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Research Report

Glucosylceramide synthase decrease in frontal cortex of Alzheimer brain correlates with abnormal increase in endogenous ceramides: Consequences to morphology and viability on enzyme suppression in cultured primary neurons

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ABSTRACT

Abnormal increase in native long-chain ceramides (*lc*Cer) in AD implicates roles in neuronal atrophy and cognitive dysfunction especially in view of divergent roles this second messenger plays in cell function. Since clearance is mediated by glucosylceramide synthase (GCS, EC 2.4.1.80) levels of the enzyme were compared for 18 samples of AD Brodmann area 9/10 frontal cortex with 11 age-matched controls. Western analysis for $_{ir}$GCS showed a significant decrease in AD brain ($p<0.01$) consistent with the hypothesis that enzyme dysfunction contributes to neuronal decay. To examine kinetics and consequences to morphology, cerebellar granule cells were treated in vitro with D-*threo*-P4 (P4). This potent inhibitor of GCS induced a time- and concentration-dependent increase in *lc*Cer parallel to loss of viability and dramatic changes in neuron/neurite morphology via caspase-independent pathways distinct from those of apoptosis or necrosis. Fluorescent labeling with NBD-sphingolipids or immunostaining with anti-synaptic or cytoskeletal markers showed unusual formation of globular swellings along neurites rich in synaptophysin that may resemble formation of dystrophic neurites in AD. Effects of the inhibitor were verified by changes in *lc*Cer mass and turnover of ^{14}[C]-acetate and -galactose or NBD-labeled anabolic products. Addition of a panel of inhibitors of other pathways confirms GCS as the major route for clearance in the present model. Pretreatment with GM_1 whose turnover is compromised was protective and pointed to useful therapeutic applications by supplementing existing membrane stores prior to GSC dysfunction.

1. Introduction

Ceramides (long-chain ceramides, C_{18-24}; *lc*Cer) serve not only as structural backbones for glycosphingolipids (GSLs), but also play dynamic roles in cell function and viability (Hannun and Obeid, 2002; Radin, 2003; Tepper et al., 1999). Recent reports for abnormal *lc*Cer increase in AD, HIV, arteriosclerosis, ageing, and stroke (Cutler et al., 2004; Han et al., 2002; Han, 2005;

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Haughey et al., 2004; Sawai et al., 2005) focus interest on links to neurodegeneration and the potential for therapy. The pathways that link this putative second messenger to neuronal function remain elusive although it is established abnormal pool size is highly detrimental to cell function (Hannun and Obeid, 2002). This has been exploited to design *lc*Cer mimetic inhibitors as antineoplastic drugs to offset multiple drug resistance (MDR) in malignant cells capable of rapid *lc*Cer detoxification (Liu et al., 1999). This suggests in the opposite sense that an abnormal rise in neurons places these at risk.

*lc*Cer clearance in neurons is mediated largely by UDP glucose:ceramide glucosyl transferase 1 (glucosylceramide synthase [GCS]; EC 2.4.1.80) with other pathways summarized in Fig. 1 playing proportionately lesser roles (Okazaki et al., 1998). It is not established whether GCS dysfunction is a significant factor in AD type neurodegeneration despite abnormal increase in *lc*Cer noted above, or a 4-fold decrease in GCS mRNA in mild cognitive impairment (MCI) with up to 8-fold decrease with disease severity (Katsel et al., 2007). The early change in message prior to appearance of severe dementia and deposition of tau NFT/amyloid histopathology according to the Braak staging (Braak and Braak, 1991) could imply a lag between this enzyme's transcription and translation. Since AD develops over decades, presumably a gradual decrease in enzyme synthesis does not contribute to histopathology until a critical threshold is reached to significantly impair *lc*Cer clearance.

To evaluate this possibility, we report here for the first time, a significant decrease in $_{ir}$GCS in frontal cortex in AD that is consistent with this hypothesis. To explore this further we report here on a replication of *lc*Cer increase using an isolated model of primary neurons with pharmacological suppression by P4 [D-*threo* P4 (*threo*-2-palmitoylamino-3-pyrrolidino-1-propanol)], a second-generation GCS inhibitor (Abe et al., 1995; Lee et al., 1999). This approach was used since GSC$^{-/-}$ cells are unavailable owing to embryonic lethality (Jennemann et al., 2005; Yamashita et al., 1999), the poor penetration of exogenous native ceramides to increase cellular levels (Simon et al., 1999), and the puzzling biphasic effects of short-chain Cer (*sc*Cer) synthetic analogs that vary with concentration and states of neuronal development (Goswami and Dawson, 2000; Hofmann and Dixit, 1998; Irie and Hirabayashi, 1998; Radin, 2003; Schwarz and Futerman, 1997; Tepper et al., 1999).

Consequently, to circumvent these issues, postnatal cerebellar granule cells (CGCs) were selected since these are a well-studied model for neuronal cell death, and become fully differentiated on culture for 7-d in vitro (DIV) that acquire electrophysiological or other properties of mature non-mitotic primary neurons (Balazs et al., 1988; D'Mello et al., 1998; Marks et al., 1998; Oberto et al., 1996). While it is recognized cerebellum is less prone to AD histopathology, this brain area suffers neuronal loss, atrophy, and gliosis with a major impact on the granular layer (Sjobeck and Englund, 2001; Wegiel et al., 1999). Thus this cell model is well suited to identify if abnormal increase in *lc*Cer before appearance of pathology impacts viability and morphology to account for altered function. In a series of studies, Han and co-workers report <3.2 fold elevation of *lc*Cer in white matter of cerebral cortex and cerebellum with

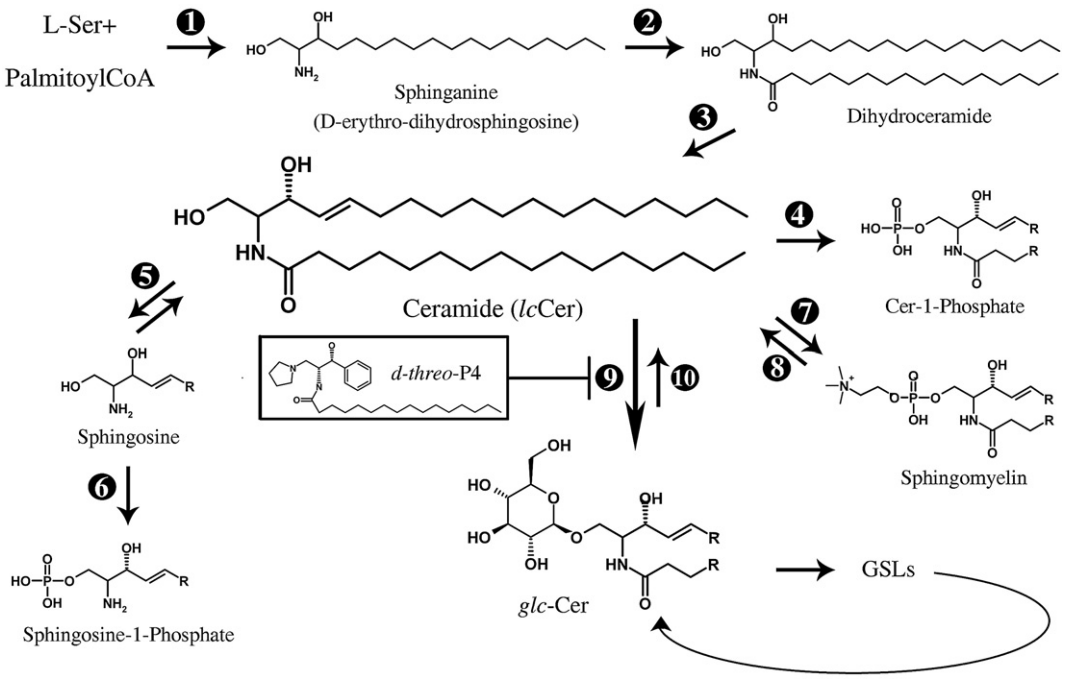

Fig. 1 – Pathways for *lc*Cer utilization in neuronal cells, and sites and agents used for pharmacological intervention. Circled numbers for enzymes followed by inhibitors utilized in this study are as follows: ❶ Serine palmitoyltransferase (Cycloserine); ❷ (Dihydro)ceramide synthase (Fumonisin B_1); ❸ Dihydroceramide desaturase; ❹ Ceramide kinase; ❺ Ceramidase (*N*-oleoyl-ethanolamine); ❻ Sphingosine kinase; ❼ Sphingomyelin synthase; ❽ Reverse reaction catalysed by SMases including Mg^{2+}-dependent *n*SMase (GW4869), *a*SMase (Desiprimine, SR55337) among others; ❾ Glucosylceramide synthase, GCS (D-*threo*-P4 shown boxed); ❿ β-Glycosidase (Conduritol B epoxide). *glc*Cer (glucosylceramide, glucocerebroside) can feed back into reformation of complex GSLs.