Sodium valproate increases glycogen phosphorylase brain isoform: looking for a compensation mechanism in McArdle disease

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ABSTRACT

McArdle disease, also termed ‘glycogen storage disease type V’ is a disorder of skeletal muscle carbohydrate metabolism originated by inherited deficiency of the muscle-specific isoform of glycogen phosphorylase (GP-MM). It is an autosomic recessive disorder caused by mutations in the PYGM gene which typically presents with exercise intolerance, i.e., episodes of early exertional fatigue frequently accompanied by rhabdomyolysis and myoglobinuria. Muscle biopsies from these patients contain subsarcolemmal deposits of glycogen. Besides GP-MM, two other GP isoforms have been described: the liver (GP-LL) and brain isoforms (GP-BB), which are encoded by PYGL and PYGB genes, respectively; GP-BB is the main GP isoform found in human and rat foetal tissues including the muscle, although its postnatal expression is dramatically reduced in the vast majority of differentiated tissues with the exception of brain and heart where it remains as the major isoform. We developed a cell culture model from knock-in McArdle mice that mimics the glycogen accumulation and GP-MM deficiency observed in skeletal muscle from McArdle patients. We treated mice primary skeletal muscle cultures in vitro with sodium valproate (VPA), a histone deacetylase inhibitor. After VPA treatment, myotubes expressed GP-BB and a dose-dependent decrease in glycogen accumulation was also observed.

Thus, this in vitro model could be useful for high-throughput screening of new drugs to treat this disease. The immortalization of these primary skeletal muscle cultures could provide a never ending source of cells to obtain this experimental model. Furthermore, VPA could be considered as a gene expression modulator allowing compensatory expression of GP-BB and decreased glycogen accumulation in skeletal muscle of McArdle patients.
INTRODUCTION

McArdle disease, also termed glycogen storage disease type V (OMIM® number 232600), is a disorder of skeletal muscle carbohydrate metabolism originated by inherited deficiency of the skeletal-muscle isoform of glycogen phosphorylase (GP-MM). It is caused by pathogenic mutations in both copies of the GP-MM encoding gene (PYGM), which is located in chromosome 11q12-11q13. Owing to their inability to use glycogen for fuelling muscle contractions, patients commonly experience exercise intolerance, which typically consists of acute crises of early exertional fatigue, muscle stiffness and contractures which, in the most severe cases, can be accompanied by rhabdomyolysis and subsequent myoglobinuria, thereby increasing the risk of renal damage (Lucia et al., 2008).

Although undetectable GP-MM activity is the common (and in fact diagnostic) observation in the differentiated cells (i.e., fibers) obtained from patients’ skeletal-muscle biopsies, cultured muscle cells derived from their muscle biopsies present GP activity (Martinuzzi et al., 1993; Meienhofer et al., 1977). Further, human primary skeletal muscle cultures obtained from patients’ biopsies do not differ from controls in that there is no excessive accumulation of periodic acid Schiff (PAS) staining material, and thus no abnormal glycogen deposits (Martinuzzi et al., 1993). Yet other authors have failed to detect GP-MM in human primary skeletal muscle cultures obtained from patients or healthy controls (DiMauro et al., 1978; Sato et al., 1977).

Several types of treatments have been studied to reduce the symptoms in patients with McArdle disease, with different and controversial results. No significant beneficial effects have been reported in patients receiving nutritional supplements such as branched chain amino acids (MacLean et al., 1998), depot glucagon (Day and
Mastaglia, 1985), dantrolene sodium (Poels et al., 1990), verapamil (Lane et al., 1984), vitamin B6 (Phoenix et al., 1998) (except in one recent case report (Sato et al., 2012)), high-dose oral D ribose (Steele et al., 1996), or high dose creatine (Vorgerd et al., 2000). Low dose creatine conferred a modest benefit on ischemic exercise in a few number of patients (Vorgerd et al., 2000). The ingestion of simple carbohydrates before engaging in strenuous exercise can, alleviate their exercise intolerance symptoms and diminish the risk of muscle rhabdomyolysis (Vising and Haller, 2003), with supervised exercise training interventions also showing to be clinically beneficial (Haller et al., 2006; Mate-Munoz et al., 2007; Santalla et al., 2014). The Cochrane review of pharmacological and nutritional treatment for McArdle disease includes the evidence from randomized controlled trials for improving exercise performance and quality of life in McArdle disease (Quinlivan et al., 2010).

Because almost 50% of Caucasian McArdle patients carry the non-sense p.R50X mutation, treatment with drugs that could potentially induce ‘read through’ of the generated premature termination codon could help to re-express GP-MM activity; however, gentamicin treatment failed to normalize phosphorus (31P) magnetic resonance (indicators of GP-MM activity in the skeletal muscles of McArdle patients (Schroers et al., 2006). Gene therapy strategies have also been evaluated either in vitro, i.e., in human and sheep myoblasts cultures deficient for GP-MM or in vivo, i.e., in the ovine model of McArdle disease: while in myoblast cultures GP-MM activity was restored after the transfection with PYGM cDNA (Pari et al., 1999), intramuscular injection of adenovirus and adeno-associated vectors containing GP-MM expression cassettes in the ovine McArdle model only produced GP-MM functional activity in the surroundings of the injection site, and its expression diminished with time probably as a consequence of an immune response (Howell et al., 2008).
Additionally to GP-MM, two other GP isoforms have been described: the liver and brain isoforms, which are encoded by *PYGL* and *PYGB* genes, respectively. The brain isoform is the main GP isoform found in human and rat foetal tissues including the muscle, although its postnatal expression is dramatically reduced in the vast majority of tissues with the exception of the brain, where it remains as the major isoform. As described in the UCSC genome browser (http://genome-euro.ucsc.edu/index.html), both human, sheep and mouse *PYGB* genes present CpG islands in their promoters, and thus their expression might be regulated epigenetically through methylation of their promoters. In fact, postnatal down-regulation of gene expression has been reported for many genes containing CpG promoters (Numata et al., 2012). Thus, any pharmacologic treatment able to up-regulate the expression of *PYGB* in the skeletal muscle of McArdle patients could theoretically alleviate the symptoms of the disease.

Valproic acid (VPA) is a short chained fatty acid that has been used for many years in the treatment of epilepsy and bipolar disorders (McCoy et al., 1993; Sherard et al., 1980). Recent data suggest that this drug can modulate the epigenome by inhibiting histone deacetylases and activating the expression of methylated genes by stimulating active, replication-independent demethylation (Detich et al., 2003). The results of a recent study performed in McArdle sheep model, showed that enteral and intramuscular injection of VPA increased muscle expression of GP although glycogen deposits and the expression of specific GP isoforms at the muscle tissue level were not analysed (Howell et al., 2015).

As we have recently developed a *p.R50X* knock-in (KI) mice model which presents with the main clinical features of McArdle disease phenotype (Nogales-Gadea et al., 2012), we show here that primary skeletal muscle cultures derived from this
murine model constitute a valid in vitro model to analyse and evaluate potential treatments for the disease, as in contrast to what occurs with muscle cultures derived from patients, they do not present GP activity and accumulate high glycogen deposits; additionally, in these murine-derived muscle cultures we observed how VPA treatment increased the expression of Pygb as an alternative mechanism allowing to compensating, at least partly, for the lack of Pygm expression together with a reduction of polysaccharide accumulation.

RESULTS

*Homozygous p.R50X mice myotubes from primary skeletal muscle cultures accumulates glycogen.* We first analyzed the Pygm, Pygb and Pygl expression at undifferentiated (myoblasts) and differentiated (myotubes) stages development in skeletal muscle cultures derived from wild type (WT) and KI McArdle mice. Myoblasts from WT and both myoblasts and myotubes from KI mice did not have Pygm mRNA (Fig. 1A). In WT muscle cultures, Pygm mRNA levels increased with muscle differentiation (p<0.001) (Fig. 1A). No significant differences were observed in Pygb mRNA levels among myoblasts and myotubes from WT and KI muscle cultures (Fig. 1B). Neither myoblasts nor myotubes from WT and KI had Pygl mRNA (data not shown). Western blot (WB) confirmed the mRNA results as Pygm was only present in WT myotubes (Fig. 1C). Pygb was not detected by western blot analysis, in WT/KI myoblasts or in WT/KI myotubes (data not shown).

We also performed a PAS staining in skeletal muscle cultures from WT and KI mice. We observed that KI myotubes accumulated glycogen (Fig. 1G) whereas both WT and KI myoblasts and WT myotubes did not (Fig. 1D, E, F).
Myotubes treated with VPA during 72h expressed Pygb and reversed the glycogen accumulation. WT and KI myoblasts and myotubes were treated during 72 hours with VPA at 1, 2 and 5 mM. WT and KI isolated myoblasts treated with VPA did not increase Pygb mRNA expression (data not shown). However, when VPA was added to the confluent myoblasts and differentiated myotubes, we observed an increased expression of Pygb mRNA presence of mRNA, both in WT and in KI muscle cultures (p<0.01 and p<0.05 respectively). The highest amount of Pygb protein expression was observed in myotubes treated with 2mM of VPA (Fig. 2). After 72 hours of VPA treatment no presence of myotubes detached from the plate was observed, indicating that VPA was not toxic for the muscle culture (data not shown). The PAS staining on treated and non-treated myotubes, showed a reduction in glycogen accumulation in cultures treated with 2 and 5 mM VPA (Fig. 3A). The heat map represented hot colours indicative of high glycogen concentration while cold colours such as green and blue showed the zones with no glycogen accumulation (Fig. 3B). To test if a long period of VPA treatment could be toxic for the muscle cells, we changed the culture medium with or without VPA every 72 hours during 12 days. After the 12-day period, the only muscle cultures which were maintained at a healthy myotube stage were muscle cultures treated with 2mM of VPA (Fig. 4).

DISCUSSION

The primary skeletal muscle cultures derived from p.R50X KI mice represent a good cellular model of McArdle disease as they mimic the glycogen accumulation that is commonly found in the skeletal muscle from McArdle patients (Lucia et al., 2008). It has been previously reported that in human culture cells either from patients or healthy individuals, PYGM expression poorly contributed to the total glycogen phosphorylase
mRNA, while *PYGB* expression was predominant in myoblasts and *PYGB* and *PYGL* were both expressed in myotubes (Nogales-Gadea et al., 2010). As *PYGB* and *PYGL* were the main GP isoforms expressed in human cultures, an increase in glycogen deposits was not observed in skeletal muscle cultures derived from McArdle patients (Nogales-Gadea et al., 2010). On the contrary, neither *Pygb* nor *Pygl* were expressed at any differentiation stage in our WT or KI mice cultures, while GP-MM expression was restricted to WT myotubes similarly to what has been shown to occur in the skeletal muscle of human adult healthy individuals. Additionally, vacuolization and increased intracellular PAS staining was also observed in KI mice cultures. Thus, primary skeletal muscle cultures derived from KI mice, might represent a useful tool to test different pharmacologic therapies prior their evaluation in in vivo models.

A natural ovine model of McArdle disease has been described, which is characterized by an adenine for guanine substitution at the intron 19 acceptor splice site of the *Pygm* gene (Tan et al., 1997). These sheep exhibit clinical features and morphological changes at the muscle tissue level that are similar to those shown by patients (Tan et al., 1997). In this animal model, regeneration of muscle fibers after necrosis induced by notexin injection reduced glycogen storage in regenerating muscle fibers, which showed re-expression of non-muscle (liver and brain) isoforms of GP, thereby indicating the need to investigate the potential functional benefit of inducing at the muscle tissue level these normally latent isoforms (Howell et al., 2014).

VPA exerts significant inhibitory effects on the activity of glycogen synthase (GS) kinase 3 beta (GSK3β) both in vitro and also in vivo (i.e., on endogenous GSK3β) (Chen et al., 1999). As the inhibition of GSK3β might generate the accumulation of more active unphosphorylated form of the glycogen synthase enzyme, with its consequent increase in glycogen accumulation, we did not analyzed GSK3β activity in
VPA treated and untreated mice KI cells, as on the contrary, we observed a great reduction in glycogen accumulation in vitro, reaching normal levels. Additionally, it has also been observed that VPA has beneficial effects on skeletal muscle myotubes, activating Akt signalling, stimulating gene transcription, protein synthesis and promoting the survival of the cells via inhibition of apoptosis (Gurpur et al., 2009). We observed these “beneficial” effects only when muscle cultures were treated with 2mM VPA concentration during a long period of time (12 days).

VPA induces histone acetylation of H3 histones, and is involved in the regulation of methylated genes by increasing the accessibility of the enzyme *demethylase* to the DNA (Detich et al., 2003; Milutinovic et al., 2007). The potential demethylation treatment of CpG islands of *PYGB* promoter, could allow the activation and transcription of *PYGB* gene in skeletal muscle from McArdle patients as an approach to compensate for the lack of GP-MM. VPA treatment in humans could potentially have more effects on the glycogen accumulation because of the longer VPA half-life, compared with mice (Loscher, 1978).

Our results demonstrate that mice primary skeletal muscle culture is a good study model of the disease as it mimics the phenotype of the patients muscle tissue. Furthermore, VPA can enhance *PYGB* expression in vitro and may be a candidate for the treatment of McArdle disease. Additional preclinical studies are needed to optimize the drug dosage that most effective to modulate *PYGB* gene expression.

**MATERIAL AND METHODS**

All experimental procedures were approved by the animal care and use committee of the *Vall d’Hebron Institut de Recerca* (CEEA 35/04/08), and were in accordance with the European Convention for the Protection of Vertebrate Animals used for

**Mice skeletal muscle cultures**

Myogenic precursor cells were isolated from 8 weeks-old KI McAdle mice carrying the p.R50X mutation in both copies of the *PYGM* gene (Nogales-Gadea et al., 2012) and from WT mice. All the muscles from lower limbs were dissected and digested in a 0.2% pronase A (Calbiochem, Darmstadt, Germany) during 1 hour at 37°C. After two washes with Dulbecco's Modified Eagle's Medium (D-MEM, Lonza Group Ltd., Basel, Switzerland), supplemented with 10% FBS (Lonza) and 100 units/ml penicillin (Lonza), 100 μg/ml streptomycin (Lonza) and 0.25 μg/ml amphotericin (Lonza) (PSF), cells were seeded in a non-coated 100mm petri-dish in HAM-F10 media (Lonza) supplemented with 20% FBS, 2 mM glutamine (Lonza) and PSF during 1 hour to allow fibroblasts to adhere. Thereafter, supernatants were plated in 2% gelatin-coated dishes containing HAM-F10 growth media supplemented with 20 ng/ml bFGF (Peprotech, Rocky Hill, NJ, USA). 2500 cells/cm² were seeded in growth media to obtain myoblasts at day 7. To obtain myotubes 12,500 cells/cm² were seeded in growth media and after 48 hours, and the culture media was replaced by for one containing D-MEM, 5% horse serum (Lonza), 2 mM glutamine and PSF to allow myoblasts to fuse and to form myotubes. Myotubes were analyzed after 5 days in differentiation medium. The medium was changed twice a week, and the muscle cultures were examined to confirm confluent growth of myoblasts and myotubes. Each condition was performed in triplicate.
VPA treatment in cell cultures

After 48h in differentiation media where myotubes were formed, we added VPA (Sigma-Aldrich, Madrid, Spain) at different concentrations (1, 2 and 5 mM) during an additional 72-hour period.

RNA extraction and real-time polymerase-chain reaction (PCR)

Total RNA from mice skeletal muscle cultures treated or not with VPA was extracted using Trizol (Life Technologies, Madrid, Spain). A 0.5 μg of total RNA was DNAse treated (Life Technologies, Madrid, Spain) and then was reverse-transcribed into cDNA using High capacity cDNA RT kit (Life Technologies, Madrid, Spain).

Quantification of PYGM, PYGB, PYGL and GAPDH (used as an internal standard) transcripts was performed using TaqMan Universal Master Mix technology (Life Technologies). Quantitative PCR was performed in a total reaction volume of 20 μl per well. The primers used for real time PCR were designed by Applied Biosystems (Roche Molecular Systems) (Pygm Mm 00478582_m1, Pygb Mm 00464080_m1, Pygl Mm 01289790_m1 and mouse Gapdh endogenous control). The comparative CT method (ΔΔCT) for relative quantification of gene expression was used. The Student’s t test was used for statistical comparisons between the data obtained in KI vs. WT mice.

Western Blot

Cell and muscle samples corresponding to each experimental condition were trypsinized from the culture dish and homogenized with a lysis buffer containing 40mM glycophosphate, 40mM NaF, 10mM EDTA and 20mM of β-mercaptoethanol (final pH= 6). The samples were placed in boiling water for 3 minutes and centrifuged at 9500×g for 3 minutes, before 100 μg of protein was applied to each lane. Unspecific
binding sites on the blots were blocked by incubation in 5% low fat dried milk powder in a phosphate buffer saline. Thereafter primary rabbit polyclonal antibody anti-PYGB (kindly provided by Dr. K. Nowak), the primary goat anti-PYGM (kindly provided by Dr. Martinuzzi), mouse anti-GAPDH (Ambion, Life technologies) or anti-β-actin (Sigma-Aldrich) were added. Peroxidase-conjugated anti-rabbit (Jackson Immunoresearch, West Grove, PA, USA), peroxidase-conjugated anti-goat (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and peroxidase-conjugated anti-mouse secondary antibodies (Dako, Glostrup, Denmark) were applied when using anti-PYGB antibody, anti-PYGM antibody and anti-GAPDH and anti-β-actin respectively.

Periodic acid Schiff (PAS) staining in cultured cells

Both cell cultures treated or not treated with VPA were incubated with 1% of periodic acid (Sigma-Aldrich) in acetic acid for 30 minutes. Cells were washed in 0.1% sodium metabisulfite (Sigma) in 1mM hydrochloric acid. Thereafter they were incubated in a Schiff solution (Merck, Darmstadt, Germany) for 15 minutes after 0.1% sodium metabisulfite in 1mM hydrochloric acid wash, and cells were observed in an inverted Olympus FSX100 microscope.

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Author contribution: NdL and TP conceived and designed the experiments. NdL, JMG and AB performed the experiments and analysed the data. AL, MAM, JA, RM and ALA contributed to manuscript edition by thoroughly revising the manuscript and providing critical and intellectual suggestions. NdL, ALA and TP wrote the manuscript.
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**FIGURES**

![Figure 1](image_url)

**Figure 1.** *Differential glycogen phosphorylase expression in mice primary skeletal muscle cultures.* Only WT myotubes expressed *Pygm* mRNA (A). Myoblasts and myotubes from WT and KI expressed *Pygb* mRNA with a high variation and no statistically significant differences between WT and KI (B). Presence of both *Pygm*
transcript and protein levels was observed only in KI myotubes (C). PAS staining of WT myoblasts (D), WT myotubes (F), KI myoblasts (E) and KI myotubes (G): only KI myotubes accumulated high glycogen levels (G). Abbreviations: KI, knock-in; WT, wild-type. Symbols: *p<0.05 for the comparison KI vs. WT, **p<0.001 for the comparison of KI vs. WT). Scale bar 50 µm.

Figure 2. VPA treatment increase Pygb expression in mice skeletal muscle cultures. (A) After myotubes were formed, muscle cultures were treated with different concentrations of VPA during 72 hours. (B) In both cell cultures (WT and KI), Pygb mRNA increased in treated cultures (**p<0.01, *p<0.05). (C) Pygb protein was also detected with western blot analysis in WT and in KI cell cultures. Abbreviations: D-MEM, Dulbecco's Modified Eagle's Medium; KI, knock-in; VPA, valproic acid; WT, wild-type.
**Figure 3.** Reduced glycogen accumulation in KI myotubes after treatment with VPA (A, B). We observed a gradual reduction in glycogen content as myotubes were treated with growing VPA concentrations. (B) Red areas correspond with sites of major glycogen accumulation while ‘cold’ (green, blue) represent sites of low PAS staining in myotubes. Scale bar 50 µm.

**Figure 4.** Long term VPA treatment of muscle cultures. After 12 days of treatment only cultures treated with 2mM VPA showed a healthy myotubes.
TRANSLATIONAL IMPACT

Background

McArdle disease is a disorder of skeletal muscle carbohydrate metabolism originated by inherited deficiency of muscle glycogen phosphorylase (GP-MM). This enzyme catalyzes and regulates the breakdown of glycogen into glucose-1-phosphate in muscle fibers. Thus, patients are unable to obtain energy from their muscle glycogen stores and, as such, present with exercise intolerance, typically manifested as acute crisis of undue, early exertional fatigue, muscle stiffness and contractures, which in the more severe can be accompanied by rhabdomyolysis and subsequent myoglobinuria.

There are three in vivo animal models of the disease (two naturally occurring and one generated by us): the first animal described was a Charolais calf, the second was a Merino sheep in Western Australia and the third was a recently developed knock-in (KI) mice model carrying the commonest mutation in PYGM gene (p.R50X), which closely mimics the human disease phenotype. There is yet not a reported cellular model that mimics in vitro the main biochemical and histological phenotype that is typically observed in the differentiated muscle cells (i.e., fibers) obtained from patients’ muscle biopsies, that is: absence of GP-MM and glycogen accumulation.

Results

We have analysed the expression of different GP isoforms (brain (GP-BB), liver (GP-LL) and GP-MM) in cultured cells at different stages of differentiation which were previously obtained from skeletal muscles of KI (GP-MM deficient) or wild-type mice. We have observed that GP-MM was the only GP isoform that was expressed in vitro in the differentiated cells obtained from wild-type mice, while GP-MM was not expressed in the differentiated cells from the KI mice. In addition, only differentiated cells from
the KI mice accumulated glycogen in their cytoplasm, a characteristic (and in fact
diagnostic) trait that occurs in the fibers of muscle biopsies obtained from McArdle
patients. This in vitro model was used to assess if sodium valproate (VPA) can reverse
the muscle phenotype from a McArdle-like to a normal histological and biochemical
profile. VPA activated the expression of only one GP isoform, GP-BB, in differentiated
muscle cultures, yet this isoform appeared to have functional consequences as it
decreased intracellular glycogen accumulation.

Implications and future directions

These results demonstrated that mice primary skeletal muscle cultures from McArdle KI
mice represent a useful model in vitro, allowing to use high-throughput screening
techniques for testing new drugs aiming at restoring GP activity, at least partially, in the
skeletal muscle tissue of McArdle patients.

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