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Research Paper

Paradoxical Inhibition of Glycolysis by Pioglitazone Opposes the Mitochondriopathy Caused by AIF Deficiency

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ABSTRACT

Mice with the hypomorphic *AIF-Harlequin* mutation exhibit a highly heterogeneous mitochondriopathy that mostly affects respiratory chain complex I, causing a cerebral pathology that resembles that found in patients with AIF loss-of-function mutations. Here we describe that the antidiabetic drug pioglitazone (PIO) can improve the phenotype of a mouse Harlequin (Hq) subgroup, presumably due to an inhibition of glycolysis that causes an increase in blood glucose levels. This glycolysis-inhibitory PIO effect was observed in cultured astrocytes from Hq mice, as well as in human skin fibroblasts from patients with AIF mutation. Glycolysis inhibition by PIO resulted from direct competitive inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Moreover, GAPDH protein levels were reduced in the cerebellum and in the muscle from Hq mice that exhibited an improved phenotype upon PIO treatment. Altogether, our results suggest that excessive glycolysis participates to the pathogenesis of mitochondriopathies and that pharmacological inhibition of glycolysis may have beneficial effects in this condition.
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1. Introduction

Mitochondrial (mt) disorders represent an expanding group of diseases characterized by wide variability in clinical presentation and course (Turnbull and Rustin, 2015). Our understanding of these pathologies remains limited despite the elucidation of a huge number of the underlying gene defects. With a few exceptions (*e.g.* primary CoQ_{10} deficiency), no therapy can currently be offered to the patients and hardly any clinical trial has led to reliable and convincing conclusions (Koopman et al., 2016). This depends in part from the difficulty to collect sufficiently large cohorts of patients with a homogeneous genetic defect and similar clinical presentation. In this context, the use of animal models may provide a clue for identifying and testing therapies. However, in spite of extensive studies particularly on murine models, very few candidate drugs have shown some positive effects in subsequent human trials (Hackam and Redelmeier, 2006). A number of reasons have been advocated to account for this failure, and one of them may be disparity of clinical phenotypes between humans and mice (Rice, 2012). In addition, the extreme variability in clinical presentation and course is observed even within the same family, which suggests a relevant, albeit still unclear, role of additional genetic, epigenetic and environmental factors in the natural history of these conditions (Jain et al., 2016). Accordingly, the genetic background should be taken into account in modelling specific mt diseases (Benit et al., 2010 #4255). For instance, studies on mouse models are usually carried out on highly selected, inbred, isogenic individuals. This may be useful in the elucidation of disease mechanism or in investigating the function of a disease gene, but is inadequate to test drug efficacy in a clinically relevant (and hence heterogeneous) setting. In addition to these genetic considerations, the nursing conditions, such as cage constraint, reduced exercise, idleness, and *ad libitum* feeding, and the improper conception and execution of some studies (Couzin-Frankel, 2013), all concur to explain why so many murine models fail to yield convincing results in pre-clinical studies.

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In a more than five-year-long study, we used a non-isogenic respiratory chain (RC) deficient mouse strain, namely the *Harlequin* (Hq) mouse, to test a set of drugs. We previously attributed the Hq mouse phenotype to a partial defect of RC complex I (CI) activity (Vahsen et al., 2004), due to a retroviral insertion in the X-linked *AIF* (apoptosis inducing factor) gene, leading to the formation of a hypomorphic allele (Klein et al., 2002). Noticeably, the complete inactivation of *AIF* in genetically-engineered mice (Pospisilik et al., 2007) or deleterious mutations in humans (Ghezzi et al., 2010) have a wider impact on the RC, affecting also complexes III and/or IV. The knockout, knockdown or hypomorphic mutation of the *AIF* causes a defect in CHCHD4-dependent RC biosynthesis in human cell lines in vitro, as well as in mice in vivo (Hangen et al., 2015). Similarly, mutations that occur in the human gene encoding for AIFM1 and that affect the binding of AIF to CHCHD4 cause mitochondriopathies that manifest as a severe X-linked mt encephalomyelopathy in infants (Meyer et al., 2015).

A spectacular inter-individual variability in time of onset and severity characterizes the Hq disease in the CW/BL genetic background (Bénit et al., 2008). We previously reported these variable features associated with the partial loss of CI activity in the Hq mouse and showed the positive effect of a high-fat diet on the disease course (Schiff et al., 2011). In a further attempt to identify disease-attenuating drugs in Hq mice, we selected three drugs postulated to have different mechanisms of action, being either PPAR-α (bezafibrate; BZ) or PPAR-γ (pioglitazone; PIO) agonist or having an antioxidant effect (melatonin; ML), this latter *a priori* not linked to the activation of the melatonin-specific receptor. These three drugs have been previously reported as having a potential action against mitochondriopathies, ML (Reiter et al., 2007), BZ (Wenz et al., 2008) and PIO (Pinto et al., 2016). Here, we report that PIO may improve the Hq phenotype, presumably through the inhibition of excessive glycolysis.

2. Materials and Methods

2.1. Housing and Treatment of Mice

Hemizygous Hq males (*Hq*/Y) were obtained by mating heterozygous (*Hq*/X) females with WT males obtained from the Jackson Laboratory (Bar Harbor, ME). We used F1 mice bred from founders having a mixed genetic background (B6CBACaAw-J/A-Pdcd8*Hq*/J). Mice were housed with a 12 h light/dark cycle with free access to food and water. To avoid any bias, one month-old litters were assigned to predefined (before birth) groups. Four groups were studied for melatonin (ML; Sigma M525; 4 mg/ml H_2O) trialing (Hq or WT fed R03 pellets (control diet) *minus* or *plus* ML in bottle water; six groups for trialing bezafibrate (BZ; Sigma B7273) and pioglitazone (PIO; ChemPacific 112529-15-4) in the pellets (Hq fed R03; R03 *plus* BZ or PIO; estimated 0.925 g/kg/d and 3 mg/kg/d respectively). WT mice were fed R03, R03 *plus* BZ or *plus* PIO. We checked that WT and Hq mice consumed similar food amounts per day. Customized pellets were prepared by SAFE (Augy, France).

2.2. Genotype Determination

Mice were genotyped using multiplex PCR with two primers for sex determination (SRY: 59-TGGGACTGGTGACAATTGTC-39 and 59-GAGTACAGGTGTGCAGCTCT-39), two for the wild-type *Aif* allele (*Aif* 1F: 59AGTGTCCAGTCAAAGTACCGG-39; *Aif* 1R: 59-CTATGCCCTTCTCC ATGTAGTT-39), and one for the *Aif* allele (*Aif* RV: 59-CCCGTGTATCCAATAAAGCCTT-39) (Bénit et al., 2008).

2.3. Ethics Statement

Details of the mouse study were approved by the Robert Debré-Bichat Ethics Committee on Animal Experimentation (http://www.bichat.inserm.fr/comite_ethique.htm; Protocol Number 2010-13/676-003) in accordance with the French and European Laws on animal protection.

2.4. Phenotype Evaluation

Locomotor ability, balance and coordination, of WT and Hq mice were assessed on a Rotarod device (Imetronic; Pessac, France). The mice were trained for 3 consecutive days after a previous days of training at 4, 5 and 6 months, at increasing speeds (4–40 rpm). The latency before fall from the rod was recorded. Dystonia was measured using the tail suspension test (Glynn et al., 2005). When suspended by its tail, a WT mouse tries to escape by splaying its hind limbs away from the trunk, while an Hq mouse holds its hind limbs against its trunk. If the hind limbs were 10 s splayed outward, it was assigned a score of 2. If one or both hind limbs were retracted for >50% of the time, it received a score of 1. If its hind limbs were entirely and consistently retracted, it received a score of 0. Muscular strength of the forelimb was measured by a grip strength meter (Ugo Basile SRL, Varese, Italy). Each mouse was given four successive trials and a mean grip strength reading was calculated. Blood glucose was measured by a trained unique investigator between 2 and 3 pm using OneTouch Vita - glucometer (LifeScan, France) in mice with free access to food and water. Blood was collected either from the tail or from the heart at sacrifice in 6 month-old mice.

2.5. Mouse Astrocytes and Human Skin Fibroblasts

Astrocytes were prepared from meninges-free cerebellum of 6–7 days old mice. Astrocytes were plated into culture flasks in DMEM containing glucose (1 g/l) and 10% fetal calf serum at 37 °C in a 5% CO_2. Upon confluence, flasks were shaken (180 rpm × 30 min; Rocking Orbital shaker, VWR) to remove contaminated microglia cells. Astrocytes are then detached from the culture flash by trypsin and pelleted at 1500 g × 5 min.

Skin fibroblasts were derived from two healthy individuals, and two patients (P1 and P2) harboring R201del missense mutations in AIF (Ghezzi et al., 2010). Fibroblasts were grown either in DMEM glucose (4.5 g/l), 6 mM glutamine, 10% FCS, 200 μM uridine, penicillin/streptomycin (100 U/ml) *plus* 10 mM pyruvate, or in selective medium (DMEM containing glutamine but no glucose, uridine nor pyruvate). For cell counting and size determination, cells were at 50% confluence (d = 0). After 5 days, cell density (cell number per cm^2) was estimated from representative phase-contrast photographs taken on an LSM 5 Exciter optic microscope (Eclipse TE300 Nikon, France) (×4). Cell density (cell number per cm^2) was estimated in 54 identical areas for each condition. Cell size (length and width) was determined on the same representative photographs for a minimum of 90 cells. Width was estimated at the area adjacent to nuclei.

2.6. Immunocytochemistry

Immunocytochemical studies were performed on mouse cultured astrocytes adherent to coverslips (Schildge et al., 2013) and stained with Abcam primary antibodies (GAPDH ab8245; GFAP ab7260) and fluorescent-tagged secondary antibody (mouse, AlexaFluor 555 or rabbit, AlexaFluor 488; Thermofisher Scientific, France). Cells were imaged using a 20× Plan-Apochromat (aperture 0.8)/63x EC Plan-Neofluar oil-immersion lens (aperture 1.3) of a Zeiss Axio Observer inverted microscope (Carl Zeiss, Jena, Germany) equipped with an AxioCam MRm camera.

2.7. Immunoblot Analyses

Western blot analysis was performed either on supernatants (200 g × 5 min) of tissue homogenates in 20 mM Tris buffer (pH 7.2), 250 mM saccharose, 40 mM KCl, 40 mM EGTA and 1 mg/ml bovine

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