Reduced protein kinase C delta association with a higher molecular weight complex in mitochondria of Barth Syndrome lymphoblasts

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Abstract

The protein kinase C delta (PKC δ) signalosome exists as a high molecular weight complex in mitochondria and controls mitochondrial oxidative phosphorylation. Barth Syndrome (BTHS) is a rare X-linked genetic disease in which mitochondrial oxidative phosphorylation is impaired due to a mutation in the gene TAFAZZIN which results in reduction in the phospholipid cardiolipin and an accumulation of monolysocardiolipin. Here we examined if PKC δ association with a higher molecular weight complex was altered in mitochondria of BTHS lymphoblasts. Immunoblot analysis of blue native-polyacrylamide gel electrophoresis mitochondrial fractions revealed that PKC δ associated with a higher molecular weight complex in control lymphoblasts but this was markedly reduced in BTHS patient B lymphoblasts in spite of an increase in PKC δ protein expression. We hypothesize that the lack of PKC δ within this higher molecular weight complex may contribute to defective mitochondrial PKC δ signaling and thus to the bioenergetic defects observed in BTHS.

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Introduction

Barth Syndrome (BTHS) is a rare X-linked genetic disease caused by a mutation in the TFAZZIN gene localized on chromosome Xq28.12 [1-3]. BTHS is characterized by cardiomyopathy, skeletal myopathy, growth retardation, neutropenia, and frequently 3-methylglutaconic aciduria. The TFAZZIN gene encodes the protein tafazzin. Tafazzin is a transacylase enzyme involved in the remodeling of the mitochondrial phospholipid cardiolipin (CL) [4,5]. BTHS mutations in TFAZZIN result in reduced CL and elevated monolysocardiolipin [1-3, 6]. In several studies Epstein-Barr virus transformed patient lymphoblasts have been used to examine BTHS metabolic pathology [6-9].

Protein kinase C delta (PKC δ) is a signaling kinase that regulates many cellular responses and is controlled via multi-site phosphorylation [10-13]. The PKC δ pathway adjusts the fuel flux from glycolytic sources to the intensity of mitochondrial respiration, thus controlling mitochondrial oxidative phosphorylation. In mitochondria, the PKC δ signalosome exists in a high molecular weight complex, which includes cytochrome c as the upstream driver of PKC δ , the adapter protein p66Shc as a platform and retinol [12, 14]. All four components are required for activation of PKC δ signaling in mitochondria. The expression of PKC δ and its association with a higher molecular weight complex in BTHS cells had never been investigated. We previously demonstrated that PKC δ phosphorylation was altered on several sites in BTHS patient lymphoblasts [15]. Since altered phosphorylation may affect PKC δ activation, we examined if PKC δ association with a higher molecular weight complex was altered in mitochondria of BTHS lymphoblasts.

In this study we demonstrate for the first time that PKC δ is associated with a higher molecular weight complex in lymphoblast mitochondria and that this is reduced in BTHS patient B lymphoblast mitochondria compared to age-matched control lymphoblasts in spite of an increase in PKC δ protein expression. We hypothesize that the lack of PKC δ within this complex may contribute to defective mitochondrial PKC δ signaling and thus to the bioenergetic defects observed in BTHS.

Materials and Methods

Epstein-Barr virus transformed BTHS lymphoblasts (Patient 618: Exon 2, c. 171 del. A, frameshift) and age-matched control lymphoblasts (Patient 3798) were a generous gift from Dr. Richard Kelley, Kennedy Krieger Institute, Baltimore, MD., and cultured as previously described [9]. Electrospray ionisation mass spectrometry (ESI-MS) coupled with high performance liquid chromatography (HPLC) mass spectrometry of cardiolipin (CL) and monolysocardiolipin (MLCL) from cell lysates was performed described [16]. Mitochondrial fractions were isolated using the mitochondrial isolation kit from Abcam (Toronto, ON, Canada, Catalog number ab110170). Mitochondrial protein content was determined using the M protein assay kit (Mississauga, ON, Canada). For Blue-Native polyacrylamide gel electrophoresis (BN-PAGE) analysis, mitochondrial protein (80 μ g) was treated with 0.2% digitonin and then separated on a 3-12% gradient gel as described [9].

Immunoblot analysis of the gel was performed using anti-PKC δ antibody (1:1000) (Abcam, Toronto, ON, Canada) as described [17]. PKC δ was visualized using the Amersham Enhanced Chemiluminescence Western blotting detection system (VWR,

Mississauga, ON, Canada). Band intensity was quantified using Image J software. Citrate synthase activity was measured using the citrate synthase assay kit (Sigma-Aldrich, Oakville, ON, Canada, Catalog number CS0720). Data are expressed as mean $\pm$ standard deviation of the mean. Comparisons between control and BTHS patient lymphoblasts were performed by unpaired, two-tailed Student *t* test. A probability value of $p < 0.05$ was considered significant.

Results

The number of each major fatty acyl substituent on individual CL and MLCL molecular species is indicated in **Table 1** and a representative chromatograph of the different CL molecular species between age-matched control and BTHS lymphoblasts is indicated in **Figure 1**. All major molecular species of CL were significantly reduced in BTHS lymphoblasts compared to age-matched control lymphoblasts (**Figure 2A**). Accompanying this reduction in CL molecular species was a general, but not significant increase in most major MLCL species. In contrast, a >20-fold increase ($p < 0.01$) in trioleoyl-MLCL (mass/charge (*m/z*) 1192) molecular species was observed in BTHS lymphoblasts compared to age-matched control lymphoblasts (**Figure 2B**).

BTHS patient lymphoblasts exhibited abnormally increased mitochondrial mass [7, 8]. To confirm this, mitochondrial fractions were prepared and citrate synthase activity determined. Citrate synthase activity was elevated 20% ($p < 0.05$) in BTHS lymphoblasts compared to age-matched control cells (**Figure 2C**). Thus, the reduction in CL, increase in MLCL and MLCL/CL ratio and increase in citrate synthase activity was consistent with that observed in BTHS patient B lymphoblast cells.

We previously observed that PKC δ phosphorylation was altered on several sites examined in BTHS lymphoblasts [15]. Since altered phosphorylation may affect PKC δ activation [13], we examined if this was associated with altered PKC δ associated with higher molecular weight complex in mitochondria of BTHS patient lymphoblasts. Mitochondrial fractions were subjected to BN-PAGE followed by immunoblot analysis for determination of PKC δ levels. The two upper bands indicated on the left of the blot are molecular mass markers at 1236 kDa and 1048 kDa, respectively (**Figure 3**). The level of PKC δ located on the gel at a predicted molecular mass near 77.5 kDa was elevated 1.5-fold ($p < 0.05$) in BTHS lymphoblasts compared to age-matched control cells. In contrast, the level of PKC δ located on the gel at approximately 480 kDa was reduced 72% ($p < 0.01$) in BTHS lymphoblasts compared to age-matched control cells. Thus, BTHS lymphoblasts exhibit elevated expression of PKC δ but reduced PKC δ association with a higher molecular weight complex in mitochondria.

Discussion

Protein kinase C delta (PKC δ) is a signaling kinase that regulates a plethora of cellular responses [10-13]. In this study, we examined whether the association of PKC δ with other proteins (which results in the formation of higher molecular weight complexes) was altered in mitochondria of BTHS lymphoblasts. We demonstrate that PKC δ associates with a higher molecular weight complex in lymphoblast mitochondria and that PKC δ higher molecular weight complex formation in mitochondria is reduced in BTHS patient lymphoblasts.

BTHS is a rare X-linked genetic disease and is the only known disease in which the specific biochemical defect is a reduction in CL and accumulation of MLCL [1-3]. We observed a reduction in all major molecular species of CL in BTHS lymphoblasts accompanied by a >20-fold elevation in trioleoyl-MLCL. Previous studies demonstrated an increase in abnormal mitochondrial mass in BTHS patient lymphoblasts [7, 8]. We confirmed this observation in our BTHS patient lymphoblasts through an increase in mitochondrial citrate synthase activity.

BTHS lymphoblasts exhibit elevated oxidative stress and increased reactive oxygen species [8, 9]. Oxidative stress is known to induce expression of PKC δ [18]. We observed increased protein expression of PKC δ in mitochondria of BTHS lymphoblasts compared to controls. The elevated PKC δ levels observed might serve as a compensatory mechanism to increase ATP production in BTHS cells through PKC δ signaling [12]. Additionally, elevated expression of PKC δ promotes mitochondrial proliferation [18]. As indicated above abnormal proliferation of BTHS lymphoblast mitochondria has been previously observed [7, 8]. Phosphorylation of PKC δ is required for its activation [13]. We previously observed alteration in phosphorylation of PKC δ in BTHS lymphoblasts [15]. Altered phosphorylation of PKC δ might contribute to an attenuated mitochondrial PKC δ signaling in these cells.

The molecular components that mediate PKC δ signaling in mitochondria are beginning to emerge. Mitochondria contain a high molecular weight functional complex, which includes cytochrome *c* as the upstream driver of PKC δ , and it uses the adapter protein p66Shc as a platform with vitamin A (retinol) [12, 14, 19]. All four partners are required for functional PKC δ signaling. BN-PAGE immunoblot analysis of mitochondrial

proteins not only has the advantage of probing for expression of individual proteins but may additionally be used to detect if these proteins are associated with higher molecular weight complexes. Using this approach we observed reduction in PKC δ association with a higher molecular weight complex in mitochondria.

The PKC δ /retinol complex signals the pyruvate dehydrogenase complex for enhanced flux of pyruvate into the Krebs's cycle [12, 14]. Interestingly, in the UK BTHS NHS clinic almost half of BTHS boys examined were vitamin A deficient (Nicol Clayton: https://www.youtube.com/watch?v=wNDr_oCTJ7A). However, supplementation with vitamin A did not increase plasma levels. This was not because tissue levels were low but possibly due to increased levels of vitamin A (retinyl esters) in chylomicrons. It is possible that this unique observation is coupled to defective mitochondrial PKC δ signaling which might contribute to a reduced Krebs's cycle ATP production through alteration in the mitochondrial PKC δ /retinol signaling complex and contribute to the multitude of bioenergetic defects observed in BTHS.

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Author contributions

E.M.M and G.C.S. performed experiments. H.M.Z. and G.M.H wrote the manuscript. All authors read and edited the manuscript.

Conflict of interest statement

The authors of this study have no conflict of interest

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Figure Legends

Figure 1. Comparison of CL spectra between age-matched control and BTHS lymphoblasts.

Representative chromatograph of the major CL species quantified using ESI-MS coupled with HPLC mass spectrometry of age-matched control (Green solid line) and BTHS lymphoblasts (Blue dashed line).

Figure 2. CL levels are reduced and trioleoyl-MLCL and citrate synthase activity elevated in BTHS lymphoblasts.

Quantification of the major CL (**A**) and MLCL (**B**) fatty acyl molecular species in age-matched control and BTHS lymphoblasts as described in Materials and Methods. **C.** Mitochondrial fractions were prepared from age-matched control and BTHS lymphoblasts and citrate synthase activity determined as described in Materials and Methods. Data represent the mean $\pm$ SD, n=3. #p<0.001, **p<0.01, *p<0.05, ns, not significant.

Figure 3. BTHS lymphoblasts exhibit reduced PKC δ association with a higher molecular weight complex in mitochondria.

Mitochondrial fractions were prepared from age-matched control and BTHS lymphoblasts and subjected to BN-PAGE followed by immunoblot analysis of PKC δ . **A.** Age matched control (lanes 1, 3, 5 and 7); BTHS lymphoblasts (lanes 2, 4, 6 and 8). Molecular mass markers are in the first lane and indicated on the left. **B.** Quantification of PKC δ .

Table 1. Major CL and MLCL species identified in age-matched control and BTHS lymphoblasts.

m/z	Number of each fatty acyl substituent on individual CL molecular species							
	16:0	16:1	18:0	18:1	18:2	18:3	20:4	22:6
1422		1			3			
1424		1		1	2			
1426		1		2	1			
1428		1		3				
1448					4			
1450				1	3			
1452				2	2			
1454				3	1			
1456				4				
1472					3		1	
1474				1	2		1	
1476				2	1		1	
1478				3			1	
1496					3			1
1498				1	2			1
1500				2	1			1
1502				3				1

Monolysocardiolipins

1186					3			
1188				1	2			
1190				2	1			
1192				3				

Figure 1

Figure 2

A

B

C

Figure 3

A

B