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Galactose and Hexose 6-Phosphate Dehydrogenase Support the Myelin Metabolic Role.

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ABSTRACT

We have previously reported that myelin is a site of an extra-mitochondrial oxidative phosphorylation. Isolated myelin conducts glucose oxidation,expressing glycolytic and Krebs cycle enzymes.Galactose, whose metabolism is high in brain, is known to incrementthe cell oxidative burst. It is also a component of the glycosyl-moiety of brain lipids.

Here we studied galactose metabolism and hexose6-phosphate dehydrogenase (H6PD), a membrane-boundenzyme active on many phosphorylated and non-phosphorylated hexoses,in myelin. In the presence of galactoseboth ATP synthesis and oxygen consumption by isolated myelin werehigher than in the presence of other respiring substrates. Western blot analysisand spectrophotometric assays show the functional expression of H6PD in isolated myelin. H6PD is proposed to producereducing equivalents that would directly feed the respiratory Complex I expressed in myelin.Galactose may be an exclusive oxidative substrate for the myelinated parts of the nervous system.

KEYWORDS Galactose; Hexose 6-phosphate Dehydrogenase; Myelin Sheath; Oxidative Phosphorylation.

INTRODUCTION

Myelin plays an important role in the axonal survival (Nave, 2010) asits loss causesaxonal degeneration and imbalance in energy supply (Lubetzki and Stankoff, 2014). We have shown that isolated myelin (IM), a subcellular fraction naturally devoid of mitochondria, conducts an extra-mitochondrial oxidative phosphorylation (OXPHOS), utilizing several respiratory substrates (Ravera et al., 2015a, 2015b, 2014, 2011, 2009; 2013a, 2013b). IM expresses the enzymes of glycolysis, Krebs cycle and the Electron Transfer Chain (ETC) (Ravera et al., 2015b, 2014, 2011, 2009; 2013a, 2013b). ATP produced by myelin could sustain the energy request of the axon, with the involvement of connexons(Adriano et al., 2011; Ravera et al., 2015a).

Gal metabolism in brain was reported to beconsistent(Roser et al., 2009). Gal can boost the mitochondrial OXPHOS (Perciavalle et al., 2012), also augmentingOXPHOS proteins expression(Rossignol et al., 2004). Galis a substrate of hexose 6-phosphate dehydrogenase (H6PD, E.C. 1.1.1.47),a bi-functional microsomal enzyme startingan endoluminal pentose phosphate pathway (Ozols, 1993). H6PD isdistinct from cytosolic Glucose-6-phosphate dehydrogenase (G6PD), as it canoxidize several phosphorylated and non-phosphorylated hexoses (Hewitt, et al., 2005) and can also use NAD+ as substrate (Beutler and Morrison, 1967), producing NADH or NADPH+ H+.Interestingly, H6PD gene has been indicated as a risk gene for Multiple Sclerosis (MS)(Alcina et al., 2010), suggesting that this enzyme plays an important role in myelin. Here we studied the role of galactose and H6PD in myelin energy metabolism.

MATERIALS& METHODS

Myelin isolation

Isolated Myelin (IM) was obtained from bovine forebrain by the 'floating up' sucrose gradient method(Ravera et al., 2009).Protease inhibitor cocktail (Sigma–Aldrich) 50 µg/ml 5-fluorouracil and 20 µg/ml ampicillin were present throughout isolation.

Electrophoretic separation of IM and SemiquantitativeWestern Blot

Denaturing electrophoresis was performed on a gradient 4-16% SDS-PAGE(Silvia Ravera et al., 2013). After blot, nitrocellulose membrane were labeled with specific antibodies (Ab_s), o.n at 4°C, following the dilution indicated on datasheets. Binding of secondary HPR-conjugated Ab_swasrevealed by enhanced chemiluminescence (Roche), and acquired with ChemiDoc (BioRad) whichconverted bandsinto a densitometric trace. Signals were compared to the signal of stained protein lane, as Relative Optical Density (R.O.D.)(Ravera, et al., 2009).

O_2 consumption assay in IM

IM respiratory rates were measured with an amperometric O_2 electrode in a closed chamber (1.7 ml volume), magnetically stirred at 37 °C, as previously described(Ravera, et al., 2009, 2011, 2013a, 2013b, 2014). Substrates were: 5 mM pyruvate plus 2.5 mMmalate, or 5 mMGlu or5 mMGal, plus inhibitors of Complex I and III (40 M rotenone and 50 µM antimycin A, respectively).

ATP synthesis assay in IM

ATP synthesis in IM was measured at 37°Cover2 min on 20 µg ofIM proteinby the luciferin/luciferase method (Ravera, et al., 2009, 2011).The metabolic substrates were: 5 mM pyruvate

plus 2.5 mM malate, 5 mMGluor 5 mM Gal.

Spectrophotometric assay of H6PD activity

H6PD activitywas assayed at 37°C on 50 µg of IM, following the reduction of NADP at 340 nm, and expressed as mIU/mg of protein (nmol/min/mg of protein). NADPH molar extinction coefficient was considered 6.22 mM^{-1} cm^{-1}. Substrates (10 mM) were:Gal, Glu, Gal6-phosphate, Glu6-phosphate, 2-deoxyGlu, 2-deoxyGlu 6-phosphate.

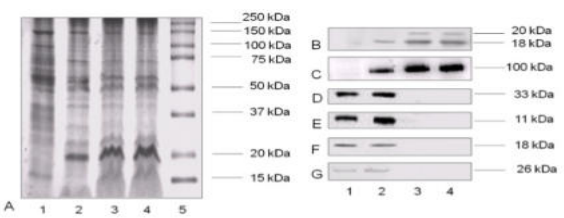
Complex I activity

To asses if NADPH (NADH) produced by H6PD could use directly by the myelin OXPHOS machinery, Complex I was assayed spectrophotometrically at 420 nm, following the reduction of ferricyanide, after the addition of 20 mM galactose.

RESULTS

Isolated myelin characterization

Figure 1 is a WB analysis of forebrain-derived samples and mitochondria enriched fractions(control). Panel A shows the protein pattern of the samples. Panels B and C reportthat the signal ofMBP and MAG, two typical myelin proteins,increased in parallel with myelin isolation. Panels D-G show that ANT, TIM, TOM, and AK3wereundetectable in IM, suggesting the absence of mitochondrial contamination in IM. Panel H shows the densitometric analysis of signals normalized against the protein signal of each lane(mitochondria and IM do not display anycommon housekeeping protein).



H

	Mitochondria enriched fraction (Lane 1)	Forebrain Homogenate (Lane 2)	Crude Myelin (Lane 3)	Isolated Myelin (Lane 4)
Coomassie (Panel A)	14.5	13.9	13.4	13.1
MBP (20 kDa) (Panel B)	0	0	2.5	2.9
MBP (18 kDa) (Panel B)	0	4.2	6.8	8.2
MAG (Panel C)	0	25.3	36.9	43.1
ANT (Panel D)	22.2	20.1	0	0
TIM (Panel E)	21.8	23.4	0	0
TOM (Panel F)	5.6	4.9	0	0
AK3 (Panel G)	2.7	2.4	0	0

Figure 1:Characterization of isolated myelin (IM). Panel A: protein pattern (lane 1, mitochondria-enriched fraction; lane 2, forebrain homogenate; lane 3, crude myelin fraction; lane 4, isolated myelin fraction; lane 5, molecular weight markers), stained with Colloidal Blue Coomassie. Panels B-G : semiquantitative WB of MBP, MAG, ANT, TIM, TOM and AK3, respectively, representative of at least ten experiments. Densitometry is in Panel H, expressed as Relative Optical density (R.O.D.).

OXPHOS activity in IM

Figure 2 shows O_2 consumption by IM in the presence of Pyr/Mal(Panel A), Glu (Panel B) or Gal(Panel C), sensitive to Rotenone, Complex I inhibitor.Mean O_2 consumption is in Panel D.An ATP synthetic activity was assayed in the presence of Pyr/Mal, Glu or Gal (Figure 3).Gal stimulated the highest respiratoryratesas well as the highestATP production, consistently with the report that Galis able to enhance the OXPHOS activity in mitochondria (Aguer et al., 2011).

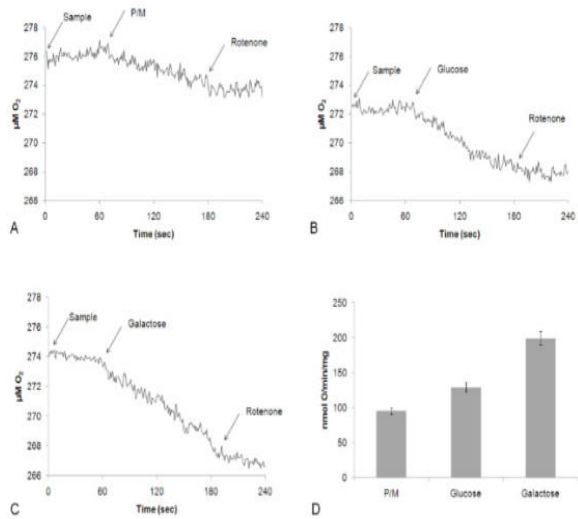


Figure 2: Oxygen consumption in IM. Amperometric tracings of respiratory rates in IM, induced by Pyr/Mal, Glu or Gal. In each case the respiration was sensitive to rotenone (Panels A-C). Panel D shows a comparison of the O_2 consumption in the presence of Pyr/Mal, Glu or Gal. Data are expressed as nmol O/min/mg± S. D.Each panel is representative of at least six experiments.

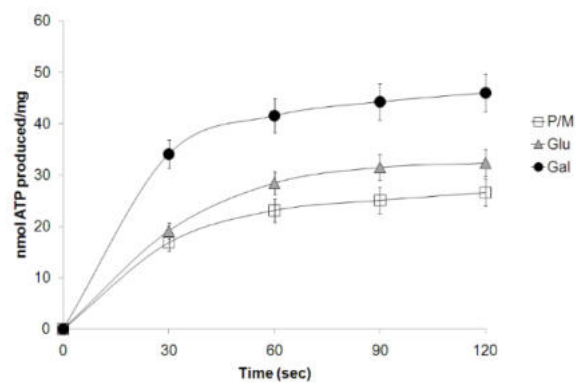


Figure 3: ATP synthesis in IM. Time course of ATP synthesis in IM, with Pyr/Mal(squares), Glu (triangles) or Gal (circles). Data are expressed as nmol ATP produced/mg of total proteins ± ST DV and represent six experiments.

H6PD activity in IM

WB (Panel A) shows that H6PDsignal was present in all samples(Figure 4).Densitometry shows that the signal in IM is similar to that in forebrain homogenates, suggesting a major contributionfrom the sheath to H6PD expression in brain. NADP was reduced by all substrates(Glu 6-phosphate (G6P), Gal 6 – phosphate, Glu, Gal, 2-deoxyGlu or 2-deoxyGlu 6-phosphate), even thoughNADPH formation in the presence of Glu 6-phosphate was higher with respect to that obtained with the other substrates (G6PD activity). Similar data wereobtained using NAD^+ in place of NADP (data not shown).

Moreover, the Complex I assay in the presence of Gal and NADP (Figure 5) demonstrates that the NADPH produced by H6PD could sustain directly the OXPHOS metabolism, bypassing glycolysis and Krebs cycle.

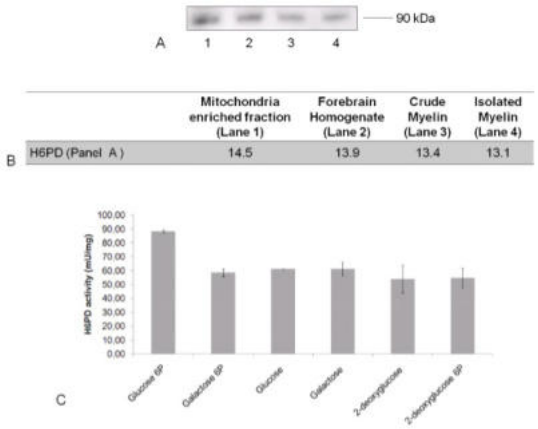


Figure 4: H6PD activity in isolated myelin. Panels A and B show the WB analysis against H6PD and the relative densitometric analysis, respectively. Panel C reports the activity of H6PD in the presence of several substrates. Data are expressed as mU/mg of total proteins ± ST DV and are representative of at least five experiment.

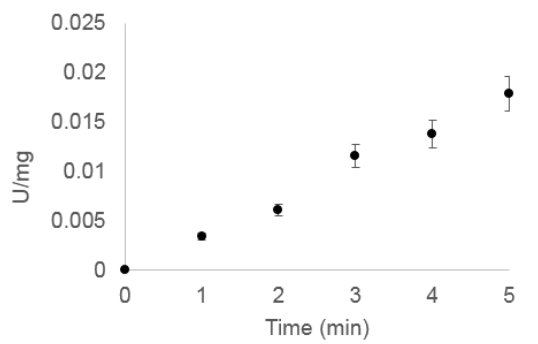


Figure 5: Complex I activity after stimulation with Galactose. The graph shows the time course of the Complex I activity, in the presence of Gal and NADP. This data suggests a direct link among H6PD and ETC in IM.

DISCUSSION

In this paper,a Gal-induced extra-mitochondrial OXPHOSand the role of H6PD were investigatedin IM.IM effectively utilizes Gal as a substrate for aerobic metabolism.Gal passes the blood brain barrier, entering the nerve cell across insulin-independent GLUT3 (Olson and Pessin, 1996). The presence of H6PD, a microsomal enzyme, in IM is consistent with the hypothesis that the OXPHOS machinery is transferred from mitochondria to myelin through a fusion with endoplasmic reticulum (S Ravera et al., 2013a).

As both H6PD and Complex I are expressed in myelin, H6PD may be coupled to the respiratory Complex I,so that NAD(P)H produced (Beutler and Morrison, 1967)by its activity on Gal would enter directly in the ETC, sustaining the extra-mitochondrial OXPHOS in myelin (see Figure 6)(Ravera et al., 2014, 2011, 2009; S Ravera et al., 2013a, 2013b).

Figure 6: Galactose pathway in myelin sheath. Possible Gal pathway in myelin and oligodendrocytes. Gal would be oxidized by H6PD to galactonate, and to xylulose. Pyruvate would be used by Krebs cycle in myelin (S Ravera et al., 2013a), while G3P may be utilized for the synthesis of lipids. Enzymes are identified following the Enzyme Classification (EC) numbering.

Measurements of galactose metabolites conducted in various rat organs of revealed that especially brain can take up and metabolize galactose (Roser et al., 2009). D-gal appears to be a hormetic sugar.In fact, low oral doses are beneficial on learning and memory, and Gal was supposed to represent an "alternative source of energy in brain" (Salkovic-Petrisic et al., 2014), while subcutaneous administration of high doses (up to 500 mg/Kg) of D-gal in rats cause severe brain oxidative stress (Lei et al., 2008). The fact that excess D-gal increases free radical production in brain is consistent with D-gal boosting OXPHOS in brain, but less with the general belief that it is transformed into Glu in liver (Bauer et al., 1976).

In conclusion, Galwould be an exclusive aerobic metabolic substrate specific for myelin, where it would be converted by H6PDto NADH/NADPH and respiring substrates, functionally coupled to the ETC complex I, ectopically expressed in myelin.

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