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Dysfunction of peroxisomes in twitcher mice brain: A possible mechanism of psychosine-induced disease ☆

Ehtishamul Haq [a], Miguel A. Contreras [a], Shailendra Giri [a], Inderjit Singh [a], Avtar K. Singh [b,*]

[a] *Department of Pediatrics and The Children's Research Institute, Medical University of South Carolina, Charleston, SC 29425, USA*
[b] *Department of Pathology and Laboratory Medicine, Medical University of South Carolina and Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29425, USA*

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Abstract

Psychosine (galactosylsphingosine) accumulates in the brain of Krabbe disease (KD) patients as well as twitcher mice, a murine model of KD, resulting in loss of oligodendrocytes and myelin. This study documents progressive loss of peroxisomal proteins/functions and induction of expression of inflammatory cytokine TNF-α in twitcher brain. The observed decrease in peroxisomal proteins was accompanied by decreased level of peroxisome proliferator-activated receptor-alpha (PPAR-α), one of the transcription factors required for expression of peroxisomal protein genes. The role of psychosine in down-regulation of PPAR-α activity was further supported by decreased PPAR-α mediated PPRE transcriptional activity in cells transfected with PPAR-α and PPRE reporters. The psychosine-induced down-regulation of PPAR activity and cell death was attenuated by $sPLA_2$ inhibitor. Therefore, this study provides the first evidence of peroxisomal abnormality in a lysosomal disorder, suggesting that such dysfunction of peroxisomes may play a role in the pathogenesis of Krabbe disease.

Keywords: Psychosine; Krabbe disease; Peroxisomes; Twitcher mice; Neuroinflammation

Krabbe disease (globoid cell leukodystrophy) is an inherited demyelinating disease caused by deficiency of the lysosomal enzyme galactocerebrosidase [1]. Galactocerebrosidase catalyzes the hydrolysis of galactose from several glycosphingolipids, including galactocerebrosides and psychosine (galactosylsphingosine) [2]. Excessive accumulation of psychosine has been proposed to be a causative lipid for the loss of oligodendrocytes and myelin in Krabbe disease; however, the mechanism of psychosine-mediated loss of oligodendrocytes is not understood well. According to the "psychosine hypothesis," psychosine accumulates mainly in the brain of Krabbe patients and twitcher mice (*twi*/*twi*; an authentic murine model of Krabbe's disease) and psychosine toxicity is believed to cause loss of oligodendrocytes [3]. Twitcher (*twi*/*twi*) mice are clinically normal until 20 days of age; after which their growth slows down and they develop typical neurological signs including tremors and hind leg weakness. Disease progression is rapid and the mice rarely survive beyond 37–40 days of age [4]. The nature of a causal relationship between psychosine accumulation-globoid cell formation and disappearance of oligodendrocytes, and demyelination/dysmyelination is not understood.

The oligodendrocyte is a myelin-forming cell, and plasmalogens are a major component of the myelin sheath, and by far the most abundant ether lipids that constitute almost 70% of the total phosphatidylethanolamine fraction in human brain white matter [5]. Plasmalogens are speculated to participate in diverse physiological processes such as modulation of membrane fluidity, facilitation of membrane fusion, mediation of signal transduction processes, and protection against oxidative stress [6]. The first two enzymes for

☆ *Abbreviations:* Alkyl-DHAP-syn, alkyl-dihydroxyacetonephosphate synthase; ACO, acyl Coenzyme A oxidase; DHA, docosahexaenoic acid; DEDA, 7,7-dimethyleicosadienoic acid; Con, control; Twi, twitcher.

* Corresponding author. Fax: +1 843 792 7130.
E-mail address: singha@musc.edu (A.K. Singh).

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the synthesis of plasmalogens are localized in peroxisomes, therefore loss of peroxisomes or these enzymes is known to result in decreased synthesis of myelin [7]. A major constituent of plasmalogens in brain is docosahexaenoic acid (DHA), and DHA is also synthesized in peroxisomes [8]. The observed loss of peroxisomal functions (VLC fatty acid β-oxidation and synthesis of plasmalogens) in psychosine-treated C_6 glial cells indicates that loss of peroxisomal functions may play a role in the pathobiology of Krabbe disease [9]. The deficient synthesis of plasmalogens, a peroxisomal function, in twitcher mice brain [9] may lead to dysmyelination/demyelination. Peroxisomes are also a major site for the turnover of very long chain (VLC) fatty acids and eicosanoids [10,11], therefore, impairment of peroxisomal functions may lead to higher bioavailability of these lipids, thus extending their detrimental effects.

The importance of peroxisomes in human health is further emphasized by the identification of a group of genetic disorders called peroxisomal disorders, most of which affect the CNS and are fatal [12–14]. These disorders can be subdivided into two groups depending on whether there is a generalized biogenesis defect or loss of a single enzyme which compromises peroxisomal function. Peroxisomal metabolic derangement of very long chain fatty acids in patients with X-adrenoleukodystrophy is known to cause secondary neuroinflammatory disease-mediated loss of oligodendrocytes and demyelination and associated morbidity and mortality [15,16]. The expression of proinflammatory cytokines (TNF-α and IL-6) in brain from Krabbe patients and twitcher mice was reported recently [17,18]. TNF-α is a well-established pro-inflammatory mediator of the immune process and is essential for the maintenance of CNS homeostasis. However, its over expression leads to the development of chronic CNS inflammation and degeneration [19]. We have also previously reported the induction of iNOS and NO-mediated toxicity in Krabbe brain [20]. Proinflammatory mediators (cytokines and LPS) are known to down-regulate the expression of peroxisomal proteins in liver hepatocytes [21] and alter the structure/function of peroxisomes in liver of rats treated with a sublethal dose of LPS [22,23].

Recently, we have also reported that treatment of C_6 glial cells with psychosine down-regulates the peroxisomal proteins/functions and that combination of psychosine and cytokines down-regulates peroxisomal proteins/functions further [9]. Here, we report that in twitcher mice brain progressive accumulation of psychosine is associated with a progressive increase in the levels of proinflammatory cytokine TNF-α and a concomitant decrease in two important proteins of peroxisomes (acyl-CoA oxidase for fatty acid β-oxidation and alkyl-DHAP synthase for biosynthesis of plasmalogens) as well as decreased expression of peroxisomal proliferator-activated receptor-alpha (PPAR-α), a ligand-activated transcription factor for expression of peroxisomal genes [24]. However, on the other hand there were no changes in the mRNA, protein or activity of catalase, another peroxisomal protein. The observed decrease in peroxisomal proteins and PPAR-α protein in twitcher mice brain and psychosine-mediated inhibition of PPAR-α-dependent transcriptional activity in C_6 glial cells indicate that there is psychosine-mediated down-regulation of the expression of peroxisomal proteins in brain of the Krabbe disease animal model. The studies described here also document a role of $sPLA_2$-mediated signaling events in psychosine-induced down-regulation of PPAR-α activity and apoptotic death of oligodendrocytes.

Materials and methods

Experimental procedures

Animals. All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina. Twitcher heterozygote breeding pairs (C57BL/6J $twi^{-/+}$) were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained at the University animal facility. *Twi*/*twi*$^{(-/-)}$ and normal age-matched siblings wild type $^{(+/+)}$ were identified by genotyping with genomic DNA extracted from the clipped tails by use of a DNA Isolation Kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). Genotyping was performed as reported earlier [9].

Western blot analysis. Western blot analysis for alkyl-DHAP-synthase, catalase, ACO, and PPAR-α was performed using standard procedures. Brains were harvested and homogenized in lysis buffer (50 mM Tris–HC1 (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and 0.5% Nonidet P-40) containing a protease inhibitor cocktail (Sigma–Aldrich, USA). The lysates were clarified by centrifugation at 10,000*g* for 15 min at 4 °C. Equal amounts of supernatant protein were subjected to SDS–PAGE (4–20% Tris–HCl Criterion gradient Gel; Bio-Rad Laboratories Hercules, CA) and electrophoretically transferred to a High-Bond nitrocellulose membrane (Amersham Life Science, Arlington Heights, IL) [25]. After blocking with Tween 20 Tris-buffered saline (TTBS; 20 mM Tris–HCl (pH 7.6), 137 mM NaCl, and 0.05% Tween 20) containing 5% nonfat milk for 1 h at room temperature, the membranes were incubated for 1 h at room temperature or overnight at 4 °C with the primary antibodies at 1:2000 dilution in blocking buffer (5% nonfat milk inTTBS). The membranes were then washed three times for 10 min each in TTBS and incubated with an appropriately diluted horseradish peroxidase-labeled secondary antibody (1:10,000) in blocking buffer for 1 h at room temperature. The membranes were washed three times, reacted with ECL reagent (Amersham Life Science), and subjected to autoradiography. Expression of β-actin was used as an internal standard for equal protein loading.

Immunohistochemistry. Expression of alkyl-DHAP synthase and TNF-α was detected by immunohistochemical analysis using specific antibodies. Paraffin-embedded sections from the formalin-fixed brain tissues were stained for alkyl-DHAP synthase and TNF-α [26]. In brief, the brain sections were deparaffinized, sequentially rehydrated in graded alcohol, and then immersed in PBS (pH 7.4). Slides were then microwaved for 10 min in antigen unmasking solution (Vector Labs), cooled, and washed two times for 5 min. in PBS containing 0.05% Tween 20 (PBS-T). Sections were blocked in immunohistochemical grade 1% bovine serum albumin in PBS containing 0.1% Triton X-100 for 45 min. Sections were incubated overnight with rabbit polyclonal TNF-α antibody (1:100, Santa Cruz Biotechnology, CA, USA); rabbit polyclonal alkyl-DHAP-synthase antibody (1:100, custom-made). After incubation, they were rinsed three times for 5 min. in PBS-T. Sections were then incubated with anti-rabbit HRP reagent for 30 min. at room temperature and washed three times for 5 min in PBS-T. Slides were then incubated for 5–10 min in biotinyl tyramide amplification reagent (Perkin-Elmer, Boston, MA, USA) and washed again three times for 5 min in PBS-T. Slides were then incubated with Streptavidin-Texas red fluorophore (Perkin-Elmer, Boston, MA, USA) for 30 min. at room temperature. The slides were then washed three times for 5 min. in PBS-T and mounted for fluorescence microscopy. The sections were examined under a fluorescence microscope (Olympus BX-60) with an

Olympus digital camera (Optronics, Goleta, CA) at 40× magnification for TNF-α and a 60× oil objective for alkyl-DHAP synthase. Images were captured and processed using Adobe Photoshop 7.0.

Quantitative real-time PCR. RNA was isolated from brain samples using Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). cDNA was synthesized from RNA by using the iscript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. For quantitative real-time PCR, a PCR master mixture was prepared using the SYBRGreen PCR Master Mix (Applied Biosystems, Foster City, USA) and aliquoted into microplate wells together with 1 μl template and 250 nM of each primer for a final volume of 25 μl per reaction. All samples were run in triplicate. Thermal cycling conditions were as follows: activation of AmpliTaq gold DNA polymerase at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 30 s and 58–60 °C for 1 min. The expression of target gene was normalized with 18 s rRNA. The primers were designed using the Premier Biosoft International software (Bio-Rad) and synthesized from Integrated DNA Technologies (IDT, Coralville, IA). The sequence of primers was: 18s rRNA, forward primer (Fw) 5′-CGTCTGCCCTATCAACTTTCG-3′ and reverse primer (Rv) 5′-GCCTGCTGCCTTCCTTGG-3′; catalase, Fw 5′-CAGG TGCGGACATTCTAC-3′ and Rv 5′-GCGTTCTTAGGCTTCTCAG-3′; alkyl-DHAP synthase, Fw 5′-CCAAGAACGGCAAGAAGTTATG-3′ and Rv 5′-TTATGCTCCAGACTTACTCCAAGG-3′; PPAR-α, Fw 5′-C TGGGTCCTCTGGTTGTC-3′ and Rv 5′-TTGGCTCTCCTCTAAGT TCC-3′; Acyl-CoA oxidase (ACO), Fw 5′-CCGCCGCCACCTTCAATC-3′ and Rv 5′-TCTTCTTAACAGCCACCTCGTAAC-3′; IL-6, Fw 5′-GAGGATACCACTCCCAACAGACC-3′ and Rv 5′-AAGTGCATCAT CGTTGTTCATACA-3′; and TNF-α, Fw 5′- CATCTTCTCAAAATTC GAGTGACAAA-3′ and Rv 5′-TGGGACTAGACAAGGTACAACC C-3′.

Enzymatic assay for catalase. Enzymatic activity of catalase was determined using hydrogen peroxide as substrate in a colorimetric assay (Shimadzu spectrophotometer UV-1601) in the presence of Triton X-100. The substrate that remained after a period of incubation at 4 °C was detected by formation of a colored complex with titanium oxysulfate which was determined at 405 nm [26]. Protein concentration was determined according to the method of Bradford using bovine serum albumin as protein standard [27].

Cell culture. C_6 rat glioma cells were obtained from ATCC (Manassas, VA), and MO 3.13 cells were a kind gift from Catherine Waters (Biological Sciences, University of Manchester, UK). The cells were maintained in DMEM F12 containing 10% FBS and 10μg/ml gentamicin. All cultured cells were maintained at 37 °C in 5% CO_2/95% air. At 80% confluency the cells were incubated with serum-free DMEM F12 medium for 24 h prior to incubation with psychosine treatment. Serum-starved MO 3.13 and C_6 cells had greater oligodendrocyte properties as characterized and described in our earlier publications [25,28]. The viability of the cell was assayed by Trypan blue dye exclusion. C_6 cells and oligodendrocytes remained viable up to 72 h at a concentration of 10 μM of psychosine. Psychosine was first dissolved in DMSO and mixed in the medium at a DMSO concentration 0.1% of the medium.

Caspase 3 assay and DNA ladder. Caspase-3 activity assay and DNA laddering were done as mentioned in our previous publication [25].

Plasmids. The peroxisome proliferator-response element (PPRE)-containing reporter plasmid (J6-thymidine kinase (TK)-Luc) and PPAR-α expression vector were provided by Dr. B. Staels (Institut Pasteur de Lille, Lille, France) [29]. PPAR-α-GAL4 chimera and the reporter plasmid (upstream activating sequences)$_5$-TK-CAT were provided by Dr. Steven A. Kliewer [30].

Transcriptional assays. MO 3.13 oligodendroglial cells or C_6 glial cells were transiently transfected with PPRE-luciferase reporter gene (1.5 μg/well) or PPAR-α-Gal with β-gal (0.1 μg/well) in the presence or absence of cDNAs (as indicated in figures, 0.5 μg/well) by lipofectamine-2000 (Invitrogen Life Technologies), as described previously [31]. pcDNA3 was used to normalize all groups to equal amounts of DNA. Luciferase activity and CAT were determined using a luciferase assay kit (Promega) and CAT ELISA kit (Roche Diagnostics).

Results

Developmental expression of TNF-α and peroxisomal enzyme alkyl-DHAP synthase

In normal brain, the levels of psychosine are undetectable and due to the deficiency of galactocerebrosidase its accumulation in twitcher mice can be detected starting post-natal day 7 with exponential increase during the life span of the mice [32]. Recently, we documented that psychosine treatment down-regulates the functions of peroxisomes/proteins in C_6 cells and these effects were further amplified when cells were treated with a combination of psychosine and pro-inflammatory cytokines [9]. To further establish the relationship between psychosine, peroxisomes, and the neuroinflammatory disease process in Krabbe brain, we examined the post-natal expression of alkyl-DHAP-synthase (a peroxisomal enzyme) and TNF-α during post-natal development in brain of twitcher mice. Fig. 1A shows that TNF-α expression, studied by immunohistochemistry, increased in twitcher brain sections from post-natal day 25 whereas no TNF-α was detected in brains of control animals. Alkyl-DHAP-synthase was detected in control brains of all ages but in twitcher mice brains its levels progressively decreased after post-natal day 21 (Fig. 1B). We further assessed protein levels of alkyl-DHAP-synthase and catalase, two peroxisomal proteins, during development by Western blotting analysis (Fig. 1C). Alkyl-DHAP synthase protein remained consistent during post-natal development in control brain whereas in agreement with immunohistochemistry studies the levels of alkyl-DHAP synthase progressively decreased during post-natal development. On the other hand, no change was observed in the levels of catalase, during development indicating differential regulation of peroxisomal proteins (Fig. 1C).

Levels of peroxisomal proteins and TNF-α at post-natal day 32 in control and twitcher brain

We further assessed three important functions of peroxisomes by measuring the protein and mRNA levels of catalase, an enzyme responsible for detoxification of H_2O_2 in peroxisomes, acyl-CoA oxidase, a rate-limiting enzyme of peroxisomal fatty acid β-oxidation system in peroxisomes, and alkyl-DHAP synthase, a rate-limiting enzyme for synthesis of plasmalogens. As shown in Figs. 2A–C, there were no differences in the activity, protein, and mRNA levels of catalase in twitcher brain as compared to age-matched controls. The levels of alkyl-DHAP synthase and acyl-CoA oxidase were significantly decreased in twitcher brain as compared to age-matched controls at both protein (Fig. 3A) as well as mRNA levels (Figs. 3B and C). There was a significant increase in the levels of TNF-α mRNA in twitcher brain as compared to age-matched controls (Fig. 3D). These observations also indicate that regulation

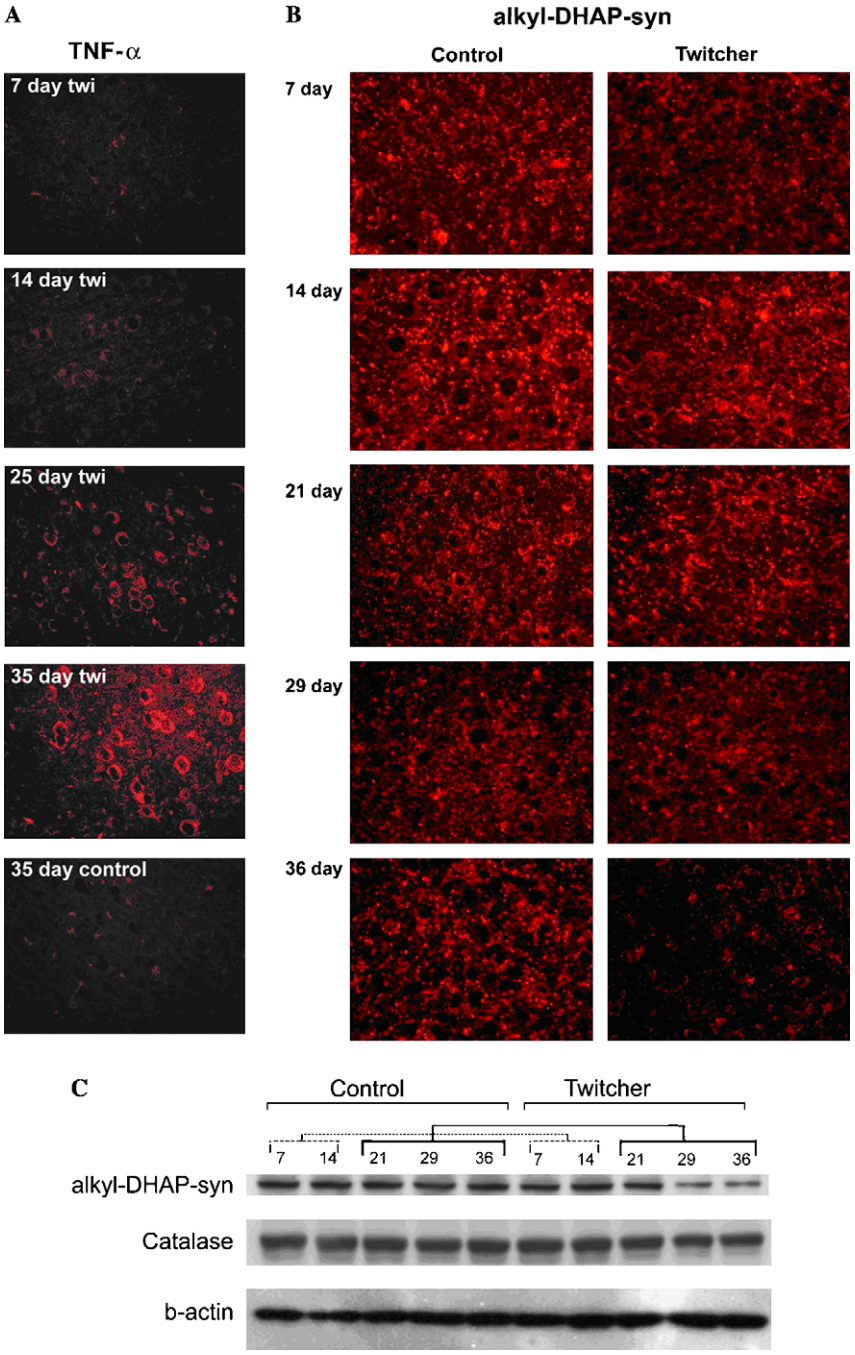

Fig. 1. Down-regulation of peroxisomal enzyme alkyl-DHAP-synthase in twitcher mice brain. Immunohistochemical and Western blot analysis in twitcher mice brain was performed as mentioned in Materials and methods. Immunohistochemistry for TNF-α (A) and alkyl-DHAP-synthase (B) in twitcher brain sections was performed using antibodies against TNF-α and alkyl-DHAP-synthase at different stages of twitcher development. Western blot analysis of alkyl-DHAP synthase and catalase (C) levels in brain homogenates. For Western blot analysis, brains were isolated from twitcher and age-matched controls at different time points of age, and were frozen at −70 °C untill use. β-actin was used for equal protein loading.

of expression of catalase differs from the two other peroxisomal proteins studied.

Psychosine inhibits the peroxisomal proliferator-activated receptor alpha-dependent transcriptional activity

It is well established that PPAR-α in combination with retinoic acid receptor (RXR) regulates the expression of mRNA for peroxisomal proteins by acting on peroxisomal proliferation response elements (PPRE) present in the promoter region of the genes of peroxisomal proteins [33]. Agonists of PPAR-α are known to cause proliferation (increased biosynthesis) of peroxisomes. As shown in Fig. 4, the levels of PPAR-α were significantly reduced in twitcher brain of 35-day-old mice as compared to age-matched controls both at the mRNA and proteins levels.

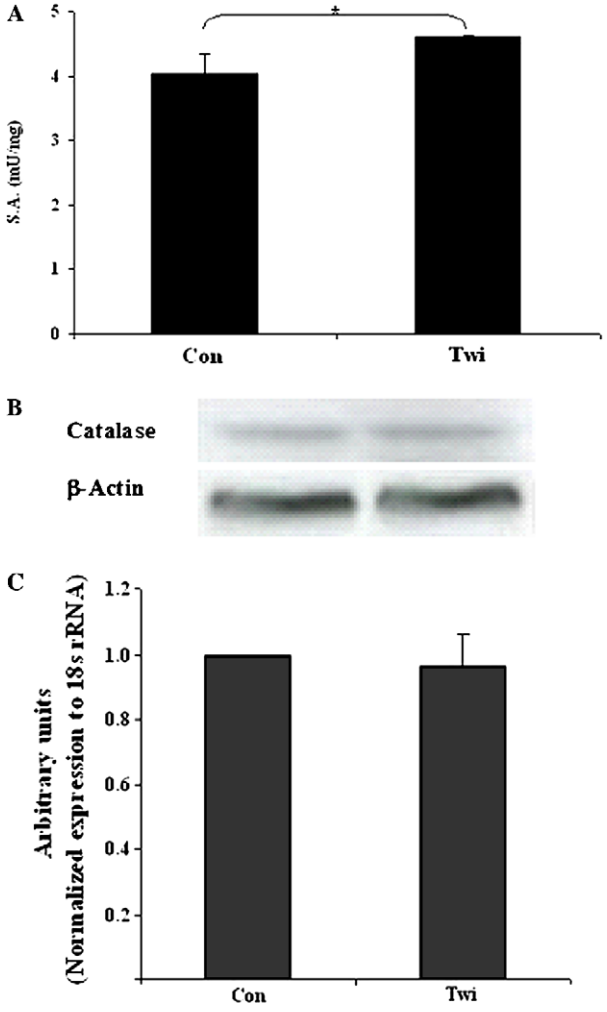

Fig. 2. Catalase in the brain of control and twitcher mouse brain homogenate. Activity (A), protein (B), and mRNA levels (C) of catalase were measured in the brain homogenates from twitcher and age-matched control mice at 35-day age as described under Materials and methods. Values are average + SD; $^{*}P < 0.03$ ($n = 3$).

These decreased levels of PPAR-α in the twitcher brain may be responsible for the decreased levels of peroxisomal proteins in twitcher brain.

To further understand the mechanism of psychosine-mediated loss of peroxisomal functions, we investigated whether psychosine mediates its effect via down-regulation of PPAR-α-mediated transcriptional activity, in C_6 glial cells transfected with a PPRE-luciferase reporter construct. The basal level of PPRE-reporter activity was significantly higher as compared to cells treated with reporter parent vector (PGL3-basic). Treatment of these cells with psychosine down-regulated the PPRE-reporter activity in a dose-dependent manner (Fig. 5A). Further to confirm that psychosine down-regulates PPAR-α-mediated induction of PPRE activity, we co-transfected C_6 glial cells with an expression vector of PPAR-α gene and PPRE-reporter, and examined the effect of psychosine on its reporter activity (Fig. 5A). Co-transfection of PPAR-α significantly induced PPRE-reporter activity as compared to pcDNA3 transfected reporter activity, which was down-regulated by psychosine treatment (Fig. 5A). To further strengthen the conclusion of psychosine-induced down-regulation of PPAR-α transcriptional activity, we employed a chimeric receptor system in which the putative ligand-binding domain of the PPAR-α is fused to the DNA-binding domain of the yeast transcription factor galactose-responsive gene 4 (GAL4). pCMV-GAL4-binding domain (without insert) and UAS (upstream activating sequences)$_5$-TK-CAT were transfected as control to detect the basal levels of chloramphenicol acetyltransferase (CAT) activity. Transfection of C_6 glial cells with PPAR-α-Gal showed significantly higher CAT reporter activity as compared to basal activity (Fig. 5B). The inhibition of PPAR-α-dependent CAT reporter activity by psychosine treatment further confirms that psychosine down-regulates PPAR-α-dependent transcriptional activity in brain glial cells. Taken together these observations indicate that psychosine-mediated inhibition of PPAR-α transcriptional activity is responsible for the observed decreased levels of peroxisomal proteins and peroxisomes in twitcher mice brain.

Secretory phospholipase-A_2 inhibitor 7,7-dimethyleicosadienoic acid inhibits psychosine-mediated cell death and reverses psychosine-mediated inhibition of PPAR-α

While studying the mechanism of psychosine-induced cell death, we have recently found that psychosine upregulates generation of lysophosphatidylcholine (LPC) and arachidonic acid (AA) by activating secretory phospholipase-A_2 (sPLA$_2$) in oligodendroglial cell line, MO 3.13. Further we wanted to examine whether sPLA$_2$ inhibitor (DEDA; 7,7-dimethyleicosadienoic acid) will reverse the effects of psychosine on PPAR-α. Oligodendroglial MO 3.13 cells were treated with DEDA (10 μM) in the presence or absence of psychosine (20 μM) for 48 h and cell viability was determined by MTT assay. In agreement with our previous observations [25], psychosine reduced cell viability significantly, and this effect of psychosine was completely reversed by DEDA, whereas DEDA alone had no effect on cell viability (Fig. 6A). Further to examine its effect on DNA fragmentation, psychosine- and DEDA-treated cells were lysed in DNA lysis buffer after 48 h of treatment and DNA fragmentation was analyzed. Similar to cell viability observation, psychosine-induced DNA fragmentation, an important hallmark of apoptotic cell death, was blocked by DEDA treatment in a dose-dependent manner (Fig. 6B). Cell death by psychosine was further confirmed by caspase-3 activity as reported earlier [25], psychosine treatment of MO 3.13 cells for 48 h significantly induced the caspase-3 activity, and DEDA (10 μM) completely reversed the caspase-3 activation by psychosine (Fig. 6C). To determine the effect of DEDA on psychosine-mediated down-regulation of PPAR-α transcriptional activity, we transfected MO 3.13 oligodendroglial cells with PPAR-α-Gal chimeria along with Gal4-CAT reporter

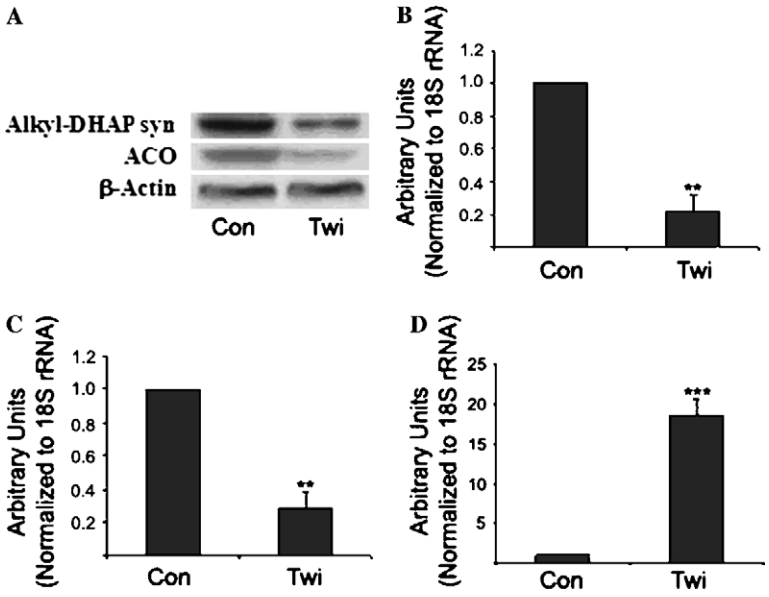

Fig. 3. Western blot and real-time PCR analysis. Western blot analysis for peroxisomal enzymes alkyl-DHAP-synthase and acyl-CoA oxidase (ACO) was performed in twitcher brain homogenates (A). Results are representative of two different experiments. Real-time PCR analysis of alkyl-DHAP-synthase (B), ACO (C) and TNF-α (D) in the 35-day-old brain tissue of twitcher and age-matched control was performed as described under Materials and methods. The copies of mRNA for TNF-α have increased in *twi*/*twi* when compared to those in control (D), while copies of mRNA for alkyl-DHAP-synthase and ACO have decreased in *twi*/*twi* when compared to those in control (B,C). $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to respective control.

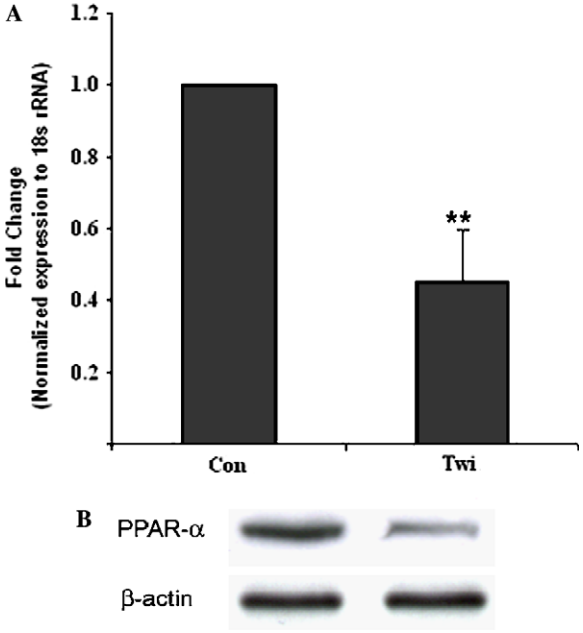

Fig. 4. Peroxisomal proliferator activated receptor alpha (PPAR-α) in brain of twitcher and control mice. Real-time PCR analysis for mRNA (A). Western blot analysis of PPAR-α (B) in the brain homogenates from twitcher and age-matched control mice at 35-day age. $^{**}P < 0.01$ as compared to control.

and examined the effect of psychosine in the presence or absence of DEDA on PPAR-α transcription. Transfection of MO 3.13 cells with PPAR-α-Gal showed significantly higher CAT reporter activity as compared to basal activity, and PPAR-α-dependent CAT reporter activity was significantly inhibited by psychosine treatment, and that pre-treatment with different concentrations of DEDA completely reversed the inhibitory effect of psychosine (Fig. 6D). These findings document the role of $sPLA_2$ in psychosine-induced down-regulation of PPAR-α transcriptional activity and apoptotic loss of oligodendrocytes and that inhibition of sPLA2 by DEDA attenuated both the loss of PPAR-α transcriptional activity as well as loss of oligodendrocytes.

Discussion

Pathognomonic accumulation of psychosine reported under the psychosine "hypothesis" and subsequent induction of proinflammatory cytokines TNF-α and IL-6 [17,18] and iNOS and possibly $ONOO^-$ [20] are reported to play a role in the pathobiology of Krabbe disease [20]. The observed apoptotic cells in Krabbe [34] and twitcher brains [35], and psychosine-induced apoptotic cell death of oligodendrocytes in culture document the possible role of psychosine in cellular signals related to apoptotic cell loss [25]. Recently, we had observed decreased levels of plasmalogens and increased levels of VLC fatty acids (two metabolites of peroxisomes) in twitcher brain indicating a possible role for peroxisomes in the pathobiology of twitcher brain [9]. Therefore, we evaluated the structure/function of peroxisomes in twitcher brain during development. Twitcher mice are normal until post-natal day 20, subsequently develop neurological disease including tremor with rapid disease progression, and rarely survive beyond 37–40 days of age [4]. Our immunohistochemical studies for TNF-α document

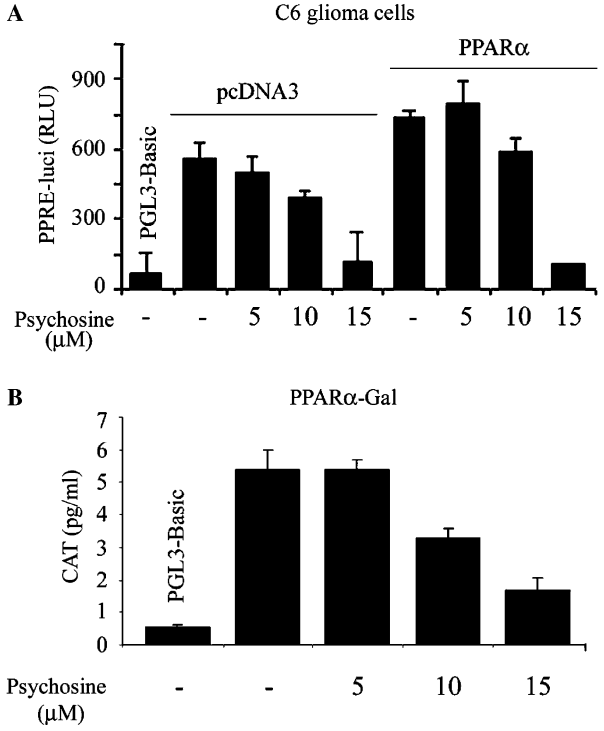

Fig. 5. Psychosine inhibits PPAR-α-mediated transcriptional activity in C6 glial cell. C6 glial cells were transiently transfected with PPRE-luc vector (1.5 μg/well) and pCMV-β-gal (0.1 μg/well) along with or without PPAR-α expression vector. After 24 h, cells were treated with different concentrations of psychosine (5–15 μM) and luciferase activity was normalized with respect to β-Gal activity (A). Total content of DNA was normalized with pcDNA. PGL3-Basic was transfected as a control to detect the basal levels of luciferase activity. Data are means ± SD of three different values. To examine the effect of psychosine on the *trans* activation of PPAR-α, C_6 glial cells were co-transfected with PPARs-GAL4 chimeras and the reporter plasmid (upstream activating sequences)$_5$-TK-CAT. After 48 h, cells were treated with different concentrations of psychosine (5–15 μM) for 24 h. Cell extracts were subsequently assayed for CAT by ELISA (Roche) (B). pCMV-GAL4-binding domain (without insert) and (upstream activating sequences)$_5$-TK-CAT were transfected as a control to detect the basal levels of CAT activity (B; *first bar*).

increased expression starting from day 25 to 35 days in twitcher brains whereas no such expression of TNF-α was observed in control mice brains (Fig. 1). We evaluated three important functions of peroxisomes (biosynthesis of plasmalogens, β-oxidation of fatty acid and detoxification of H_2O_2) in brain of twitcher mice. These studies report that during post-natal development with progression of disease in twitcher brain there was reduction of peroxisomal proteins representing important metabolic function (acyl-CoA oxidase for fatty acids β-oxidation and DHAP-synthase for synthesis of plasmalogens) (Figs. 1–3), documenting that peroxisomal functions are compromised in twitcher brain. On the other hand, there was no change in the protein and enzyme activity of catalase indicating a selective alteration in peroxisomes (Fig. 2). This study underscores the role of compromised peroxisomal functions in the psychosine-induced pathobiology in Krabbe and twitcher brains.

Peroxisomes were identified by de Duve based on the identification of H_2O_2 producing enzymes D-amino acid oxidase and H_2O_2 degrading enzyme catalase in the gradient fractions from cellular extracts [36]. Over the last 40 years, more than 70 enzymes/proteins have been identified in peroxisomes [14,37]. The major metabolic functions of peroxisomes include H_2O_2 detoxification, synthesis of plasmalogens, bile acids, and DHA, and catabolism of VLC fatty acids and metabolites of arachidonic acid (eicosanoids and prostaglandins) [11,38,39]. Treatment of C_6 glial cells with psychosine decreased peroxisomal functions [9]. Therefore, it will be of interest to know the alterations in peroxisomes in Krabbe disease (oligodendrocytes are affected by galacto-psychosine accumulation) vs. peroxisomes in Gaucher disease (neurons are affected by glucosylpsychosine accumulation); two lipids, glucosylpsychosine and galactosylpsychosine, are metabolized predominantly in neurons and oligodendrocytes, respectively. Peroxisomes are vital for survival because loss of peroxisomal function(s) leads to fatal diseases [12,14,37]. The observed decrease/loss of peroxisomal proteins for fatty acid β-oxidation and synthesis of plasmalogens in twitcher brain indicates that loss or deficiency of these peroxisomal functions may play a role in the pathobiology of twitcher/Krabbe disease. Zellweger patients lack peroxisomes and these patients die during early childhood and deficient activity for peroxisomal degradation of VLC fatty acids leads to fatal childhood adrenoleukodystrophy [15,40]. Since arachidonic acid metabolites (prostaglandins and eicosanoids) are β-oxidized to shorter chain metabolites in peroxisomes [11,39], the resulting increased half-life of these bioactive lipids may also contribute to the pathobiology of the psychosine-induced disease.

Krabbe and twitcher diseases are characterized by the pathognomonic accumulation of psychosine, induction of neuroinflammatory disease, degeneration and/or dysfunction of myelin producing oligodendrocytes, and demyelination [32]. Previously, our laboratory has reported that psychosine treatment of glial cells alters the cellular redox leading to reduction of peroxisomal functions/proteins and that these effects were magnified when cells were incubated with psychosine in the presence of inflammatory cytokines [9], indicating psychosine/cytokine-mediated down-regulation of peroxisomes. The expression of peroxisomal proteins is regulated by the transcription factor PPAR-α, and activators of PPAR-α are known to cause proliferation of peroxisomes [33]. PPAR-α as a dimer with retinoic acid receptor (RXR), following activation by respective ligands, regulates the expression of mRNA for peroxisomal proteins by acting on peroxisomal proliferator response elements (PPRE) present in promoter regions of the peroxisomal genes [33]. Therefore, to understand the psychosine-mediated reduction of peroxisomal proteins we examined the levels of PPAR-α in twitcher brain. The observed reduction of amount of PPAR-α in twitcher brain prompted us to test the hypothesis that psychosine accumulation in twitcher/Krabbe brain down-regulates

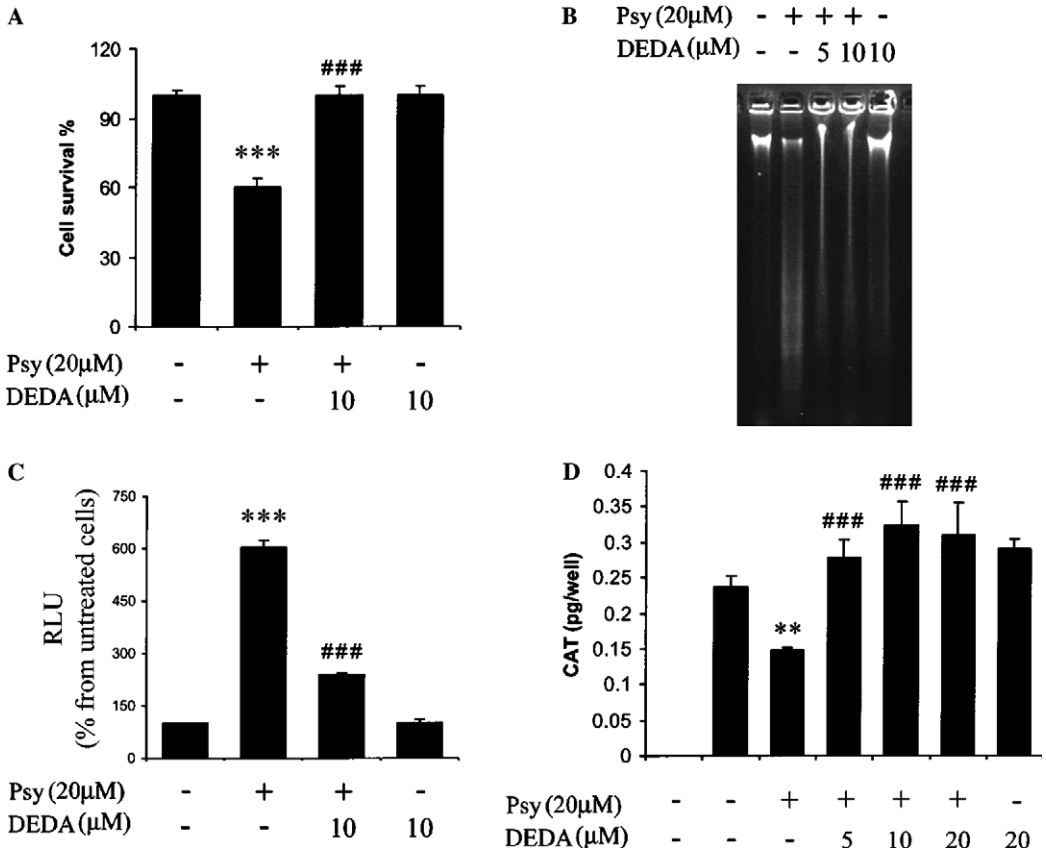

Fig. 6. sPLA2 inhibitor (DEDA) inhibits psychosine-mediated cell death and reverses psychosine-mediated inhibition of PPAR-α transcriptional activity in oligodendrocyte cell line (MO 3.13). MO 3.13 cells were treated with DEDA (10 μM) in the presence or absence of psychosine (20 μM) for 48 h. Cell viability was determined by MTT assay (A). The experiment is a representative of three individual experiments done in triplicate. Psychosine- and DEDA-treated cells were lysed in DNA lysis buffer after 48 h of treatment and DNA extracts obtained were electrophoresed on a 2% agarose gel (B). The gel is a representative of two individual experiments. MO 3.13 cells were treated with DEDA for 30 min followed by psychosine for 48 h and caspase-3 activity was measured (C). The experiment is a representative of three individual values. To examine the effect of DEDA on psychosine-mediated PPAR-α transcriptional activity, MO 3.13 cells were co-transfected with PPAR-α-GAL4 chimeras and the reporter plasmid (upstream activating sequences)$_5$-TK-CAT. After 24 h, cells were treated with different concentrations of DEDA for 30 min prior to the addition of psychosine (20 μM) for 24 h. Cell extracts were subsequently assayed for CAT by ELISA (Roche) (D). pCMV-GAL4-binding domain (without insert) and (upstream activating sequences)$_5$-TK-CAT were transfected as a control to detect the basal levels of CAT activity (D; *first bar*). Data are means of three values ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated cells and $^{###}P < 0.001$ compared to psychosine-treated cells.

PPAR-α-dependent gene expression of peroxisomal proteins. Accordingly, psychosine treatment of C_6 glial cells decreased the PPRE-luciferase and PPAR-α-Gal reporter activities in C_6 glial cells transfected with PPRE-luciferase and PPAR-α-Gal reporter (Fig. 5). Therefore, these observations indicate that psychosine-mediated down-regulation of PPAR-α transcriptional activity and the resulting reduced peroxisomes/peroxisomal functions and related alterations in cellular levels of peroxisomal metabolites may in turn up-regulate the inflammatory disease. In fact, excessive accumulation of VLC fatty acid as a result of deficiency of peroxisomal function of VLC fatty acid metabolism is known to result in secondary neuroinflammatory disease in childhood ALD where expression of inflammatory mediators lead to loss of peroxisomes and dysmyelination [41,42]. Our recent findings provide evidence for the upregulation of $sPLA_2$ by psychosine in MO 3.13 oligodendroglial cell line, and release of LPC and AA, and we found that DEDA a specific $sPLA_2$ inhibitor was able to restore the cell viability and inhibit apoptosis induced by psychosine (Figs. 6A–C). And to test whether DEDA would have any effect on PPAR-α transcription, we transfected MO 3.13 oligodendroglial cells with PPAR-α-Gal chimera along with Gal4-CAT reporter. The observation that DEDA could completely restore the PPAR-α-dependent CAT activity to normal levels is a promising finding, which could open the new avenues for treatment of twitcher/Krabbe disease.

In summary, this study provides several lines of evidence that peroxisomes and their functions are compromised as a result of psychosine accumulation and owing to the indispensable role of peroxisomes in many biological processes may in turn be responsible for oligodendrocyte loss and demyelination observed in twitcher and Krabbe brain. This study describes for the first time the lipid mediated down-regulation of peroxisomal transcriptional activity and thus dysfunction of peroxisomes in a lysosomal disorder where dysfunction of peroxisomes may play a role in sphingolipid

(psychosine)-mediated pathobiology. The observed inhibition of loss of PPAR-α activity and cell death by inhibitor of $sPLA_2$ may hold a promising therapy for Krabbe disease.

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