J Inherit Metab Dis (2011) 34:345–355
DOI 10.1007/s10545-010-9260-x

GALACTOSEMIA

Is prenatal myo-inositol deficiency a mechanism of CNS injury in galactosemia?

Gerard T. Berry

Received: 23 September 2010 / Revised: 23 November 2010 / Accepted: 26 November 2010 / Published online: 19 January 2011

Abstract Classic Galactosemia due to galactose-1-phosphate uridyltransferase (GALT) deficiency is associated with apparent diet-independent complications including cognitive impairment, learning problems and speech defects. As both galactose-1-phosphate and galactitol may be elevated in cord blood erythrocytes and amniotic fluid despite a maternal lactose-free diet, endogenous production of galactose may be responsible for the elevated fetal galactose metabolites, as well as postnatal CNS complications. A prenatal deficiency of myo-inositol due to an accumulation of both galactose-1-phosphate and galactitol may play a role in the production of the postnatal CNS dysfunction. Two independent mechanisms may result in fetal myo-inositol deficiency: competitive inhibition of the inositol monophosphatase1 (IMPA1)-mediated hydrolysis of inositol monophosphate by high galactose-1- phosphate levels leading to a sequestration of cellular myo-inositol as inositol monophosphate and galactitol-induced reduction in SMIT1-mediated myo-inositol transport. The subsequent reduction of myo-inositol within fetal brain cells could lead to inositide deficiencies with resultant perturbations in calcium and protein kinase C signaling, the AKT/ mTOR/ cell growth and development pathway, cell migration, insulin sensitivity, vescular trafficking, endocytosis and exocytosis, actin cytoskeletal remodeling, nuclear metabolism, mRNA export and nuclear pore complex regulation, phosphatidylinositol-anchored proteins, protein phosphorylation and/or endogenous iron "chelation". Using a knockout animal model we have shown that a marked deficiency of myo-inositol in utero is lethal but the phenotype can be rescued by supplementing the drinking water of the pregnant mouse. If myo-inositol deficiency is found to exist in the GALT-deficient fetal brain, then the use of myo-inositol to treat the fetus via oral supplementation of the pregnant female may warrant consideration.

Communicated by: Estela Rubio-Gozalbo

Competing interest: None declared.

G. T. Berry (✉)
Division of Genetics, Children's Hospital Boston,
Center for Life Sciences Building,
Room 14070 3 Blackfan Circle,
Boston, MA 02115, USA
e-mail: Gerard.Berry@childrens.harvard.edu

Hereditary Galactosemia (OMIM 230400) is a rare inborn error of carbohydrate metabolism (Fridovich-Keil and Walter 2008; Elsas 2010; Berry and Walter 2011). The classic form of the disease is life-threatening in the newborn period, associated with multiorgan involvement and is due to a severe deficiency of the enzyme, galactose-1-phosphate uridyltransferase (GALT, EC 2.7.7.12). As a consequence, galactose-1-phosphate, as well as its precursor, galactose, builds up to millimolar concentrations in target tissues such as brain and liver, but only when the neonates ingest gram quantities of dietary lactose. However, even when patients with zero residual GALT activity have been placed on a lactose-restricted diet from birth, they usually manifest long-term complications including language/speech defects, alterations in cognition and behavior/mood, and, in women, primary ovarian insufficiency (POI) (Kaufman et al. 1981; Komrower 1982; Waisbren et al. 1983; Waggoner et al. 1990; Nelson et al. 1991; Schweitzer et al. 1993; Holton 1996; Guerrero et al. 2000; Robertson et al. 2000; Webb et al. 2003; Gubbels et al. 2008; Berry 2008; Potter et al. 2008). Furthermore, patients on a lactose-restricted diet still manifest chronically elevated, albeit not millimolar, levels of galactose-1-phosphate in erythrocytes and galactitol in plasma and urine (Donnell et al. 1963; Schweitzer et al. 1993; Berry et al. 2011; Bosch 2006). This may be primarily due to endogenous de novo

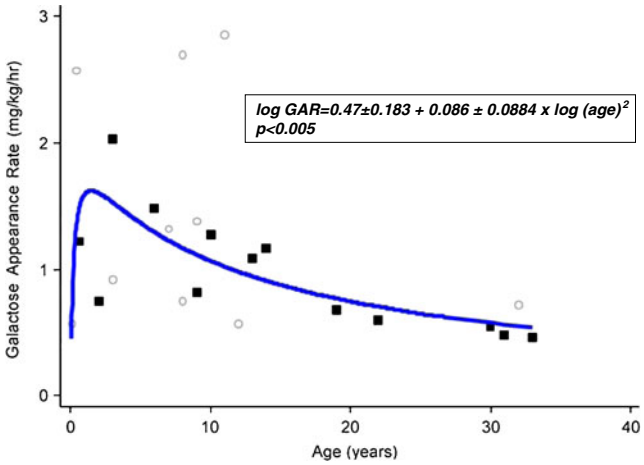

Fig. 1 The galactose appearance rate or apparent endogenous galactose production rate in patients with galactosemia of different ages was determined by the continuous intravenous infusion method (Berry et al. 2004). The patients with a Q188R/Q188R genotype are shown as the ■ symbols while patients with other genotypes are shown as ○ . The regression analysis of this data showed that the log of the galactose appearance rate was highly significantly ($p<0.005$) negatively correlated with the log of age^2 (Berry et al. 2004). The regression equation that produced the best fit is noted in the figure. From Berry et al. 2004

synthesis of galactose (Gitzelmann 1969; Berry et al. 1995a, 2004; Schadewaldt et al. 2004; Huidekoper et al. 2005). Endogenous production rates are higher in infants and young children compared to adolescents and adults (Berry et al. 2004; Schadewaldt et al. 2004). In fact, the rate of endogenous galactose production per body weight when plotted versus age suggest a typical pediatric developmental growth curve (Fig. 1). Therefore, the rate of endogenous synthesis may be much higher in prenatal fetal tissues than after birth. In support of this notion, elevated levels of galactose-1-phosphate and/or galactitol have been detected in fetal tissues, amniotic fluid and/or cord blood (Schwarz 1960; Komrower 1982; Irons et al. 1985; Jakobs et al. 1988). The data raise the following question: are the diet-independent complications in galactosemia due to endogenous galactose synthesis in the fetal-placental unit?

Evidence for myo-inositol deficiency

I hypothesize that prenatal myo-inositol deficiency in the fetal central nervous system due to an accumulation of both galactose-1-phosphate and galactitol leads to permanent nervous system dysfunction. As discussed below, galactose-1-phosphate is a substrate for inositol monophosphatase1 or IMPase1 (see Figure 4). In high concentrations, galactose-1-phosphate may inhibit myo-inositol recycling in developing neurons following agonist-induced signal transduction events. Intracellular galactitol via osmoregulatory perturbations may lead to reduced myo-inositol transport into fetal neurons. High concentrations may therefore lead to trapping of myo-inositol as inositol monophosphate. Independently, galactitol accumulation may reduce myo-inositol transporter activity. Both pathways may lead to myo-inositol deficiency in certain target cells. The first data to appear in the literature to support a deficiency of myo-inositol in the brain of a patient with galactosemia was that of Wells et al. in 1965 (Wells et al. 1965). A Caucasian female infant died at 27 days of age: the brain galactitol level was 12.90 μmol/gram tissue (wet weight) and the myo-inositol was 5.70 μmol/gram tissue. Control brain tissue from a male infant who died of unrelated disease at 30 days of age revealed a myo-inositol level of 7.29 μmol/gram tissue. Galactitol was undetectable. Largely representing phosphatidylinositol (PtdIns) lipid-bound myo-inositol was 1.30 and 0.62 μmol/gram tissue in the control and galactosemic brains, respectively (Quan-Ma et al. 1966). This group reported a second patient with galactosemia, a male infant who died at 23 days of age (Quan-Ma et al. 1966). The myo-inositol level of 1.13 μmol/gram tissue was reduced by 90% compared to the 30% reduction in galactosemic infant #1. The brain galactitol content was 22.18 μmol/gram tissue. Please note that the content of myo-inositol is higher in the brain of the fetus than at any other time in life. The reason is unclear. Also there is an almost linear decline in brain myo-inositol content from at least 32 weeks gestational age until term (Kreis et al. 2002). Using a 1.5 Tesla system for brain MRS, Kreis et al. showed that the myo-inositol concentration in mmol/kg wet weight in the occipital gray matter was 12.0±1.4 (mean±SD) in the preterm newborn (n=9) at 34±1 weeks (mean±SD) gestational age (Kreis et al. 2002). By 38±4 weeks gestational age (n=70), it was 7.8±2.2 mmol / kg wet weight in composite brain tissue. Usually, galactitol is undetectable in the brain of a term newborn infant using a 1.5 Tesla magnet indicating that it is below millimolar concentrations. The composite brain level of myo-inositol continues to decline in post-natal life until it reaches the value of approximately 4 mmol/kg wet weight. This level is maintained in adulthood until middle age at which point a decline ensues. The murine fetal, term and adult values in brain tissue resemble those seen in humans (Buccafusca et al. 2008).

We finally had an opportunity to perform an in vivo brain MRI/MRS for assessment of brain galactitol/myo-inositol content in a 9 day old male infant with classic galactosemia and a Q188R/Q188R genotype in 2001 (Berry et al. 2001). The infant was encephalopathic and had brain white matter edema. Diet therapy had been started at 7 days of age. The galactitol to creatine-containing compounds ratio was 2.12 and 2.42 in the basal ganglia and occipital cortex voxels, respectively, while the characteristic galactitol doublet at 3.67 and 3.74 ppm was absent in 8 and 9 day old control infants. If we assume that the

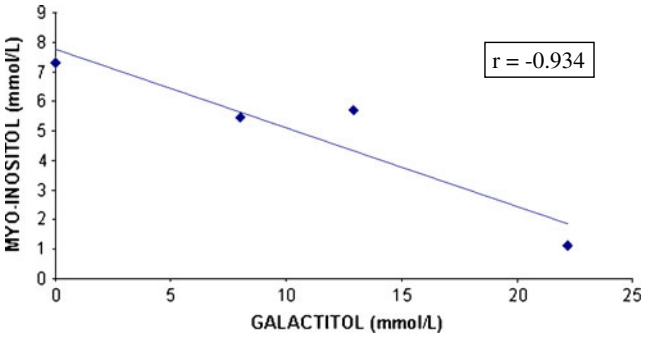

Fig. 2 The apparent human brain tissue levels of myo-inositol and galactitol are expressed as mmol/L. The regression analysis of this data showed that the myo-inositol level was negatively correlated with the galactitol level (r = -0.934)

content of creatine-containing compounds detected by proton magnetic resonance using the Siemens 1.5 Tesla scanner in the brain of the newborn with galactosemia was normal, then the galactitol level in occipital gray matter was approximately 8 μmol per gram tissue. This is not unlike the level detected in post-mortem tissue of infant #1. Compared to control infants, the brain myo-inositol content was reduced by 22-29% in the infant with galactosemia (Ins/Cr ratios of 0.83 vs 1.07 and 1.21 vs 1.71). When the patient was studied again at 20 months of age, myo-inositol content was normal and no galactitol peak was evident. We further studied three more newborn infants with classic galactosemia on a lactose-restricted diet (6, 13 and 15 days of age) with evidence of galactitol peaks and diffuse white matter MRI signal abnormalities (Wang et al. 2001). Eight additional patients ranging in age from 1.3 to 57 years of age on lactose-restricted diets were tested. As in the adult study of Möller et al. (Möller et al. 1995), most of the patients did not exhibit a galactitol peak and myo-inositol content was within normal limits. However, two of these subjects, ages 1.3 and 27 years old, had a galactitol to creatine-containing compounds peak ratio of 0.24 and 0.25, respectively. A severely ill, untreated 6 month old infant with classic galactosemia underwent brain MRI/MRS (Otaduy et al. 2006). In addition to widespread white matter abnormalities, the galactitol to creatine-containing compounds peak in parieto-occipital white matter was markedly increased at 14.30 (controls: 0) while the myo-inositol ratio was reduced to 0.19 (controls: 0.48±0.07). Following treatment and at 2 years of age, the myo-inositol/creatine normalized at 0.50 and the galactitol peak was not evident. However, marked brain atrophy, more evident in the frontal lobes was present. While the white matter signal intensities had almost completely resolved, there were enlarged sulci, dilatation of the lateral ventricles and residual lesions in the basal temporal lobes and peri-ventricular frontal regions. This patient is a classic example of the galactosemic survivor with massive post-natal brain injury super-imposed on the theoretical "common" pre-natal insult.

In an attempt to better delineate the relationship between brain galactitol and myo-inositol content, I have assembled all of the available data drawn from both post-mortem and in vivo brain MRI/MRS studies and, with some assumptions, converted all of the results to mmol/L. Only data where both values were available were used. The results are shown in Fig. 2, in which the concentration of galactitol is compared to the concentration of myo-inositol in each pair. When galactitol is "zero", the normal myo-inositol concentration in the brain of the newborn infant is 8 mmol/L. There is a highly significant inverse correlation between the galactitol and myo-inositol levels in the brains of the patients with classic galactosemia. In other words, as the content of galactitol rises, myo-inositol levels decrease. As discussed below, this is not surprising, and quite expected based on the polyol literature (Berry 1995).

Physiology and biochemistry of myo-inositol

Myo-inositol plays two roles in mammalian physiology (Fig. 3). The first and most well known is to serve as the precursor of the membrane-bound inositolphospholipids important in signal transduction events (Nishizuka 1988; Berridge and Irvine 1989;). The second is to function as a "non-perturbing" organic osmolyte, "buffering" changes in extracellular osmolality (Thurston et al. 1989; Kwon et al. 1992; Bersudsky et al. 1994). The key and obligatory enzymatic reaction in the synthesis of phosphoinositides is phosphatidylinositol synthase (Paulus and Kennedy 1960). Following the conversion of myo-inositol to phosphatidylinositol (PtdIns), the major inositol-containing lipid, PtdIns is sequentially phosphorylated to phosphatidylinositol-4-phosphate (PtdIns-4-P) and then to phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2). Almost all of the PtdIns-4,5-P_2 that is involved in membrane signaling events, especially in the CNS, contains arachidonate in the *sn*1 position and stearate in the *sn*2 position. Following agonist-induced receptor activation, PtdIns-4,5-P_2 is hydrolyzed by a phospholipase C to myo-inositol 1,4,5-trisphosphate (PtdIns-1,4,5-$P_{3)}$ and diacylglycerol). Subsequently, the water soluble second messenger, Ins-1,4,5-P_3, binds to an Ins-1,4,5-P_3 receptor on an internal membrane to activate a calcium channel resulting in a transient burst in cytosolic calcium activity (Berridge and Irvine 1989). The other hydrolytic product, diacylglycerol, activates protein kinase C (PKC) at the plasma membrane (Nishizuka 1988). This canonical calcium signaling and PKC pathway is utilized in many tissues to transmit hormone and neurotransmitter signals and is highly conserved across many species.

It is likely that a severe deficiency of myo-inositol in cells would lead to an impairment in signal transduction involving multiple pathways (Deranieh and Greenberg 2009). The most notable one would be the PtdIns-4,5-P_2 and Ins-1,4,5-P_3 pathway affecting calcium signaling and PKC activation. As

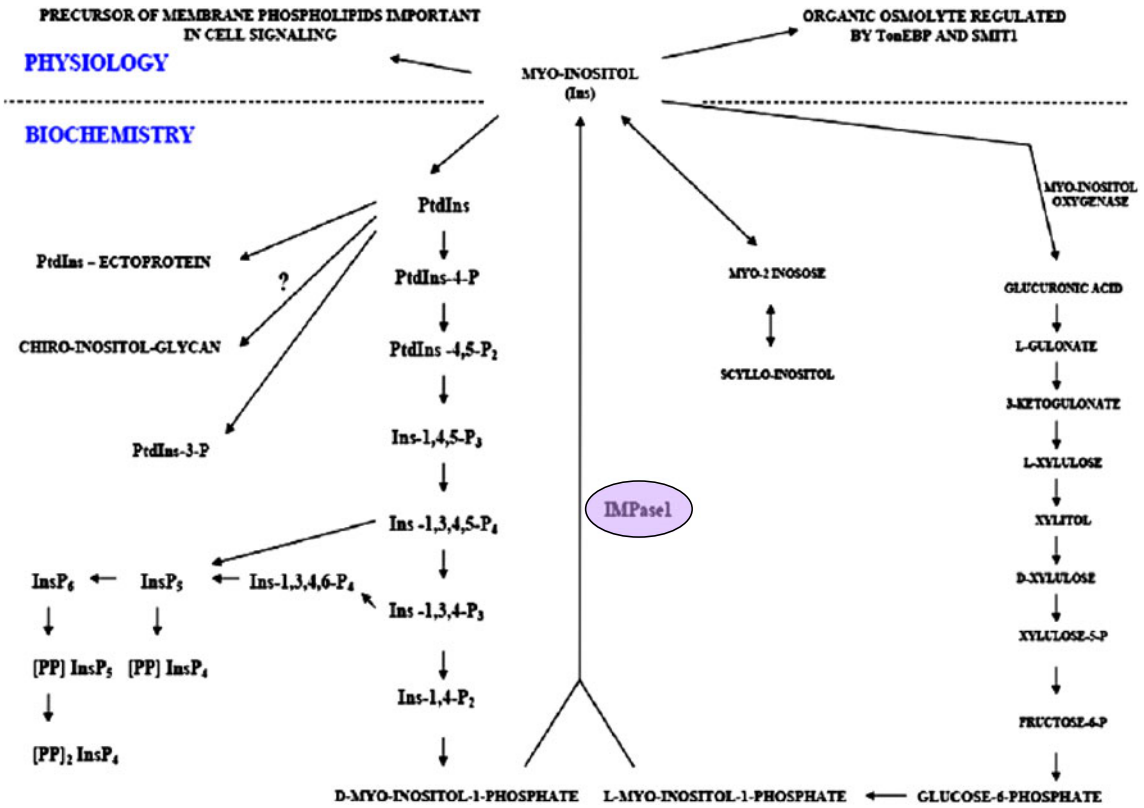

Fig. 3 The two known roles of myo-inositol (Ins) in mammalian physiology and the key biochemical pathways are depicted. Please note that we did not include all of the known or putative reactions such as the phosphatase and kinase activities that mediate degradation and/or isomer conversion of Ins-polyphosphates. The enzyme, phosphatidylinositol synthase, is responsible for the conversion of myo-inositol and CDP-DAG to phosphatidylinositol and CMP, and is the only pathway for conversion of Ins to polyphosphoinositides and myo-inositol polyphosphates in mammals. The same enzyme responsible for the conversion of glucose-6-phosphate to l-myo-inositol-1-phosphate also catalyzes the reaction in which mannose-6-phosphate is converted to neo-inositol-1-phosphate. Neo-inositol is further dephosphorylated to yield free neo-inositol, present in brain cells in very low concentrations. From Buccafusca et al. 2008

shown in Fig. 3, both PtdIns-4,5-P_2 and Ins-1,4,5-P_3 may be further metabolized and phosphorylated to higher polyphosphate species. Thus, a significant deficiency of PtdIns may lead to a reduction in PtdIns-3,4,5-P_3, Ins-1,3,4,5-P_4, Ins-1,2,3,4,5-P_5, Ins-1,2,3,4,5,6-P_6 (phytate) and [PP]Ins P_5 just to name a few of the inositide species. A reduction in PtdIns may lead to a deficiency of PtdIns-3,4,5-P_3 and PtdIns-3-P, i.e., a failure of the PI-3 kinase signaling systems (Engelman et al. 2006). The

Table 1 Inositides and cell function

Inositides	Cell function
PtdIns-4,5-P_2 and Ins-1,4,5-P_3	Calcium and protein kinase C signaling (Nishizuka 1988; Berridge and Irvine 1989)
PtdIns-3,4,5-P_3	PI3K/AKT/mTOR cell growth and development pathway, cell survival, cell migration, tumorigenesis, insulin sensitivity (Brachmann et al. 2005; Taniguchi et al. 2006, Yuan and Cantley 2008; Liu and Bankaitis 2010)
PtdIns-4,5-P_2, PtdIns-3,4,5-P_3, Ptd-3,4-P_2	Vesicular trafficking, endocytosis, exocytosis (Cremona and De Camilli 2001; Di Paolo and De Camilli 2006; Clague et al. 2009; Majerus and York 2009)
PtdIns-4,5-P_2, PtdIns-3,4,5-P_3	Actin cytoskeletal reorganization; Cilia (Di Paolo and De Camilli 2006; Bielas et al. 2009)
PtdIns-4,5-P_2, PtdIns-3,4,5-P_3 and Ins-1,2,3,4,5,6,-P_6	Nuclear metabolism/mRNA export and nuclear pore complex regulation (Cocco et al. 2009; Okada and Ye 2009)
[PP] $InsP_5$	Protein phosphorylation (Saiardi et al. 2004; Bhandari et al. 2007; Shears 2009)
PtdIns-anchored proteins	Alkaline phosphatase, 5′-nucleotidase, acetylcholinesterase, folate receptor, Thy-1 antigen (Sharom and Lehto 2002; Loretscher and Lavery 2002)
Chiro-inositol-containing glycan	Insulin signaling (Larner et al. 2003; Lin et al. 2009)
Ins-1,2,3,4,5,6-P_6	Endogenous iron “chelation” (Hawkins et al. 1993; Irvine 2005)

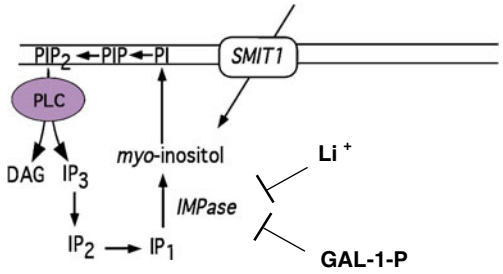

Fig. 4 The inositide cycle pathway and associated enzymes and transporter. Li^+, lithium; PI, phosphatidylinositol (aka PtdIns); PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; DAG, diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; IP_2, inositol bisphosphate; IP_1, inositol monophosphate; gal-1-P, galactose-1-phosphate; IMPase, inositol monophosphatase; and SMIT1(SLC5A3), sodium/myo-inositolco-transporter1. Modified from the Shaldubina et al. 2006

PI-3 kinase pathway including AKT and PTEN are major factors in cancer development (Yuan and Cantley 2008; Liu and Bankaitis 2010). In addition to plasma membrane agonist-induced receptor-mediated signaling, PtdIns-4,5-P_2 and PtdIns-3,4,5-P_3 play an important role in vesicle trafficking including fission and fusion as well as clathrin-mediated endocytosis at the pre-synaptic nerve terminal (Di Paolo and De Camilli 2006). Both phosphoinositides and inositol polyphosphates affect nuclear metabolism including chromatin remodeling, mRNA editing and nuclear pore export (Cocco et al. 2009; Okada and Ye 2009). Also, PtdIns deficiency may lead to impaired synthesis of ectoproteins that are anchored to the plasma membrane via PtdIns such as alkaline phosphatase, 5′-nucleotidase, acetylcholinesterase and Thy-1 (Sharom and Lehto 2002; Loretscher and Lavery 2002). Please refer to Fig. 3 for other related pathways and metabolites that may be perturbed because of a primary deficiency of Ins and PtdIns. Also note that there are other pathways that may function in mammalian cells to produce the inositol polyphosphates including the diphosphoinositol polyphosphates (Irvine 2005; Bhandari et al. 2007; Shears 2009). However, the utilization of these pathways and the magnitude of flux through them in man is unknown. The impact of these disturbances on cell physiology and signaling is summarized in Table 1. To further emphasize the importance of inositide metabolism in cellular homeostasis, several genetic diseases involving inositide pathway enzymes are known to exist. They are Lowe syndrome, myotubular myopathy, Charcot-Marie-Tooth type 4B1 disease, Francois-Neetens fleck cornea dystrophy, Joubert syndrome and cancer (Attree et al. 1992; Majerus and York 2009; Bielas et al. 2009; Laporte et al. 1996; Bolino et al. 2000; Li et al. 1997, 2005; Bader et al. 2005; Weishart and Dixon 2002). To summarize, my hypothesis is that the neurons and/or glial cells of the fetus with classic GALT gene mutations will manifest multiple signaling and vesicle trafficking defects because of the secondary deficiency of myo-inositol.

A mouse model for severe fetal myo-inositol deficiency

In order to answer the question whether myo-inositol deficiency is important for the fetal and newborn mammal, we created a murine knockout model of myo-inositol deficiency (Berry et al. 2003). The first experimental issue we encountered was the decision as to which system to employ to deplete cells of myo-inositol. Since almost all mammalian tissues or cell types that maintain a high concentration gradient for myo-inositol, e.g., human adult plasma with 10-40 μM myo-inositol vs human adult kidney tissue with~4 mM myo-inositol, display a sodium- and energy-dependent active transport system for myo-inositol, we chose ablation of an active myo-inositol transporter to accomplish this task. Furthermore, we had shown that fetal endothelial cells in culture possessed a high affinity sodium-dependent myo-inositol transport system suggesting that a similar active transporter may be operative in mammalian fetal tissues that display high millimolar levels of myo-inositol (Berry et al. 1993). We cloned the first human Na+/myo-inositol cotransporter (SLC5A3) gene that encodes SMIT1 in 1995 (Berry et al. 1995b), characterized its structural organization and promoter that is under osmoregulatory control (Mallee et al. 1997) and fine mapped its location to human chromosome 21q22.1 (Berry et al. 1996).

The SLC5A3 organization in all mammals is unusual in that the gene is made up of only 2 exons and the large exon 2 contains an intron-free coding region plus an extremely long 3′ UTR (Mallee et al. 1996, 1997; Rim et al. 1997; McVeigh et al. 2000). Furthermore, the SLC5A3 gene is embedded within a much larger gene, the mitochondrial ribosomal protein subunit 6 (MRPS6) gene, with which it shares exon 1 (Buccafusca et al. 2008). These structural features of SLC5A3/MRPS6 are present in all mammals and the genomic coexistence is evolutionarily conserved after the genesis of fish (Buccafusca et al. 2008). We hypothesized that the SLC5A3 gene was responsible for the myo-inositol concentration gradient in the mammalian fetus as this gene is highly expressed in adult kidney, placenta and adult brain in that order (Berry et al. 1995b). To test this hypothesis, we generated a murine knockout mouse with a homozygous targeted ablation of the exon 2 coding region of the SLC5A3 gene (Berry et al. 2003). All of the homozygous SLC5A3 knockout mice died within 20 minutes after birth due to, at least in part, apneic episodes and hypoventilation. This is the most severe myo-inositol deficiency state ever created in a mammal as the whole embryonic day 18.5 (E18.5) fetus and fetal brain manifest myo-inositol levels that are reduced by 82% and 92-96% respectively (Berry et al. 2003; Buccafusca et al. 2008).

In this almost pure metabolic model, the entire necropsy including EM analysis of brain is normal (Buccafusca et al. 2008). The only physiological disturbance identified to date is a unique electrical discharge emanating from the brainstem

respiratory control center that can explain the cause of death (Berry et al. 2003). The perturbation in respiratory rhythmogenesis is also resistant to acute correction with exposure of the isolated brainstem to pharmacologic concentrations of myo-inositol (Buccafusca et al. 2008). However, administration of pharmacologic amounts of myo-inositol via the drinking water of the pregnant female carrier mouse before E9.5 will rescue the lethal phenotype 100% of the time (Buccafusca et al. 2008; Chau et al. 2005). The knockout mice require no myo-inositol treatment beyond the weaning period to survive, but as adults with myo-inositol deficiency they manifest abnormal brain behavioral tests which mimic those seen in rodents exposed to lithium, a drug used to treat mood disorders (Buccafusca et al. 2008; Agam et al. 2009). In summary, we have created a unique mammalian model of fetal myo-inositol deficiency so severe that it recapitulates the 90% degree of brain myo-inositol deficiency detected in the infant with galactosemia in the post-mortem state. We have also demonstrated that the SMIT1 protein is responsible for the maintenance of the myo-inositol concentration gradient in the murine fetal-placental unit. Furthermore, the lethal nature of the loss of SLC5A3 gene expression is probably due to myo-inositol deficiency as myo-inositol supplementation of the maternal drinking water is uniformly effective in allowing the knockout newborn mice to survive.

Mechanisms behind myo-inositol deficiency in the human fetus with galactosemia

There are two mechanisms to explain a reduction in myo-inositol levels in brain cells of the fetus with a severe impairment or loss of GALT-mediated conversion of galactose-1-phosphate to UDPgalactose. The first involves the accumulation of the GALT substrate itself. The second is due to the accumulation of galactitol secondary to increased activity of enzyme, aldose reductase, that converts galactose to galactitol. This assumes that intracellular galactose levels are elevated either because of endogenous synthesis of galactose and/or galactose uptake from extracellular fluid.

In 1997, Parthasarathy et al. showed that galactose-1-phosphate may act as a substrate for the myo-inositol monophosphatase (IMPA1) enzyme (Parthasarathy et al. 1997). Bhat later hypothesized that IMPA1 inhibition by galactose-1-phosphate may be the mechanism of galactose-1-phosphate toxicity in patients with galactosemia (Bhat 2003). Slepak et al. reported that galactose-1-phosphate is a competitive inhibitor of myo-inositol-1-phosphate for IMPA1 (Slepak et al. 2007). Of great interest, IMPA1 is the target of lithium action (Alllison and Stewart 1971; Hallcher and Sherman 1980). Patients with mood disorders may benefit from the use of lithium and Sherman et al. originally hypothesized that the mechanism was related to the deficiency of neuronal myo-inositol that is created when myo-inositol is sequested as myo-inositol-1-phosphate due to IMPA1 inhibition by lithium (Fig. 4). Berridge and colleagues produced comparable biochemical data in blowfly salivary gland and brain slices (Fain and Berridge 1979; Berridge et al. 1982), and a similar explanation was generated, the "inositol depletion hypothesis", arguing that the beneficial effect of lithium was due to the creation of a myo-inositol deficiency which would limit or reduce the synthesis of PtdIns and perturb PtdIns-4,5-P_2-related signal transduction events (Berridge et al. 1989).

It has been known for many years now that in any tissues or cells in which galactitol accumulates, it is likely that myo-inositol levels will drop in parallel (Berry 1995). Originally thought to be due to an inhibitory effect of galactitol on myo-inositol transport, it is more likely related to transcription factor modulation of gene expression. The question remains: why do myo-inositol levels in certain cells such as astrocytes, ocular lens fiber cells and endothelial cells go down when intracellular galactitol goes up? The answer may lie in the area of osmoregulatory control (Miyakawa et al. 1999; Dahl et al. 2001; Handler and Kwon 2001: Woo et al. 2002). Myo-inositol, sorbitol, taurine, glycerophosphorylcholine and betaine are all organic osmolytes (Burg et al. 1997). Certain mammalian cells use one or more to buffer changes in extracellular osmolarity (Jeon et al. 2006). When extracellular osmolality increases, these "non-perturbing" osmolytes increase in concentration because of activation of osmotic pressure- or osmolality-sensing proteins in the cytosol (Ferraris and Burg 2006). An example is the tonicity-responsive enhancer/osmotic responsive element-binding protein, TonEBP/OREBP (Miyakawa et al. 1999; Dahl et al. 2001). Upon activation, this Rel protein translocates to the nucleus and, along with an activator protein-1(AP-1), increases transcriptional activity of the SLC5A3 gene, thus increasing the production of SMIT1 transporters and, finally, myo-inositol levels (Irarrazabal et al. 2008). It does this by binding to multiple TonEBP/OREBP cognate DNA elements known as osmotic response elements (OREs) or tonicity enhancer-responsive elements (TonEs). In addition to an AP-1 binding site in the antisense direction, there are five 11 bp enhancers, spaced over 50 kb in the 5′ region of the SLC5A3 gene (Rim et al. 1998). In keeping with the theme that this is a family of genes that are not infrequently turned on or off in unison in certain tissues with osmosensing cells such as kidney, a 7 bp AP-1 site and similar 11 bp enhancer elements which bind TonEBP/OREBP are also present in the 5′ region of the aldose reductase gene whose product is responsible for the synthesis of both sorbitol and galactitol (Irarrazabal et al. 2008). The galactosemic condition presents a unique situation in which a surfeit of intracellular galactose results in activation of aldose reductase leading to increased intracellular accumulation of galactitol that is largely trapped within the cell because of the lack of

effective natural transporters to effect efflux. I hypothesize that the consequence of this reverse osmotic inbalance is a reduction in transcriptional activators such as TonEBP/OREBP and/or AP-1 leading to a reduction in SLC5A3 gene transcription and a depression in myo-inositol levels in an attempt to try to maintain osmotic equilibrium. A similar but not identical mechanism may pertain to the development of astroctye myo-inositol deficiency when glutamine levels rise as a consequence of hyperammonemia (Gropman et al. 2008).

The consequence of this osmotic dysequilibrium may be detrimental to cell function and ultimately tissue function, i.e., cataracts and white matter edema. However, aldose reduction activity is more enriched in human ocular lens tissue than in brain. It is possible that in the absence of exogenous intake of galactose, i.e., lactose ingestion during the newborn period, the brain cells require the accumulation of both galactose-1-phosphate and galactitol to develop a physiologically relevant reduction in myo-inositol that affects cell signaling. The perfect and most unfortunate time for this to occur would be in the galactosemic fetal brain during development when the endogenous production of galactose per body weight is at an all time maximum. In support of this hypothesis, chronic diet-independent CNS complications almost never occurs in the patient with galactosemia due to galactokinase (GALK EC2.7.1.6) deficiency (OMIM 230100) because of the lack of prenatal galactose-1-phosphate accumulation in brain cells. Yet, pseudotumor cerebri may develop in post-natal life in these rare patients with sufficient lactose exposure (Litman et al. 1975).

How do we remediate the pre-natal toxicity of galactose-1-phosphate and galactitol?

The hypothesis of prenatal myo-inositol deficiency due to galactose-1-phosphate and galactitol toxicities is based on the outstanding work and conceptual ideas of Professor Komrower (Komrower 1982). Yet, it is a hypothesis that requires proper testing. To properly address the question of whether myo-inositol is reduced in the brain of the galactosemic fetus at a time in gestation when myo-inositol needs to be maintained at the highest levels ever detected in the lifespan of a human, an international consortium of investigators would be needed to conduct a clinical research study with sufficient power. With an adequate number of subjects, we may be able to finally determine whether the fetus with galactosemia manifests galactitol accumulation in brain tissue and a concominant reduction in myo-inositol. Theoretically, this could be accomplished by performing magnetic resonance spectroscopy (MRS) in conjunction with brain MRI on the fetus of a carrier pregnant women at risk for delivering an infant with classic galactosemia. Ideally, the imaging would need to be done in a special well-equipped maternal-fetal center, especially those that engage in fetal surgery. Several articles have appeared in the literature demonstrating the capability of performing MRS on the brain of the fetus in utero at different gestational ages (Kok et al. 2001; Kok et al. 2002; Heerschap et al. 2003; Girard et al. 2006a, b; Pugash et al. 2009; Charles-Edwards et al. 2010). The range of normal levels of metabolites such as myo-inositol, N-acetylaspartate, creatine-containing and choline-containing compounds at different gestational ages are being formulated. I do not think it is feasible at this period in time to employ MRS to measure galactose-1-phosphate in fetal brain. It is to be a target of future studies.

This model of neurotoxicity is very appealing for a number of reasons. First, a recent publication in Nature Neuroscience provided unexpected evidence that the gene which encodes the IMPA1 protein, the target of both galactose-1-phosphate and lithium, has the most abundant transcript of all of the mRNA species found in axons of developing sympathetic neurons (Andreassi et al. 2010). A nerve growth factor-responsive element in the long 3′UTR of the IMPA1 mRNA is used to target this species to the nerve terminal for local translation. In vitro elimination of nerve growth factor (NGF) or localization of the long IMPA1 mRNA to the axon results in axonal degeneration. Please see Fig. 4 for a simplified scheme of a putative signaling cycle involving IMPA1 and myo-inositol. Second, independent genetic regulation of various genes and their proteins could play a role in the expression of the proposed neurotoxicity in addition to the constitutional factors that may govern the rate of endogenous galactose production and turnover in the neuron. An example would be the L- myo-inositol-1-phosphate synthetase enzyme with its encoding gene functioning as a modifier gene in galactosemia, a Mendelian disorder with complex genetic diseases traits. These types of modifiers could be tested in a large cohort of subjects homozygous for the Q188R gene defect and with variable and diverse long-term outcomes. Third, metabolite replacement therapy with myo-inositol may be feasible as evidenced by our murine SLC5A3 knockout model.

Obviously, the most logical therapeutic approach would be corrective gene therapy. When should gene transfer be performed? It would need to be at a gestational age before the window of toxicity, i.e., before when prenatal damage is induced by galactose-1-phosphate and galactitol. Thus, the timing of the GALT deficiency-induced prenatal damage would need to be defined. And, the genotype of the fetus would need to be identified via choronic villus DNA mutation analysis before gene therapy could be performed. This, of course, would be necessary if the gene transfer were to be delivered via transplantation of re-programmed stem cell-like autologous somatic cells subjected to gene transfer ex vivo. The next

possibility would be enzyme replacement therapy with recombinant GALT enzyme protein. But how do we package the GALT enzyme into a suitable vector and assure that it is delivered to the cytosolic compartment in brain and ovarian cells? Both of the above two therapies still appear to be many years away from their being of practical use in the clinical setting. Another very attractive modality would be substrate reduction therapy, i.e., a galactokinase inhibitor administered to the pregnant woman with an affected fetus (Tang et al. 2010). For this to be feasible the GALK inhibitor would need to be very specific for this kinase alone, as well as safe for pregnant women. Another therapeutic possibility is the administration of an aldose reductase inhibitor to block galactitol synthesis (Berry 1995). In fact, both agents could be given simultaneously to the pregnant woman. However, in both of the above instances, the question of safety of either therapy, alone or in combination, will probably preclude their use for the foreseeable future.

As a consequence of these many difficulties and obstacles, metabolite replacement therapy with myo-inositol may need to be considered if the fetus with galactosemia proves to be deficient in myo-inositol in brain tissue. However, because pharmacological amounts of myo-inositol would probably need to be administered to the pregnant woman, this form of treatment would need to be approached very carefully, if at all. In its favor, myo-inositol, which in the past has been likened to a vitamin-like substance, is very safe and has been given to newborn infants, children, adolescents and adults without side effects (Salway et al. 1978; Arendrup et al. 1989). Furthermore, the safe human consumption of gram quantities has been shown to increase brain myo-inositol levels. Even though a suitable animal model of galactosemia, especially a large animal model, is not available, it may prove to be necessary to test the safety of myo-inositol consumption in non-human primates (Lai et al. 2009). In this discourse, I have attempted to bring to light an aspect of the galactosemic story that has not received widespread attention. Parts of this review that concern fetal metabolism and non-invasive imaging are speculative in nature, but I felt justified in sharing my thoughts about the complicated pathophysiology of galactosemia with my fellow colleagues in the field of inborn errors of metabolism as so many years have gone by since the discovery of this enigmatic disease and, aside from dietary lactose restriction in infancy, little in the way of a therapeutic advance has been achieved.

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