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Psychosine-induced alterations in peroxisomes of Twitcher Mouse Liver

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Abstract

Krabbe's disease is a neuroinflammatory disorder in which galactosylsphingosine (psychosine) accumulates in nervous tissue. To gain insight into whether the psychosine-induced effects in nervous tissue extend to peripheral organs, we investigated the expression of cytokines and their effects on peroxisomal structure/function in twitcher mouse liver (animal model of Krabbe disease). Immunofluorescence analysis demonstrated TNF- α and IL-6 expression, which was confirmed by mRNAs quantitation. Despite the presence of TNF- α , lipidomic analysis did not indicate a significant decrease in sphingomyelin or an increase in ceramide fractions. Ultrastructural analysis of catalase-dependent staining of liver sections showed reduced reactivity without significant changes in peroxisomal contents. This observation was confirmed by assaying catalase activity and quantitation of its mRNA, both of which were found significantly decreased in twitcher mouse liver. Western blot analysis demonstrated a generalized reduction of peroxisomal matrix and membrane proteins. These observations indicate that twitcher mouse pathobiology extends to the liver, where the induction of TNF- α and IL-6 compromise peroxisomal structure and function.

Keywords

TNF- α ; IL-6; neuroinflammation; Krabbe disease; twitcher mouse; peroxisomes; psychosine; sphingolipid; catalase; liver

Introduction

Krabbe disease (globoid cell leukodystrophy) is an autosomal recessive genetic disorder caused by deficiency of the lysosomal enzyme galactosylceramidase [1], and it is characterized by accumulation of galactosylsphingosine (psychosine; also a substrate of the enzyme) in the central nervous system (CNS) [2] and other peripheral tissues [3]. The endogenously synthesized psychosine that accumulates become cytotoxic [4], and exerts a lethal effect in the CNS, specially on oligodendrocytes, resulting in early demyelination [5]. Pathological features of the disease include myelin degeneration, astrocytic gliosis, axonal loss, and the presence of multinucleated giant (globoid) cells [1,6]. Many advances in the understanding of the pathobiology of Krabbe disease have been in part possible to the availability of the twitcher

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mouse, an authentic murine model of the human disease [7]. The observed increased expression of the inflammatory cytokine Tumor Necrosis Factor- α (TNF- α) in brains from both Krabbe and twitcher [8,9], the inflammatory cytokine Interleukin-6 (IL-6), and the major histocompatibility antigens class I and II in twitcher brain [9-11], in addition of the presence of inducible nitric oxide synthase (iNOS)-expressing cells [12] and tunnel positive cells (apoptotic cells) in postmortem brain samples of Krabbe patients [13], indicate that local production of inflammatory cytokines may play a role in the apoptotic loss of oligodendrocytes in Krabbe and twitcher brains [14,15]. Studies from our laboratory indicate that psychosine enhances the expression of proinflammatory cytokine as well as that of iNOS, and hence the production of nitric oxide (NO) in primary cells culture [12]. These data support psychosine-induced cytokines as a possible mechanism of progression of the pathobiology of Krabbe disease.

Peroxisomes are subcellular organelles which perform critical cellular functions through a specific β -oxidation system [16]. Some of the compounds metabolized by the peroxisomal system include long chain (LC) and very long chain fatty acids, LC dicarboxylic acids, 2-methyl branched fatty acids and monoenoic acids, bile acid intermediates, the side chain of some xenobiotic compounds, and arachidonic acid metabolites (eicosanoids), among others [16]. In addition, peroxisomes produce and detoxify hydrogen peroxide generated by the many oxidases that contain [17], and participate in the biosynthesis of important structural membrane constituents, such as the n-3 polyunsaturated fatty acid docosahexaenoic acid and plasmalogens [18,19]. Furthermore, their importance is underscored by the identification of fatal neurological diseases as a result of alterations of peroxisome biogenesis or proteins dysfunction [20,21]; and the role that peroxisomal metabolism play in brain myelination [22-24], brain cell homeostasis [25-27], and inflammation [28-30].

Recently, we have reported that psychosine-induced inflammation in the brain of twitcher mouse correlates with the loss of peroxisomal functions, suggesting that peroxisomal impairment may contribute to progression of the pathobiology of Krabbe disease [29]. Since psychosine also accumulates in peripheral tissues [3], the objective of this study was to investigate whether the accumulation of psychosine in liver might be detrimental to peroxisomal functions. Our results indicate that accumulation of psychosine in twitcher liver induce expression of proinflammatory cytokines (TNF- α and IL-6), which impairs peroxisomal structure/functions. In addition, they indicate that the psychosine-induced inflammatory process observed in the nervous tissue extends to liver and possibly to other organs.

Materials and Methods

Reagents

All reagents were of analytical grade or the highest purity commercially available. Buffered Formalin 10% (w/v) was from EMS (Fort Washington, PA, USA). Bovine serum albumin, 3, 3'-diaminobenzidine, Tween-20 and iodoacetamide, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrocellulose membranes and non-fat milk powder were from BioRad Laboratories (Hercules, CA, USA).

Animals

Animal work was performed under a protocol approved by the Institutional Animal Care and Use Committee. Twitcher heterozygote breeding pairs (C57BL/6J *twi*^{+/-}) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the University animal facility. *Tw*^{-/-} (*twi*) and age-matched littermates wild type *Wt*^(+/+) (*wt*) were identified using tail genomic DNA [29] and used for this study at postnatal day 32-33.

Immunofluorescence

Expression of cytokines (TNF- α , IL-6) was detected by immunofluorescence analysis on sections from formalin-fixed liver samples [29]. Sections were incubated with anti-TNF- α antibody or anti-IL-6 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) and the immuno-complex detected with biotinyl tyramide amplification reagent (Perkin Elmer, Boston, MA, USA), and Streptavidin-Texas red fluorophore (Perkin Elmer). The slides were examined under a fluorescence microscope (Olympus BX-60), and the images were acquired using an Olympus digital camera (Optronics, Goleta, GA, USA) and rendered using Adobe Photoshop 7.0.

Analysis of liver sphingolipid by HPLC-MS

Liver samples from *wt* and *twi* (100 mg wet tissues) were quick-frozen in liquid nitrogen, and stored at -70°C until further analysis. Quantitative HPLC-tandem mass spectrometry analysis of bioactive sphingolipids was performed on extracts derived from *wt* and *twi* livers (University Lipidomics Core facility) [31].

Morphological examination of peroxisomes

Processing of samples for electron microscopy was performed as described earlier [32], using two small fixed pieces (1×1 mm) of fresh *wt* and *twi* livers. The fixed tissues were incubated in alkaline 3,3'-diaminobenzidine [33] and infiltrated with Embed-812. Thin sections were counterstained with uranyl acetate and lead citrate, and the ultrastructural examination was performed with a Hitachi 12-A electron microscope.

Assay of catalase activity

Catalase activity was determined using hydrogen peroxide in a colorimetric assay [29]. The substrate that remains after incubation was detected by formation of a complex with titanium oxysulfate and read in a Shimadzu UV-1601 spectrophotometer at 405 nm (Columbia, MD, USA). Protein concentration was determined according to the method of Bradford [34].

Protein analysis by SDS-PAGE and western blot

Liver homogenates and total membranes (containing integral membrane proteins) obtained by carbonate treatment of liver homogenates [32] were resolved by SDS-gel electrophoresis (4-16% gradient gel, Criterion, BioRad), transferred to nitrocellulose membranes, and western blot analysis performed as described previously [29]. Antibodies used were: anti-catalase (RBI, Natick, MA, USA); anti-peroxisomal 3-oxo-acyl-CoA thiolase (custom made against residues 388-402 of the rat protein); anti- β -actin (custom made against residues 361-375 of the bovine protein); anti-PMP-70 (custom made against C-terminal residues 644-659 of the human protein), and anti-ALDP (MAB2162, Chemicon, Temecula, CA, USA). Blots were developed by treatment with Lumi-Phos WB reagent (Pierce Biotechnology, Rockford, IL, USA) and exposure to CL-Xposure films (Pierce).

Messenger RNA analysis by quantitative real-time PCR

RNA isolation and quantitative real-time PCR were performed as described previously [29]. Thermal cycling conditions (iCycler, BioRad) were: activation of DNA polymerase at 95 °C for 10 min; 40 cycles of amplification at 95 °C for 30 sec, and 58-60 °C for 1 min. The expression of target gene was normalized to 18S rRNA. Primers (IDT, Coralville, IA, USA) were: 18S rRNA, forward primer (F)5'-CGTCTGCCCTATCAACTTTCG-3' and reverse primer (R)5'-GCCTGCTGCCTTCCTTGG-3'; catalase, F5'-CAGGTGCGGACATTCTAC-3' and R5'-GCGTTCTTAGGCTTCTCAG-3'; TNF- α , F5'-CATCTTCTCAAATTCGAGTGA CAAA-3' and R5'-

TGGGACTAGACAAGGTACAACCC-3'; IL-6, F5'-GAGGATAC CACTCCCAACAGACC-3' and R5'-AAGTGCATCATCGTTGTCATACA-3'. All samples were run in triplicate.

Statistical Analysis

Data represent the mean $\pm$ SD for (n) number of experiments. Statistical analysis was performed by two tailed *t* test for unpaired observations (InStat 3, GraphPad Software Inc., San Diego, CA, USA). The difference at $p < 0.05$ was considered statistically significant.

Results

Psychosine accumulates in brain and liver of twitcher mouse

Mass spectrometric analysis of *wt* and *twi* brain and liver samples indicates that psychosine accumulates in *twi* brain and liver at levels that are near 90 and 46 times the levels present in *wt* tissue samples, respectively (Table 1). These results are consistent with previous published analysis [3,35,36], and support the notion that globoid cell leukodystrophy is a generalized psychosine storage disease.

Cytokine (TNF- α and IL-6) expression in twitcher liver

Psychosine accumulation in the twitcher animals induce TNF- α and IL-6 expression in the nervous tissue [9]. Therefore, we investigate the expression of those cytokines in liver samples of *wt* and *twi* mice by immunofluorescence. Using specific antibodies against TNF- α and IL-6, the analysis demonstrated the expression of both cytokines in *twi* (Fig. 1B, D), but not in *wt* liver (Fig. 1A, C). These finding were further supported by the quantitation of mRNA levels for these cytokines by the polymerase chain reaction (RT-PCR). The analysis of total mRNA extracted from *wt* and *twi* livers, indicated that mRNA for both cytokines were upregulated by 3.8 and 3.9 fold respectively in *twi* animals (Fig. 1E).

Sphingolipid homeostasis in twitcher liver

It is well documented that TNF- α is important in the pathophysiology of hepatocytes [32,37]. TNF- α induced changes in sphingolipid homeostasis play an important role in hepatocyte cell death [38,39]. To evaluate the effects of the observed increase in levels of TNF- α and IL-6 on liver sphingolipid homeostasis, we analyzed the total content and the fractions containing-saturated and unsaturated fatty acids of sphingomyelin (SM) and ceramide (CM) present in hepatic tissue from *twi* and *wt*, for alteration in these lipids (Fig. 2A-F). Mass spectrometry analysis was used to determine the concentration of SM, CM and sphingosine/sphingosine-1-phosphate (Fig. 2A, B). In general, there was a significant increase in the levels of total SM (46%, $p < 0.03$; Fig. 2A) and no significant changes in the levels of total CM, sphingosine or sphingosine-1-phosphate in *twi* liver (Fig. 2B). A detailed analysis of the SM and CM species, based on the composition of saturated and unsaturated fatty acids, indicated significant fluctuation between these species. In SM, the fractions containing the fatty acids C14:0, C16:0; C18:0 and C26:0 showed a significant increase in *twi* liver as compared to those in *wt* liver (Fig. 2C). Concordantly, the monounsaturated species C18:1; C20:1 and C26:1, were also found significantly increased (Fig. 2D). On the other hand, in CM fractions containing the fatty acids C18:0 and C18:1 showed significant increase and the species C22:0 and C24:1 a significant decrease (Fig. 2E-F); the rest of the species were not affected in a significant manner. Interestingly, the dehydro-C16:0 (DHC16:0) specie, an indicator of the *de novo* CM synthesis, was found significantly reduced (Fig. 2E). The observed significant increase in total SM fraction indicates that psychosine-induced cytokines do not activate the synthesis of ceramide, the sphingosine-derived intermediate of cell death, in *twi* mouse liver.

Peroxisome morphology in mouse liver

Previous reports indicated that cytokines have a negative effect on peroxisomal structure/function [32,40]. To understand the possible mechanism of alterations in peroxisomes, *wt* and *twi* liver samples stained for peroxisomes with catalase-dependent diaminobenzidine (DAB) reaction, were examined by electron microscopy. In general, *twi* hepatocyte cytoplasm showed decreased glycogen deposits, swollen mitochondria, and no drastic difference in the peroxisome number as compared to *wt* (Fig. 3A, B). Higher magnification analysis (80,000x) showed peroxisomes of *wt* hepatocytes as particles limited by a smooth membrane surrounding a homogeneous and dark matrix (Fig. 3C), whereas peroxisomes in *twi* hepatocytes were seen as particles limited by an irregular membrane limiting a heterogeneous and less stained matrix (Fig. 3D-F). The observed reduction in DAB-stained peroxisomes in *twi* samples may be an indication of reduced enzymatic activity of catalase in hepatocytes of *twi* animals.

Peroxisomes structure/functions in twitcher mouse liver

To evaluate catalase activity in *wt* and *twi* mice livers, detoxification of hydrogen peroxide was assayed as function of catalase activity in liver homogenates. The enzymatic assay indicated a decreased of 43% ($p < 0.0003$) in the catalase activity present in liver homogenate from *twi* as compared to *wt* (Fig. 4A). To further understand the cause of decreased enzymatic activity in peroxisomes, mRNA and protein levels of catalase were determined. Analysis of mRNA by quantitative RT-PCR and western blot demonstrated a parallel reduction in mRNA (56%; Fig. 4B) as well as in protein levels in the liver of *twi* mouse, respectively (Fig. 4C). To confirm whether these findings are catalase-specific or affect other peroxisomal proteins, the protein levels of the matrix enzyme 3-oxoacyl-CoA thiolase and the membrane transporters adrenoleukodystrophy protein (ABCD1) and peroxisomal membrane protein of 70kDa (ABDC3) were determined (Fig. 4C, D). The western blot analysis indicated a significant decrease in levels of matrix and membrane proteins, indicating impairment in the expression and in the protein composition of peroxisomes in *twi* mouse liver, as compared to *wt*.

Discussion

Clinical and pathological manifestations of Krabbe disease described so far are almost exclusively restricted to the nervous system, particularly to the white matter and the peripheral nerves, as a consequence of high levels of galactosylsphingosine (psychosine) accumulation in those tissues [1,2]. Oligodendrocytes, i.e. the myelin forming cells, are most susceptible, and are specifically and rapidly lost during the early stages of brain development (myelination) due to accumulation of endogenously synthesized psychosine [5]. The psychosine analysis in various tissues of Krabbe patients and *twi* mouse indicate that levels in nervous tissue are much higher than in other organs [3]. However, as psychosine also accumulates in peripheral organs (liver, kidney and lungs amongst others) [3,35,36], globoid cell leukodystrophy has been referred to as a generalized psychosine storage disease [3].

Our data indicate that accumulation of psychosine in liver of *twi* mouse triggers the induction of an inflammatory response which in turn increases expression of proinflammatory cytokines (TNF- α , IL-6), as described in central nervous system (CNS) of these animals [9] and Krabbe brain [8]. However, differences in the levels of psychosine accumulated in liver versus brain (10-20 folds) [3,35], may account in part for the differences in pathology observed between the two organs, i.e. presence of globoid and apoptotic cells in the CNS but not in the liver. Although brain levels of psychosine are higher than liver per gram of tissue, given the size of liver, liver may represent an important psychosine reservoir [3,35]. Therefore, the effects that we observed in *twi* liver may be the result of a much lower, gradual and prolonged exposure to psychosine accumulation/cytokines induction, and may, perhaps, shed light on pathological events that occur early in the development of Krabbe/twitcher in the nervous tissue.

Alternatively, hepatocytes may be responding differently to psychosine toxicity; for example, psychosine effects on oligodendrocytes or related cells [13-15,41] is different than that seen in other cell lineages [42,43], where psychosine treatment induces multinucleate giant cell formation.

TNF- α and other factors alter endogenous cellular levels of the sphingolipid ceramide (CM), by stimulation of sphingomyelinases, a potential apoptotic signal [38]. TNF- α also induces the synthesis of molecules that protect the cell, by activating sphingosine kinase, an enzyme which converts sphingosine -the deacylated product of ceramide- to sphingosine-1-phosphate (Sph1P), a second messenger for cell survival [44]. Previous studies indicate that increased generation of CM, specially of C16:0-CM, plays a central role in TNF- α -induced apoptosis in primary hepatocytes [39]. Contrary to the expected, our analysis of sphingolipids fractions revealed no significant decrease in total sphingomyelin (SM) or accumulation of SM-derived CM in *twi* liver. However, individual variations were observed in some of the saturated and unsaturated species CM fractions (C18:0, C18:1, C22:0 and C24:1). It remains to be seen if the changes observed in individual saturated and unsaturated fatty acid species of SM and CM play a role in the progression of *twi* liver pathobiology. The results also showed that C16:0-CM as well as Sph1P levels were not modified in *twi* liver, a significant difference with the studies of TNF- α -treated primary hepatocytes [39]. These data indicate that psychosine-induced cytokines affect sphingolipid homeostasis in *twi* liver mouse, without producing significant changes in known apoptotic (C16:0-CM) or antiapoptotic (Sph1P) intermediates.

Recently, we reported a correlation between expression of TNF- α and loss of peroxisomal proteins/function in *twi* mouse brain [29]. This and other similar reports [32,40,45-48] document the negative effects of inflammatory mediators (cytokines) on peroxisomal structure/functions of liver and brain. Indeed, using three different animals models, i.e. acute endotoxemia in rat, and EAE model of multiple sclerosis and twitcher model of Krabbe disease, we have reported that mediators of inflammatory disease (cytokines, inducible-nitric oxide synthase) exert negative effects on peroxisomes, such as alteration in membrane properties and down regulation of proteins and hence, in their metabolic functions (fatty acids and eicosanoids β -oxidation; synthesis of plasmalogens and n-3 polyunsaturated fatty acids; and hydrogen peroxide detoxification) [29,30,32,47]. Our data (electron microscopy) indicate that peroxisomal population of *twi* livers is not significantly affected, suggesting that the observed decrease in peroxisomal protein, without alteration in peroxisome number, may not be related to an increase in the turnover/autophagy of the whole organelle, a mechanism that seems to regulate peroxisomal population [32,49,50]. However, it is consistent with previous findings described for CNS of animals models of neuroinflammatory disease [29,30].

In addition to the direct cytotoxic psychosine effects, the pathology of *twi* mouse is also in part mediated by activated macrophages/microglia and release of mediators of inflammation in the CNS [51]. Increased expression of MHC class II antigens, cytokines (TNF- α and IL-6), and inducible nitric oxide synthase have been reported in Krabbe and *twi* brains [8,9,11]. Indeed, nitric oxide [52] and TNF- α [53] are cytotoxic to oligodendrocytes and/or myelin *in vitro*. In culture IL-6 expression can be induced in astrocytes and microglia when treated with TNF- α or myelin debris [54,55]. Therefore, this evidence suggests that cytokines mediated inflammatory response plays a role in the pathogenesis and progression of disease in Krabbe patients and *twi* mouse. It is clear that the combined effects of psychosine and cytokines on hepatocytes and peroxisomal function compromise the homeostasis of liver and possibly of other organs, as indicate by the many reports that suggest that liver function plays an important role in brain homeostasis and vice versa [56-58].

In summary, our results indicate that the psychosine-induced inflammatory process described in the CNS of *twi* mouse also occurs in liver and possibly other organs of this animal model.

In liver, psychosine accumulation/cytokine induction produces significant derangement of peroxisomal structure and function. Therefore, a detailed view of the twitcher pathology including not only the CNS, but also inter-relationship with other organs is needed to understand the overall progression of this disease.

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Fig. 1. Immunofluorescence and mRNA analysis of cytokines expression (TNF- α and IL-6) in hepatic tissues from wild type and twitcher mice

Liver tissue sections from wild type littermates (A, C) and twitcher (B, D) were processed and analyzed for immunofluorescence as described in methods section. Primary antibodies were against TNF- α (A, B) and IL-6 (C, D) and secondary antibodies were conjugated with the fluorophore Texas Red. TNF- α and IL-6 mRNA were determined by quantitative RT-PCR (E) and are expressed as the ratio between twitcher value (TW) and the wild type value (WT) after normalization to 18S rRNA. Graph represent the average $\pm$ SD for $n = 5$ samples per group.

Fig. 2. Mass spectrometry analysis of sphingolipid in wild type and twitcher livers extracts

Levels of Sphingomyelin (SM) and ceramide (CM) (A); sphingosine (Sph) and sphingosine-1-phosphate (Sph1P) (B); and saturated (C, E) and unsaturated (D, F) fatty acid fractions of SM (C, D) and CM (E, F) fractions were determined in liver extracts from 100 mg of wet weight tissue of wild type (white bars) or twitcher (black bars), as indicated in methods section. Values in (A) were obtained from the summary of the individual SM and CM fractions (including saturated and unsaturated fatty acids fractions). Graph represent the average $\pm$ SD for $n = 5-6$ samples per group. *: $p < 0.03$ (A); *: $p < 0.03$; **: $p < 0.004$; ***: $p < 0.0001$ (C); *: $p < 0.03$; **: $p < 0.02$; ***: $p < 0.0001$ (D); *: $p < 0.04$; **: $p < 0.005$; ***: $p < 0.001$ (E); *: $p < 0.05$; **: $p < 0.0004$ (F).

Fig. 3. Ultrastructural analysis of peroxisome morphology in liver tissue from wild type and twitcher mice

Samples from wild type (A, C) and twitcher (B, D-F) livers were incubated with diaminobenzidine to stain peroxisomes and processed for electron microscopy as indicated in methods section. Arrowhead indicates peroxisomes; M indicates mitochondria. Bar represent 2 microns (50,000x mag.) in (A, B); and 100 nm (80,000x mag.) in (C-F).

Fig. 4. Hydrogen peroxide detoxification and matrix and membrane peroxisomal protein levels in wild type and twitcher liver homogenate

Enzymatic activity of catalase (A), catalase mRNA levels (B) and western blot analysis using antibodies against matrix (catalase and 3-oxoacyl-CoA thiolase)(C) and membrane (adrenoleukodystrophy protein (ABCD1) and peroxisomal membrane protein of 70kDa (ABCD3))(D) peroxisomal proteins. Enzyme activity was assayed in liver homogenates; data represent the average $\pm$ SD for $n = 8$ samples per group. WT: wild type; TW: twitcher. ***: $p < 0.0003$. Catalase mRNA was determined by quantitative RT-PCR (B) and it is expressed as percentage of wild type value (WT) after normalization to 18S rRNA. Graph represent the average $\pm$ SD for $n = 5$ samples per group. Protein levels analysis for catalase and peroxisomal thiolase was performed in liver homogenates (β -actin was used as indicator of protein loading), and for adrenoleukodystrophy protein and peroxisomal membrane protein of 70kDa was performed in total carbonate membranes, obtained as indicated in methods sections.

Table 1

Galactosylsphingosine (psychosine) concentration in brain and liver of wild type and twitcher mice determined by mass spectrometry.

	Wild type	Twitcher
	(pmole/100 mg wet weight tissue)	
Brain	5.7 ± 2.9	514.1 ± 85.9*
Liver	1.3 ± 0.4	59.3 ± 7.4*

Samples represent Mean ± SD of the analysis of 3 wild type and 5 twitcher mice (two wild type animals were at postnatal day 33 and one at postnatal day 43; twitcher animals were at postnatal day 32-33).

*
: $p < 0.0001$.