′-ATATCGTCATCCATGGCGAAC-3′; *Cyclophilin A*: 5′-CAAATGCTGGACCAAACACAA-3′ and 5′-GCCATCCAGCCATTCAGTCT-3′;*Protein phosphatase 1 regulatory subunit 3C* (*PPP1R3C or PTG*): 5′-GGCATGACGGAACTTGTCAA-3′ and 5′-TGCCTCTCGGTCCAATGAG-3′; *Glycogen Synthase 1, muscle* (*Gys1*): 5′-GCTGGACAAGGAGGACTTCACT-3′ and 5′-