Published in final edited form as:

Biochem Biophys Res Commun. 2013 August 30; 438(3): 546–550. doi:10.1016/j.bbrc.2013.07.092.

Deletion of the Basigin gene results in reduced mitochondria in the neural retina

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Abstract

Basigin-null mice are characterized as blind from the time of eye opening, with degeneration of the retina beginning at 8 weeks of age, and progressing until the entire photoreceptor cell layer is destroyed. It is likely that a metabolic deficiency underlies the blindness and degeneration phenotypes, as it has been determined that Basigin-null mice do not express the transporter protein monocarboxylate transporter 1 on the membrane of photoreceptor cells and inner segments, nor Müller cells of the neural retina, as is observed in normal mice. The purpose of the present study was to assess the health of mitochondria in normal and Basigin-null mice, specifically to determine if mitochondria within the Basigin-null mouse neural retina are metabolically active. This was achieved via a measurement of cytochrome c concentration and the expression of autophagy-specific proteins via ELISA analyses. Additionally, Mitotracker dyes were used to assess the number and relative activity of mitochondria. It was determined that cytochrome C concentrations and expression of autophagy-specific proteins were not increased in Basigin-null animals, as compared to control animals. Also, while Basigin-null mice do have metabolically active mitochondria, the amount of mitochondria was greatly reduced, when compared to control animals. The results suggest that a reduction in mitochondria is a result, rather than the cause, of the metabolic deficiency observed in Basigin-null mice, and likely occurs because of reduced metabolic activity in the absence of MCT1 expression.

Keywords

Basigin; monocarboxylate transporter; mitochondria; cytochrome c; Mitotracker dye

Introduction

Basigin gene products are intriguing members of the Immunoglobulin (Ig) superfamily that have been implicated in myriad cellular and physiological processes (reviewed in [1]). The Basigin gene is located on chromosome 10 in mice and produces two main products in the neural retina via differential splicing, originally named Basigin [2] and Basigin-2 [3]. The Basigin transcript is ~1.5 kb and the protein is 27 kDa, which becomes a 45 kDa integral

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membrane glycoprotein upon glycosylation [2]. Basigin-2 is a transcript of ~ 1.8 kb and the glycosylated protein is 55 kDa [3]. The two proteins differ in the number of extracellular Ig domains, with Basigin possessing two and Basigin-2 possessing three [3]. It should be noted that the Basigin nomenclature has recently been amended such that Basigin is now Basigin variant-2 and Basigin-2 is now Basigin variant-1 [4]. The shorter protein is expressed throughout the body, including Müller glial cells, the apical and basolateral surfaces of the retinal pigmented epithelium (RPE), and blood vessel endothelial cells within the mammalian eye, and whereas the longer protein is only expressed on photoreceptor neurons [3].

More than a decade ago, a strain of mice was generated in which the gene for Basigin was deleted by molecular means [5]. Basigin null animals have several abnormalities, including infertility in both males and females [5], lower sensitivity to irritating odors [5], reduced response to foot shock [6] and blindness [7,8].

Studies indicate that Basigin-null animals are visually impaired at the time of eye opening (2 weeks of age), despite normal retina architecture at that age [8]. At visual maturity (3 weeks of age), the photoreceptor outer segments appear less dense and shorter than those of control animals, and at 8 weeks, retinal degeneration is observed [9]. Degeneration of the photoreceptors continues such that by eight months of age, the entire photoreceptor layer is missing [7,9].

Philp et al., [10] demonstrated that Basigin gene products are necessary for the targeting of monocarboxylate transporters (MCTs) to the plasma membrane and are essential for normal retinal function. MCT 1 is the primary lactate carrier found in Müller cells and the inner segments of photoreceptors, as well as on the apical membrane of the RPE [10]. When Basigin is absent, MCT 1 expression is not observed at the plasma membrane, but rather in intracellular vesicles [10]. It was hypothesized that a lactate metabolon exists in the retina in which Basigin gene products adhere Müller cells to photoreceptors so that MCT 1 can efficiently transfer lactate from the Müller cells to the photoreceptors, which then use lactate as an energy source (reviewed in [1]). In the absence of MCT 1, lactate from Müller cell metabolism is no longer available for energy metabolism in the photoreceptors, which could lead to the blindness phenotype [10].

The purpose of this present study was to determine whether mitochondrial metabolism or overall health is compromised in the Basigin-null mouse retina. The authors hypothesized that the mitochondria in Basigin null mice photoreceptors are less active than those of littermate controls due to the lack of lactate presentation to those cells. This was assessed via a series of biochemical analyses.

Materials and Methods

Generation of mouse retina cell extracts

Basigin-null mice and heterozygous littermate controls were euthanized according to an accepted protocol (UNF IACUC #10-009) and the eyes were immediately removed. Neural retinas were isolated from individual eye cups in Phosphate-buffered Saline (PBS; 50 mM

potassium phosphate, 150 mM NaCl, pH 7.2) and the tissue was lysed in Cell Extraction Solution (10 mM Tris, pH 7.4, 100 mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 2mM Na_3VO_4 , 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate; Invitrogen/Life Technologies, Carlsbad, CA) containing 1mM phenylmethanesulfonylfluoride (PMSF; Sigma-Aldrich, St. Louis, MO) and protease inhibitor cocktail (Pierce/Thermo Scientific, Rockford, IL). The tissue was incubated on ice for 30 minutes with vortex mixing every 10 minutes, followed by centrifugation at 13,000 rpm for 10 minutes at 4°C. The protein concentrations of the cleared lysates were determined via a protein assay using Coomassie (Bradford) stain (Pierce/Thermo Scientific).

Cytochrome C assay

Cytochrome c standards were generated at 1:2 serial dilutions in Standard Diluent Buffer (Invitrogen/Life Technologies). The normal and Basigin null mouse retina cell extracts were diluted 1:10 in Standard Diluent Buffer (100 μl total volume). The standards and retina cell extracts were subjected to a Cytochrome C ELISA assay (Invitrogen/Life Technologies) such that the concentrations of Cytochrome C for each retina cell extract could be determined via a standard curve.

Autophagy assay

For detection of autophagy, ELISA was performed using the mouse retina cell extracts from normal and Basigin null animals. The extracts (diluted to 100 g/mL in PBS) were transferred to individual wells of a 96-well plate and incubated overnight at 4°C. The extracts were removed and antibodies specific for key components of autophagy induction, including RAB 24, APG1, APG16L, BECN1 and APG5L (Abgent Antibodies, San Diego, CA), diluted 1:100 in PBS, were added to appropriate wells (100 μl total volume) and incubated for 30 minutes at 37°C. The solutions were removed and the wells were washed three times with PBST (PBS containing 0.05% Tween 20). Alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody (Pierce/Thermo Scientific) was diluted 1:1000 in PBS, delivered to appropriate wells (100 μl total volume), and incubated for 30 minutes at 37°C. The solutions were removed and the wells were washed three times with PBST. Alkaline phosphatase substrate (PNPP; Pierce/Thermo Scientific) was added to all the wells (100 μl total volume) and color development was allowed to proceed. All reactions were stopped at the same time by adding 50 μl of 2N NaOH to each well. The absorbance within each well was measured at 405 nm using a Bio-Tek Powerwave XS plate reader (Biotek Instruments, Winooski, VT) and correlated to the relative concentration of autophagy target protein present in the retina cell extracts. All runs were performed in duplicate.

Mitotracker Assay

Basigin null mice and heterozygous littermate controls were sacrificed according to an accepted protocol (UNF IACUC #10-009) and the eyes were immediately removed. Neural retinas were isolated from individual eye cups in PBS and then transferred to solutions containing both Mitotracker Red FM and Mitotracker Green FM (Molecular Probes/Invitrogen/Life Technologies), each diluted 1:1000 in PBS. The tissues were disrupted by passing through a micropipette tip and incubated for 2 hours at 37°C. The tissues were pelleted by centrifugation at 13,000 rpm for 10 minutes and resuspended in PBS.

Fluorescence was measured using a Bio-Tek Fluorescence FLx800 plate reader at 550–650 nm for Mitotracker Red FM and 400 nm for Mitotracker Green FM. Relative values for total (Mitotracker Green FM) and active (Mitotracker Red FM) mitochondria within each sample were determined.

Results and Discussion

It has been proposed that a lactate shuttle exists within the retina, in which Basigin gene products interact with MCT 1 to deliver lactate from the Müller cells to the photoreceptor cells. Lactate is a monocarboxylate that serves as a source of pyruvate for metabolism in vertebrate nervous tissues, which is subsequently converted to acetyl CoA and fuels oxidative phosphorylation [11]. Basigin-null mice do not express MCT1 on the plasma membranes of Müller cells or photoreceptor cells [10]. It has therefore been proposed that in the absence of the lactate shuttle complex, as in the Basigin-null mouse retina, the photoreceptor cells are not sufficiently nourished by the Müller glial cells and they never function [1,10]. Since mitochondria are the organelles responsible for cellular respiration, the aim of this project was to assess mitochondrial health in the Basigin-null mouse retina.

Initially, mitochondrial health was assessed via a Cytochrome C expression assay. Mitochondrial cytochrome c is a water soluble protein that functions as an electron carrier in the respiratory chain. The release of cytochrome c from the mitochondria, the organelle that regulates apoptosis, precedes caspase activation and is considered an early event in the apoptotic process. These characteristics make the detection of this protein an effective tool in determining the health of mitochondria in Basigin-null animals. Basigin-null and littermate control retinas were harvested at three weeks and three months of age and protein lysates were isolated. The assay uses an ELISA format to compare Cytochrome C expression in samples relative to a standard curve of Cytochrome C. It was determined that the concentration of Cytochrome C was reduced in the Basigin null mouse retinas compared to the concentrations observed in retinas of littermate controls (Figure 1). These data suggest that apoptosis is not activated in the Basigin-null mouse retina, as compared to those of littermate controls. This is a surprising result, as it is assumed that the retinal degeneration observed in Basigin-null mice proceeds via apoptosis [9].

Autophagy, like apoptosis, is a natural and tightly regulated pathway, but involves the lysosomal degradation of whole cytoplasmic organelles and their components. Several neurodegenerative diseases have been linked to defective autophagy, including Alzheimer's disease, where a massive buildup of undigested substrate is seen within the axons and dendrites [12]. Failure of autophagosomes to recognize their correct cargo can lead to incorrect sequestration of proteins, which in turn leads to Huntington's and Parkinson's diseases [13,14]. Autophagy can be triggered by multiple forms of cellular stress, including nutrient deprivation [15]. It was proposed, based on the low levels of cytochrome c in the null mouse retinas, as compared to the controls, that mitophagy, a specialized form of autophagy in which whole mitochondria and their components are destroyed, may occur at an accelerated rate in the Basigin-null mouse retina. Therefore, the same normal and Basigin-null mouse retina lysates generated for the Cytochrome C analyses were subjected to ELISA analyses using antibodies specific for proteins known to be expressed within the

autophagy pathway. The proteins assayed are expressed at various points of autophagosome formation. Specifically, the assay measured relative amounts of APG1; which stimulates autophagy, APG16L and APG5L which are expressed during autophagic vesicle formation; BECN1, which regulates endolytic processes during late stage autophagy; and the GTPase RAB24, which is expressed during the formation and regulation of autophagic vacuoles [16]. As shown in Figure 2, no significant increase in expression of any autophagy proteins was observed in the Basigin-null mice, as compared to the littermate controls. Indeed, the expression levels of autophagy proteins APG16L, RAB24 and BECN1 were actually reduced when compared to the normal animals. It is important to note that relative levels of expression, but not the function/activity, of those proteins was assessed herein. These data do suggest, however, that autophagy is not activated in the Basigin null mouse retina.

The next step was to directly determine the relative activities of mitochondria in normal and Basigin-null mice using Mitotracker dyes (Molecular Probes/Invitrogen Corporation/Life Technologies). Retinas from Basigin-null mice and littermate controls at visual maturity and younger (post-natal days 21, 17, and 12) were isolated and mechanically dissociated in a saline buffer. The retinas were then incubated with a mixture of Mitotracker Red, which binds to metabolically active mitochondria, and Mitotracker Green, which binds all mitochondria. The red fluorescence, measured at 650 nm, provided a relative measure of mitochondria activity, since the dye is only taken up by mitochondria that possess a membrane potential. The green fluorescence, measured at 510 nm, allowed the total numbers of mitochondria to be normalized between the normal and null samples. It was determined that the percentages of active mitochondria are similar between the normal and Basigin-null samples, as shown in Figure 3A. However, the percentages of total mitochondria are reduced by ~50% in the Basigin-null mice when compared to those of control animals (Figure 3B). This is a significant finding, since the ages tested are well before retinal degeneration occurs in these animals [9], meaning that the two groups should have comparable numbers of photoreceptors. This reduction in total mitochondria was not considered statistically significant ($p=0.053$ via Student's T-test), likely due to the low number of replicates ($n=3$) and the variability in the ages tested. Basigin null mice are relatively difficult to breed [5], which limits the availability of additional animals for the study. The data do suggest; however, that altered metabolism precedes retinal degeneration in these animals.

Although the Mitotracker assay indicates a reduction in total numbers of mitochondria in Basigin-null mouse retinas, it is not known which cells of the retina have low numbers of mitochondria. A recent study in which Basigin expression within a human malignant melanoma cell line was knocked down using siRNA technology indicated that glycolytic output diminishes in the absence of Basigin [17]. Similar to what occurs in the Basigin-null mouse retina [10], the reduction in Basigin expression in the cell line led to the reduction of the expression of MCT1 at the plasma membrane [17]. Lactate produced by the Basigin knockdown melanoma cells cannot be exported from the cells and feedback inhibition reduces the rate of glycolysis [17]. It is therefore likely that Müller cells of the Basigin-null mouse retina undergo a similar phenomenon. Glycolysis rates would not likely affect total numbers of mitochondria within the same cell. It is therefore plausible to propose that the reduction in mitochondrial numbers is attributed to the photoreceptors.

To assess mitochondrial activity and numbers during postnatal development of the mouse retina, an additional assay was carried out. Retinas from normal mice at postnatal days 1, 7, 14, 21 and 28 were isolated and mechanically dissociated in a saline buffer. The retinas were then incubated with a mixture of Mitotracker Green and Mitotracker Red, as described. Figure 4 shows the results of the assay. It was determined that the number of active mitochondria within a mouse retina is greatest at visual maturity (postnatal day 21). A rather significant increase in mitochondrial numbers and activities between the second and third weeks of age was noted, which may explain the variability observed in the fluorescence for Basigin-null and littermate controls at the ages tested.

Conclusions

In conclusion, the Basigin-null neural retina has a metabolic deficiency that accounts for the blindness and retinal degeneration observed in those animals. Lactate produced by Müller cells is not transported to photoreceptors, as MCT1 is not expressed on either cell type [10]. The Müller cells likely have a reduced glycolytic output, as controlled via feedback inhibition by lactate [17]. The photoreceptor cells do not receive lactate and therefore have a reduction in oxidative phosphorylation and hence do not function [8]. Mitochondria are not being used in Basigin-null photoreceptors and therefore do not turnover at rates similar to that of normal photoreceptor cells. Neurons that do not function eventually die, which accounts for the degeneration observed [9]. It is humbling to realize how a simple metabolite can have such a tremendous impact on the health and well-being on a tissue.

Acknowledgments

This work was supported by NIH F32EY13918 and UNF Academic Affairs (to JDO) and UNF Graduate School (to KAP). We thank Dr. Cliff Ross and Dr. Michael Lentz, University of North Florida, for constructive criticisms and technical assistance.

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Abbreviations

RPE	retinal pigmented epithelium
MCT	monocarboxylate transporter
PBS	phosphate-buffered saline
ELISA	enzyme-linked immunosorbant assay

Highlights

Deletion of the Basigin gene in mice leads to a novel form of retinal dystrophy.

Deletion of the Basigin gene results in reduced numbers of mitochondria in the neural retina.

There is no change in levels of Cytochrome C or expression of autophagy-specific proteins.

It is thought that the reduction in mitochondria is due to lack of photoreceptor activity.

Figure 1. Basigin-null mouse retinas have less Cytochrome C than those of littermate controls Cytochrome C concentrations (ng/mL) in normal and Basigin-null mice retinas were determined using a Cytochrome C assay (Invitrogen Corporation). Samples of normal and Basigin-null mouse retina lysates were compared to a standard curve of known Cytochrome C concentrations to determine the concentration of cytochrome C within each sample. Runs were performed in duplicate. The average concentration at each age is plotted and the error bars indicate the standard deviation. Normal animals are indicated by the open bars, whereas the Basigin-null animals are indicated by the shaded bars.

Figure 2. Expression of autophagy-specific proteins is not increased in the Basigin-null mouse retina

Samples of normal and Basigin-null mouse retina protein lysates were compared for the relative expression of autophagy proteins via and ELISA analysis. Runs were performed in duplicate and the average absorbance at 405 nm is plotted. Error bars indicate the standard deviation for each sample. Normal mice are indicated by the open bars, whereas Basigin-null mice are indicated by the shaded bars.

Figure 3. The Basigin-null mouse retina possesses less mitochondria than that of littermate controls, at ages prior to retinal degeneration

The percentage of active mitochondria (A) were measured using Mitotracker Red FM dye (Invitrogen Corporation) and the percentage of total mitochondria (B) were measured using Mitotracker Green FM dye (Invitrogen Corporation). Runs were performed in triplicate. The value obtained for normal mouse retinas was set to 100% and those for Basigin null mouse retinas were compared. The error bars represent the standard deviations. Normal mice are indicated by the open bars, whereas Basigin-null mice are indicated by the shaded bars.

Figure 4. The mouse retina possesses the greatest number of mitochondria at retinal maturity Mitotracker Green (fluorescence at 510 nm) was used to assess of the total number of mitochondria (A) while Mitotracker red (fluorescence at 650 nm) was used to assess active mitochondria (B). Runs were performed in triplicate. The error bars represent the standard deviations.