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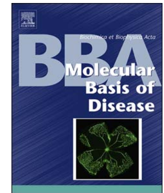

Reversal of aberrant PI3K/Akt signaling by Salubrinal in a GalT-deficient mouse model

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A R T I C L E I N F O

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A B S T R A C T

Classic Galactosemia is an autosomal recessive disorder caused by deleterious mutations in the *GALT* gene, which encodes galactose-1 phosphate uridylyltransferase enzyme (GALT: EC 2.7.7.12). Recent studies of primary skin fibroblasts isolated from the GalT-deficient mice demonstrated a slower growth rate, a higher level of endoplasmic reticulum (ER) stress, and down-regulation of the Phosphoinositide 3 kinase/Protein kinase B (PI3K/Akt) signaling pathway. In this study, we compared the expression levels of the PI3K/Akt signaling pathway in normal and GalT-deficient mouse tissues. In mutant mouse ovaries, phospho-Akt [pAkt (Ser473)] and pGsk3β were reduced by 62.5% and 93.5%, respectively ($p < 0.05$ versus normal controls). In mutant cerebella, pAkt (Ser473) and pGsk3β were reduced by 62%, 50%, respectively ($p < 0.05$). To assess the role of ER stress in the down-regulation of PI3K/Akt signaling, we examined if administration of Salubrinal, a chemical compound that alleviates ER stress, to GalT-deficient fibroblasts and animals could normalize the pathway. Our results demonstrated that Salubrinal effectively reversed the down-regulated PI3K/Akt signaling pathway in the mutant cells and animals to levels close to those of their normal counterparts. Moreover, we revealed that Salubrinal can significantly slow down the loss of Purkinje cells in the cerebella, as well as the premature loss of primordial ovarian follicles in young mutant mice. These results open the door for a new therapeutic approach for the patients with Classic Galactosemia.

1. Introduction

Classic Galactosemia (OMIM # 230400) is a potentially lethal inborn error of metabolism that results from deleterious mutations in the human *GALT* gene, which encodes the enzyme galactose-1 phosphate uridylyltransferase (GALT: EC 2.7.7.12). GALT is the second enzyme in the Leloir pathway of galactose metabolism, which catalyzes the conversion of galactose-1 phosphate (gal-1P) and UDP-glucose to UDP-galactose and glucose-1 phosphate (Fig.1) [1]. Consequently, GALT-deficiency leads to the accumulation of galactose, galactitol, and gal-1P, as well as deficiency of UDP-galactose in patient cells [2,3]. Although newborn screening programs and a galactose-restricted diet resolve the potentially lethal acute toxicity syndrome in the neonatal period, they fail to avert the long-term complications that include growth restriction, intellectual deficit, ataxia, speech dyspraxia and primary ovarian insufficiency (POI) that are frequently seen in many patients [4–6]. The molecular mechanisms of these acute and long-term complications are still poorly understood despite several hypotheses that have been presented in the literature [7–11].

Among the biochemical abnormalities detected in patients, glycosylation defects of glycoproteins and glycolipids have received significant attention [8,12–15]. It has been suggested that the observed glycosylation defects are caused by the accumulation of toxic galactose metabolites of the blocked GALT reaction, as well as deficiency of UDP-galactose (Fig. 1) [15,16]. Since glycosylation defects induced by tunicamycin intoxication can lead to endoplasmic reticulum (ER) stress [17], we and others have investigated if GALT-deficient cells exhibit an ER stress response [9–11,18]. Not only did we confirm the manifestation of ER stress in the GALT-deficient cells, we and others identified systematic changes in gene expression, which resulted in adverse alterations in many pro-survival and growth signaling pathways, including the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway [19,20]. However, the link between altered PI3K/Akt signaling and disease phenotypes in the context of GALT-deficiency

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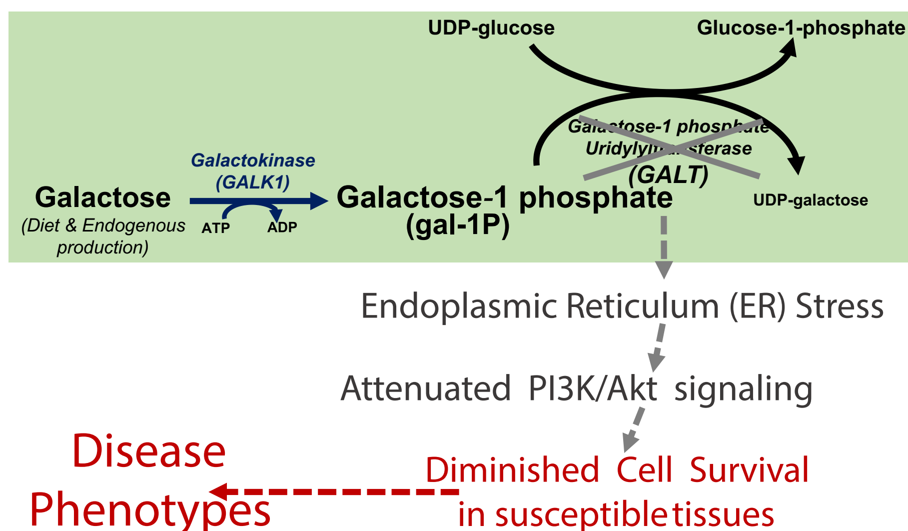

Fig. 1. Proposed pathological role of Endoplasmic Reticulum (ER) stress in disease-relevant phenotypes in the GalT-deficient mouse model. The Leloir pathway of galactose metabolism is high-lighted in green. UDP-galactose can also be produced *via* the UDP-4′ galactose epimerase (GALE) reaction (not shown). We proposed that in the absence of GALT activity, accumulation of toxic galactose metabolites leads to elevated level of ER stress, which in turns causes reduced PI3K/Akt signaling. In susceptible tissues, such attenuated PI3K/Akt signaling could lead to tissue-specific disease phenotypes.

has not been fully established. In this study, we used a mouse model of GalT-deficiency to examine the potential roles of excess ER stress and altered PI3K/Akt signaling in the development of the disease relevant phenotypes of the mutant mice. We hypothesize that elevated ER stress in GALT-deficient tissues causes aberrant PI3K/Akt signaling, which in turn plays an important role in the disease phenotypes such as subfertility and cerebellar ataxia in the GalT-deficient mouse model (Fig. 1).

2. Materials and methods

2.1. Chemicals and antibodies

Tunicamycin and Salubrinal were purchased from *Sigma Inc*. (St. Louis, MO). Antibodies against selected components of the PI3K/Akt signaling pathway were included in the Phospho-Akt Pathway Antibody Sampler Kit (*Cell Signaling Technology*, Danvers, MA). Anti-mouse BiP/GRP78 was purchased from *BD Bioscience* (San Jose, CA). Anti-mouse Hsp90 was obtained from *Santa Cruz Biotechnology Inc*. (Dallas, TX). Anti-mouse Gapdh was purchased from *Sigma Inc*. (St. Louis, MO).

2.2. Primary fibroblast and culture conditions

Primary skin fibroblasts were isolated from young adult female GalT-deficient mice [20–22] and their age-matched normal littermates using the method described by Seluanov and coworkers [23]. After confirmation of cell type identity by a Board-Certified pathologist, the isolated fibroblast strains were propagated in regular Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin at 37 °C with 5% ambient CO_2. Collagenase and cell culture media were purchased from *Invitrogen* (Waltham, MA). When indicated, cells at 70% confluence were treated with 50 μM Salubrinal dissolved in DMSO for 24 or 48 h. To induce ER stress in normal fibroblasts, tunicamycin was administered at a concentration of 2.5 μg/ml.

2.3. Animals and experimental groups

All animal studies were conducted in full compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and were approved by the University of Utah Institutional Animal Care and Use Committee. GalT-deficient mice used in this study were constructed as previously described, and were fed with normal chow at all times since weaning [21]. All mice were confirmed by genotyping (molecular and biochemical) using previously published protocols [21]. Animals were randomly assigned into the following experimental groups, each containing at least three mice.

(a) Untreated Control Group: Animals did not receive any treatment.
(b) Control Group: The animals have been administered with a vehicle solution consisting of dimethylsulfoxide (DMSO) diluted with soymilk (final concentration of DMSO was 0.6%) *per os* once daily at indicated durations.
(c) Sal Group: The animals have been administered with a mixture of solution consisting of Salubrinal (1, and 5 mg/kg) and DMSO which is then diluted with soymilk (the final concentration of DMSO was 0.6%) *per os* once a day at indicated durations.

2.4. Assessment of PI3K/Akt signaling in Salubrinal-treated fibroblasts/tissues

Fibroblast cells were lysed after the indicated incubation period and expression of PI3K/Akt signaling molecules was analyzed by Western Blot analysis as published before [20]. To harvest tissues, the animals were anesthetized by isoflurane and decapitated. After removal of the brains, the cerebella were dissected on a chilled ice plate and homogenized in ice-cold hypotonic lysis buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.4), supplemented with protease inhibitor cocktail (1 mg/ml each of aprotinin, pepstatin, and leupeptin; 100 mg/ml phenylmethylsulfonyl fluoride and 2 mM sodium orthovanadate). Ovaries were extracted and fat removed under a dissection microscope, were immediately transferred into ice-cold phosphate buffer saline (PBS) and homogenized in ice cold hypotonic lysis buffer. Homogenized samples were centrifuged at 18,000 × *g* at 4 °C for 20 min and supernatant collected. Protein concentrations were determined by a BCA protein assay kit (*Thermo Scientific*, Product # 23223) using a 96-well microplate reader.

2.5. Sample preparation for histological studies

2.5.1. Brain

The animal cranium was extracted and placed in ice-cold PBS followed by 10% formalin for 72 h. Several washes in 70% ethanol were done over a period of the next 48 h to rehydrate the tissues. The whole brain with attached cerebellum was then isolated from the skull and cut in half along the mid sagittal plane prior to tissue processing, slide preparation, and hematoxylin and eosin (H & E) staining performed by the Research Histology Core Facility of the ARUP Laboratories (Salt Lake City, UT, USA). H & E-stained sections were sent to a Board-certified pathologist at the University of Florida for the analysis of the

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