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Palmitate-activated astrocytes via SPT increase BACE1 in primary neurons by SMases

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Abstract

Astrocytes play a critical role in neurodegenerative diseases, including Alzheimer's disease (AD). Previously, we showed that saturated free-fatty acid, palmitate (PA), upregulates BACE1 level and amyloidogenesis in primary rat neurons mediated by astrocytes. However, the molecular mechanisms by which conditioned media from PA-treated astrocytes upregulates BACE1 level in neurons are unknown. This study demonstrates that serine palmitoyltransferase (SPT) in the astrocytes increases ceramide levels, which enhances the release of cytokines that mediate the activation of neural and acidic sphingomyelinase (N-SMase and A-SMase) in the neurons, to propagate the deleterious effects of palmitate, i.e. BACE1 upregulation. In support of the relevance of SPT in AD, our lab recently measure and found SPT levels to be significantly upregulated in AD brains as compared to controls (Geekiyana and Chan, 2011). Cytokines, namely TNF α and IL-1 β , released into the conditioned media of PA-treated astrocytes activate N-SMase and A-SMase in the neurons. Neutralizing the cytokines in the PA-treated astrocyte conditioned media reduced BACE1 upregulation. However, inhibiting SPT in the astrocytes decreased the levels of both TNF α and IL-1 β in the conditioned media, which in turn reduced the SMase activities and BACE1 level in primary neurons. Thus, our results suggest that the activation of the astrocytes by PA is mediated by SPT, and the activated astrocytes increases BACE1 level in the neurons, the latter is mediated by the SMases.

Keywords

Alzheimer's disease; Fatty acid; Sphingomyelinase; Serine palmitoyltransferase; Ceramide; TNF α ; IL-1 β

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1. Introduction

Alzheimer's disease (AD) is the most common dementia, affecting over 35.6 million people worldwide, with a total cost in the US of approximately \$604 billion in 2010 (Wimo and Prince, 2010). Up to now, the etiology of AD remains enigmatic and numerous hypotheses of possible mechanisms have been put forth, of which the amyloid hypothesis is the most widely accepted. The amyloid cascade hypothesis posits that amyloid beta (A β) plays an early role and triggers a cascade of events which leads to neurodegeneration (Golde et al., 2006; Pappolla et al., 2002). The protein and activity levels of BACE1 (β -site amyloid precursor protein-cleaving enzyme 1), the rate-limiting protease involved in generating A β , have been found significantly elevated in AD brain (Cole and Vassar, 2007). BACE1 expression is constitutively expressed and inducible. Its regulation is complex and cell type-dependent (Bourne et al., 2007).

Astrocytes, the most abundant glial cells in the central nervous system (CNS), are highly responsive to environmental changes. The activation of astrocytes has been attributed to the pathogenesis of several neurodegenerative diseases, including AD (Sidoryk-Wegrzynowicz et al., 2010). In further support, A β plaques are surrounded by activated astrocytes in human AD brains and in transgenic AD mouse brains (Wyss-Coray and Mucke, 2002). These activated cells secrete proinflammatory molecules, such as tumor necrosis factor alpha (TNF α) and interleukin-1beta (IL-1 β), IL-6, and nitric oxide (Krasowska-Zoladek et al., 2007). Anti-inflammatory drugs have been suggested to potentially reduce the risk of AD (Vlad et al., 2008). However, the molecular mechanism by which activated astrocytes enhances A β levels (Pra et al., 2011) and facilitates neuronal loss (Jana and Pahan, 2010) is unclear.

Diet, an environmental factor, has been suggested to play a potential role in AD (Morris and Tangney, 2010). Further, studies showed that the AD brain is characterized by high fatty acid content as compared to the healthy subjects (Roher et al., 2002). Epidemiological findings suggest that consumption of saturated free fatty acids (FFAs) may increase while unsaturated FFA may reduce the risk of AD (Takechi et al., 2009). *In vivo* animal studies further support this hypothesis, namely, mice fed a high fat and 1% cholesterol diet accelerated AD-like pathophysiological changes in their brains (Levin-Allerhand et al., 2002; Oksman et al., 2006). Our group also confirmed that PA induces AD-like changes in the primary rat neurons mediated by ceramide generated through the SPT pathway in the primary rat astrocytes (Patil and Chan, 2005; Patil et al., 2007; Patil et al., 2006). More recently we demonstrated that SPT levels are elevated in AD brain, and suppressing SPT reduced the A β expression, while overexpressing SPT increased A β expression in primary mice astrocytes expressing the human APP Swedish mutation (Geekiyana and Chan, 2011). We found previously that direct treatment of primary neurons with PA had no effect on the levels of BACE1 and A β (Patil et al., 2006). This may be attributed to the low ability of neurons to take up and metabolize long chain fatty acids (Blazquez et al., 2000; Qin et al., 2010). However, conditioned media from PA-treated astrocytes (CM-P) significantly increased the levels of BACE1 and A β in the primary rat cortical neurons. The *de novo* synthesis of ceramides produced by the astrocytes upon treatment with PA mediated the increased levels of BACE1 and A β in the primary neurons (Patil and Chan, 2005; Patil et al., 2007; Patil et al., 2006). However, the mechanisms leading to these changes in the primary neurons and mediated by the CM-P is unclear.

Recently, it was demonstrated that A β activates human astrocytes, which in turn released soluble neurotoxic substances, i.e. nitric oxide that killed the primary human neurons mediated by N-SMase. Knockdown of N-SMase decreased the ceramide level, which in turn, reduced the activation of astrocytes and neuronal loss (Jana and Pahan, 2010).

Ceramide, a product of both SMase and SPT, is three times higher in AD brain as compared to their age-matched controls (Han et al., 2002; He et al., 2008). Reducing ceramide levels decreased A β levels in neuroblastoma cells (Tamboli et al., 2005), suggesting a potential role of ceramide in AD pathology. Indeed Puglielli et al. found that ceramide stabilizes BACE1 posttranslationally to promote A β biogenesis in human neuroglioma cells (Ko and Puglielli, 2009; Puglielli et al., 2003).

Many studies have found associations between A β , SMase and ceramide (Jana and Pahan, 2004; Ju et al., 2005; Malaplate-Armand et al., 2006). For example, sphingomyelin (SM) levels are reduced while SMase levels are elevated in AD brain, and the upregulated SMase level correlated with increased A β and hyperphosphorylated tau levels (He et al., 2008). The A β peptide has been shown, in turn, to induce apoptosis of both primary neurons and glial cells through the SMase-ceramide pathway (Jana and Pahan, 2010; Yang et al., 2004).

In the present study, we set out to elucidate the mechanism by which CM-P up-regulates BACE1 level in primary neurons and establish that an environmental factor, namely elevated saturated PA, can initiate the ceramide-amyloid cascade. We demonstrate that ceramide levels are up-regulated in primary rat neurons upon culture with CM-P. Ceramide can be produced through either the *de novo* or the SMase pathways. Evidence in the literature support that inhibiting *de novo* ceramide synthesis decreases the A β production and adding ceramide exogenously increases A β production (Puglielli et al., 2003; Patil et al., 2007). More recently, we showed that ceramide and SPT expression levels are elevated in a subgroup of sporadic AD patients (Geekyanage and Chan, 2011). In this study we demonstrate how increased levels of ceramides in the astrocytes upon culture with PA leads to the upregulation of BACE1 in the primary neurons, mediated by the SMases-ceramide pathway. The elevated level of ceramides in the PA cultured astrocytes enhanced the secreted levels of TNF α and IL-1 β , which upregulated the activity of the SMases in primary neurons.

2. Materials and methods

2.1. Isolation and culture of primary rat neurons and astrocytes

All procedures were performed according to guidelines approved by the Institutional Animal Care and Use Committee at Michigan State University. Cortices from postnatal day 0 Sprague Dawley rats were used for neuronal culture according to the published methods (Zhou et al., 2009). Briefly, the brain regions were dissected, and the tissues were digested with papain (10 units/ml; Worthington, NJ, USA) and DNaseI (100units/ml; Roche, IN, USA) at 37 °C for 30 min and washed with Neurobasal A medium (all reagents are from Invitrogen, Carlsbad, CA, USA, unless otherwise specified). The mechanically separated cells were plated on poly-L-lysine (PLL, 50g/ml; Cultrex, Gaithersburg, MD, USA) coated plates at 2.5×10^5 cells/cm². Neurons were maintained in neurobasal A medium with B27, 0.5 mM glutamine, and 1X penicillin/streptomycin (P/S). The medium was changed every 2 days. The primary neurons were cultured 3–4 days prior to treatment. The purity of neurons was >90% as determined by β III tubulin immunostaining and flow cytometry.

Primary rat cortical astrocytes were isolated from newborn pups (P0–2). Cerebral cortices were removed, digested with papain and DNaseI for 30min at 37 °C, and washed with DMEM/F12. Approximately 4×10^4 cells/cm² were seeded on PLL coated plates and the cells were maintained in DMEM/F12 plus 10% fetal bovine serum and 1X P/S. The cultured medium was changed every 2–3 days. Cells were allowed to grow to confluence prior to treatment. The purity of the astrocytes monolayers were >90% as determined by GFAP immunoreactivity and flow cytometry.

2.2. Astrocyte conditioned media (CM) preparation

At 24 hr prior to treatment, the media for the primary neurons and astrocytes were changed to neuronal cell culture medium [DMEM 10313 supplemented with 10% horse serum, 10mM HEPE (Sigma, St. Louis, MO, USA), 2mM glutamine, and 1X P/S]. Astrocytes, cultured to 70–80% confluence, were treated with neuronal media containing 0.2mM PA (Sigma) plus BSA (fatty acid-free bovine serum albumin) (Millipore, Billerica, MA, USA) as a carrier protein, or BSA as control, or PA plus BSA and 2mM LCS (L-cysteine, SPT inhibitor) (Sigma). The PA/BSA molar ration was at 3:1 (Shi and Pestka, 2009). After 12 hr of incubation, the astrocytes conditioned media (CM-B, or CM-P, or CM-P+LCS) were used to treat neurons or used in combination with antibodies to neutralize the cytokines.

2.3. Quantitative real time polymerase chain reaction

mRNA was extracted using the RNA extraction kit (Qiagen, Valencia, CA, USA), then mRNA was reverse transcribed into cDNA using the cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The following primer sets (Operon, Huntsville, AL, USA) were used for PCR: N-SMase (5'-ccggatgcacactactcagaa-3', 5'-ggattgggtgtctggagaaca-3'), A-SMase (5'-tttcccgagccctgtaga-3', 5'-atctgaccacgccaatg-3'), actin (5'-ctctccagccttcctcct-3', 5'-aatgcctgggtacatggtg-3'), IL-1 β (5'-gcatccagcttcaaatctc-3', 5'-gggtctgatgtaccagttg-3'), TNF α (5'-ctactgaactcggggtgatcggtc-3', 5'-ctggtatgaagtggcaatcggtc-3'), INF γ (5'-agagcctcctcttgatattctg-3', 5'-gcttccttagctagattctggtg-3'). Amplifications of the cDNA templates were detected by SYBR Green Supermix (Bio-Rad) using Real-Time PCR Detection System (Bio-Rad). The cycle threshold values were determined by the MyIQ software.

2.4. Western blot

Whole cell extracts lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer were assayed for protein concentrations by Bradford assay (Bio-Rad). 15–30 μ g protein samples were separated by 10% Tris-HCl gel and transferred to nitrocellulose membrane. Membranes were then blocked with 5% milk or 5% BSA in 0.05% Tween 20-TBS (Tris buffered saline) (USB corporation, Fremont, CA, USA) for 1 hr and incubated with primary antibodies, BACE1 (Abcam, Cambridge, MA, USA) and actin (Sigma) overnight at 4°C. Next day, anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Thermo Scientific, Asheville, NC, USA) was added and the blots were incubated for 1 hr at room temperature. The blots were washed three times with 0.05% Tween 20-TBS, and then visualized by SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Secreted cytokines in the astrocyte supernatants were analyzed by an ELISA kit (R&D system, Minneapolis, MN, USA). The sensitivity of the assay was 5pg/ml for TNF α and IL-1 β . Optical densities were measured by Spectra MAX Plus384 plate reader at 450nm wavelength. Each sample was assayed in triplicates and three independent reactions were performed. All readings were normalized to the total cell protein content (Bradford assay), and then the data were normalized to the control (BSA treatment).

2.6. Neutralization of cytokine bioactivity

The concentration of TNF α and IL-1 β recombinant proteins (R&D system) used for neutralization were optimized based on the concentration of TNF α and IL-1 β in the CM-P and the neutralization dose₅₀ of recombinant proteins. Neutralization was performed as described in manufacturer's information sheet. Briefly, the conditioned media was collected and incubated with recombinant proteins for 2 hr at 37 °C prior to treating the cells.

2.7. Sphingomyelinase assay

Cells were lysed with the neutral (100 mM Tris-HCl, 1mM MgCl₂, pH 7.4) or acid lysis buffer (50mM sodium acetate, pH 5.0), to measure N-SMase or A-SMase activities, respectively, along with protease inhibitor cocktail (Sigma). SMase activities were measured using Amlpex Red Sphingomyelinase Assay Kit (Invitrogen) as described in the manufacturer's protocol. The total protein level in the cells was determined by Bradford method. The SMase activity levels were normalized to the total cell protein content, and then normalized to the control (CM-B treatment).

2.8. Measurement of ceramide

Primary neurons were treated with astrocyte conditioned media for the indicated time. The cells were washed twice with PBS, and the lipid was extracted as described in (Busik et al., 2009). The ceramide levels were detected by mass spectrometry using an AB 3200 QTRAP LC/MS/MS system (AB Sciex, Foster City, CA, USA). Multiple reaction monitoring (MRM) for quantitative LC/MS/MS was performed. C16 and C24 ceramide (Avanti, Alabaster, AL, USA) were used as standards. The total protein levels were measured by the Bradford method. The total ceramide levels were normalized to the protein level, and then normalized to the control (CM-B treatment).

2.9. Statistical analysis

All experiments were performed at least three times, and representative results are shown. Statistical analysis were performed by an unpaired, two tail student t-test. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

3. Results

3.1 Ceramide level is up-regulated in primary neurons with CM-P treatment

Previously we showed that conditioned media from palmitate-treated astrocytes (CM-P) upregulated the BACE1 level and amyloidogenesis in primary rat neurons. The upregulation was mediated by the increase in ceramides that were generated through the *de novo* ceramide synthesis pathway (SPT) in the astrocytes (Patil et al., 2007). It is known that ceramide can stabilize BACE1 to promote A β generation (Puglielli et al., 2003). Therefore, we examined the ceramide level in primary neurons upon CM-P treatment, and found it increased dramatically and peaked at 12hr (Fig. 1A). L-cycloserine (L-CS), a specific inhibitor of the rate-limiting enzyme SPT, was used to pre-treat the primary astrocytes. This was followed by co-treatment of the astrocytes with PA plus L-CS, denoted as CM-P+LCS. The CM-P+LCS media was subsequently used to treat the primary neurons. Upon inhibiting SPT in the astrocytes, the increased ceramide level in the neurons cultured in PA-treated astrocyte conditioned media (namely, CM-P+LCS) was reduced (Fig. 1B). Note that the direct pre-treatment of the neurons with L-CS follow by culturing in the PA-treated astrocyte conditioned media concomitantly with L-CS (namely, CM-P-LCS) did not reduce the ceramide levels (Fig. 1C). These results raised the question of whether the ceramide, produced by the astrocytes upon treatment with PA, was released into the supernatant, i.e. conditioned media (CM-P), and subsequently crossed the membrane of the neurons.

A recent study demonstrated that A β can significantly increase the ceramide level within primary human astrocytes, however the extracellular ceramide levels in the cultured media was undetectable (Jana and Pahan, 2010). This suggests that the high concentration of intracellular ceramide level in the astrocytes was not likely released into the supernatant. Furthermore, numerous studies indicate that neurons have a very low capacity to take up ceramides and long chain fatty acids, such as PA (Blazquez et al., 2000; Qin et al., 2010). Thus the rise in ceramide levels in the neurons treated with CM-P is likely generated

intracellularly. Since ceramides can be produced by either SMases or SPT, we first tested whether L-CS could inhibit SPT to reduce ceramide levels in the CM-P treated neurons. To assess this possibility, primary rat neurons were treated with 2mM L-CS for 2hrs, followed by co-treatment with 2 mM L-CS and the CM-P from the astrocytes and found this treatment with L-CS did not significantly reduce the ceramide levels in the neurons (Fig. 1C). Therefore, the *de novo* ceramide synthesis pathway in the neurons is not likely responsible for the rise in neuronal ceramide level upon CM-P treatment. Next, given that SMases are also major pathways for generating ceramides, we investigated whether the increased ceramide levels in the neurons are due to A-SMase and N-SMase, the major enzymes that hydrolyze SM.

3.2. SMases are responsible for the increased ceramide in primary rat neurons upon CM-P treatment

The mRNA (Fig. 2A, B) and activity (Fig. 2C, D) levels of A-SMase and N-SMase increased significantly in primary neurons upon CM-P treatment. Inhibiting SPT with L-CS in the astrocytes significantly reduced the mRNA (Fig. 2A, B), and activity (Fig. 2C, D) levels of the SMases and ceramide (Fig. 1B) in the neurons. This raised the possibility that the SMases in the neurons could be responsible for the ceramide rise. Nevertheless the SPT in the astrocytes initiated the path towards this upregulation of SMase expression and activity levels in the neurons.

To confirm that the increased ceramide levels in the neurons are due to the SMases, we inhibited A-SMase and N-SMase with specific inhibitors, 15uM desipramine (Sigma) and 25uM GW4869 (Cayman Chemical, Ann Arbor, MI, USA), respectively, individually and in combination. The ceramide levels in the CM-P treated primary neurons decreased significantly upon SMase inhibition (Fig. 3). Therefore the ceramide rise in the CM-P treated primary neurons is due to the SMases rather than the *de novo* synthesis pathway of SPT. Further, it indirectly confirms that the ceramide produced by the astrocytes are not likely being taken up by the neurons. Therefore, other soluble products released into the CM-P media from the astrocytes upon PA treatment are responsible for upregulating the SMase activities in the neurons. SMase activity can be induced by many different stimuli, i.e. UV or ionizing irradiation, heat shock, nerve-growth factor, Fas, TNF α or IL-1 β (Huang et al., 2001).

3.3. Palmitate induces the secretion of proinflammatory cytokines from primary rat astrocytes and neutralizing the cytokines decreases the SMase activity

Astrocytes are known to secrete proinflammatory molecules, such as TNF α , IL-1 β , IL-6, interferon (IFN)- γ and nitric oxide (NO) (Wyss-Coray and Mucke, 2002). Our lab demonstrated that PA does not induce the production of IL-6 in astrocytes upon PA treatment (Patil, 2007, Michigan State University). A recent report showed that PA induces the production of TNF α and IL-1 β in macrophages through the *de novo* ceramide synthesis pathway (Haversen et al., 2009). Further, an *in vivo* study established that IFN- γ and TNF α regulate A β plaque deposition and BACE1 expression in a transgenic AD mouse model (Yamamoto et al., 2007). Therefore we measured the mRNA levels of TNF α and IL-1 β and found them, most notably IL-1 β , to be significantly upregulated in the astrocytes treated with 0.2mM PA (Fig. 4A). Correspondingly, the protein levels of TNF α and IL-1 β were also elevated (Fig. 4B). However INF- γ was not detected (data not shown). Patil et al. demonstrated that PA increased ceramide levels in astrocytes through the *de novo* ceramide synthesis pathway (Patil et al., 2007). To determine if SPT in the astrocytes induces TNF α and IL-1 β generation upon PA treatment, we inhibited SPT with L-CS and found the levels of TNF α and IL-1 β secreted by the astrocytes decreased (Fig. 4B). Therefore, ceramide is

involved in the production of cytokines in the primary astrocytes. These cytokines, in turn, activate the SMases in the neurons.

To determine if these cytokines are involved in mediating SMase activities in the neurons, anti-rat TNF α and IL-1 β antibodies, were used to neutralize TNF α and IL-1 β in the CM-P media, prior to transferring the CM-P to the primary neurons. The A-SMase and N-SMase activities decreased significantly in neurons treated with CM-P that had the cytokines, TNF α and IL-1 β , neutralized (Fig. 5). Therefore the upregulated SMase activities in the neurons are mediated in part by TNF α and IL-1 β . In support, SMase activities in various cell types, including neurons, were reported to be activated by cytokines, i.e. TNF α and IL-1 β (Adibhatla et al., 2008; Sortino et al., 1999).

3.4. Upregulated BACE1 in primary neurons upon CM-P treatment is related to SMase-ceramide pathway in the primary neurons

Previously, we demonstrated that neurons treated with CM-P increased their BACE1 protein level, which decreased upon inhibiting SPT in the astrocytes (Patil et al., 2007). CM-P up-regulated the BACE1 protein level in the neurons in a time-dependent manner (Fig. 6). Ceramide has been reported to stabilize the BACE1 protein and increase A β generation (Puglielli et al., 2003). Inhibiting SPT reduced ceramide levels in the astrocytes, which in turn decreased neuronal ceramide generation upon CM-P treatment (Fig. 1B). Since the ceramide increase in the CM-P treated neurons are due to the SMases, we pre-treated the neurons with respective A-SMase and N-SMase inhibitors, GW4869 and desipramine, individually and in combination, followed by co-treatment of the neurons with the inhibitors and CM-P media for 12hr. The BACE1 level decreased in the CM-P cultured neurons pre- and co-treated with the inhibitors as compared to the CM-P cultured neurons without the inhibitor treatment (Fig. 7). Therefore, the ceramide produced in the neurons by the SMases enhanced the BACE1 protein level in the CM-P treated neurons. A recent report showed that ceramide can increase the expression level of acetyltransferases' (ATase1 and ATase2) and the latter posttranslationally regulates BACE1 by stabilizing the protein (Ko and Puglielli, 2009). Therefore, we measured the ATase levels in the CM-P treated neurons, and found the mRNA levels of ATase1 and ATase2 are significantly increased (Supplementary Fig. 1). Thus ATases may be involved in upregulating the BACE1 level in neurons treated with CM-P. Since TNF α and IL-1 β activate the SMases to increase ceramide production in the neurons, they should indirectly impact the BACE1 level. Neutralizing TNF α and IL-1 β in the CM-P with their respective antibodies, prior to transferring the CM-P to the primary neurons for 12 hr significantly decreased the BACE1 levels (Fig. 8). Therefore cytokines, namely TNF α and IL-1 β , released by the palmitate-treated astrocytes, activate the SMase-ceramide pathway in the neurons to thereby upregulate the BACE1 levels.

4. Discussion

Alzheimer's disease is a complex, devastating neurodegenerative disorder that results from multiple environmental and genetic factors. Epidemiological studies suggest that saturated FFAs may increase the risk of AD (Takechi et al., 2009). Feeding animals with high fat diets leads to cognitive impairment and accelerated AD-like pathology (Molteni et al., 2004). Evidence from our group suggest that PA does not directly induce AD-like pathogenesis in primary rat neurons, whereas CM-P media does, therefore implicating PA nevertheless participates in the A β biogenesis in primary neurons (Patil and Chan, 2005; Patil et al., 2006). In further support, previous studies demonstrate that the increased production of ceramide by the astrocytes through SPT played a critical role, and inhibiting SPT in the astrocytes attenuated the AD-like changes in primary neurons (Patil et al., 2007). However, the mechanism by which CM-P induced AD changes in the neurons is unknown. To address this, we investigated a possible mechanism and found the neuronal ceramide level was

significantly enhanced upon CM-P treatment, mediated by SMases rather than SPT. This is consistent with studies that have shown that the SMases levels are upregulated upon treating with amyloid peptides to increase the ceramide levels and neuronal apoptosis (Malaplate-Armand et al., 2006). Nevertheless SPT upregulation in the astrocytes is involved in upregulating the SMase activities in the neurons.

It is not surprising that activated astrocytes play a role in the AD-like pathological changes in primary neurons, given the strong neuroinflammatory response in AD brains, and that amyloid plaques are surrounded by activated astrocytes and microglia (Wyss-Coray and Mucke, 2002). Recent studies showed that overexpressing molecules from activated astrocytes in AD transgenic mouse model increased inflammation in the brain and exacerbated AD pathology (Mori et al., 2009). Activated astrocytes can release inflammatory molecules, such as cytokines, reactive oxygen species and nitric oxide (NO) (Querfurth and LaFerla, 2010). Many have reported that SMase activity can be activated by cytokines, i.e. TNF α and IL-1 β , in several different cell types, including neurons (Adibhatla et al., 2008; Sortino et al., 1999). To link astrocytes and neurons under pathological conditions, i.e. elevated FFA (i.e. PA) levels, we set out to determine which molecules released from the astrocytes upon PA treatment are involved in increasing BACE1 level and amyloidogenesis in the neurons. We found that TNF α and IL-1 β are secreted by astrocytes, and these cytokines upregulate the SMase activities. Neutralizing TNF α , IL-1 β , or both in the CM-P media decreased both the A-SMase and N-SMase activities in the neurons upon CM-P treatment. This attenuated the upregulation in the BACE1 level, thereby indicating that TNF α and IL-1 β released upon *de novo* synthesis of ceramides in the astrocytes induced an upregulation in the SMase activities in the neurons. In addition to cytokines, NO may account for the residual increase in SMase levels. Recently, a transwell study further implicated a connection between activated astrocytes and neurons in AD pathogenesis and found that NO released from the astrocytes activated the SMase-ceramide pathway to cause neuronal cell loss (Jana and Pahan, 2010).

Numerous studies have shown that SMases play important roles in the induction of apoptosis upon treatment with A β in the neurons as well as other cells in the CNS (Malaplate-Armand et al., 2006). Most of the prior cell studies have focused on a single cell type, i.e. neurons, and did not incorporate the interactions mediated by soluble factors between neurons and astrocytes. Here we demonstrate for the first time that upregulated SMase activities in the neurons are due directly to the activation of the *de novo* ceramide synthesis (namely, SPT) in the astrocytes upon PA treatment. SPT is the first and rate-limiting enzyme in *de novo* synthesis of ceramide from PA. The ceramide produced in the astrocytes released soluble factors into the CM-P to modulate the AD-like pathophysiology in the neurons. Thus SPT may play an initiating role in the AD-like pathology and the neuronal damage that results from elevated levels of saturated fatty acids arising from environmental factors, i.e. diet or head trauma.

Many studies showed that perturbed sphingomyelin metabolism is an important event in neurodegeneration, elevating ceramide levels and changing the structural and functional plasticity of the neurons (Haughey et al., 2010). Studies in AD brain suggest that the expression of several genes controlling the synthesis of ceramide are upregulated (Katsel et al., 2007), e.g. increased SMase activities (He et al., 2008) and SPT protein levels (He et al., 2008). Ceramide has been shown to stabilize BACE1 and promote A β biogenesis (Puglielli et al., 2003), including post-translational regulation of BACE1 by enhancing the acetyltransferase (ATase) activity (Ko and Puglielli, 2009). Similarly, we also found the mRNA levels of ATases are upregulated in primary neurons cultured with CM-P (Supplementary Fig. 1). Ceramide also can increase the level of the activated form of the double stranded RNA-activated protein kinase, p-PKR (Ruvolo et al., 2001). Elevated levels

of the active form of the eukaryotic translation initiation factor 2 (eIF2 α), a downstream target of p-PKR, can increase the translation of BACE1 to promote amyloidogenesis (Gil et al., 2000; O'Connor et al., 2008). We found that p-PKR level was significantly increased in primary neurons treated with CM-P (Supplementary Fig. 2). These events also could contribute to increased BACE1 level in primary neurons treated with CM-P.

Based on our findings and the published results, we proposed the following sequence of events on how elevated level of PA may initiate the A β cascade leading to the pathophysiology of AD (Fig. 9). The astrocytes in the brain metabolize PA to ceramide through the *de novo* ceramide synthesis pathway (SPT), which initiates the release of soluble molecules, i.e. cytokines, from the astrocytes. In turn, these soluble molecules activate the SMase-ceramide pathway to increase ceramide levels in the neurons. The ceramide produced in the neurons could post-translationally upregulate the BACE1 levels in the neurons to enhance A β production (Ko and Puglielli, 2009; Puglielli et al., 2003). The upregulated extracellular A β level, in turn, could act on both the astrocytes and the neurons to further enhance the intracellular ceramide levels (Jana and Pahan, 2010). This would continue to reinforce the ceramide-A β -ceramide cascade. Finally, although the cell studies do not truly recapitulate the *in vivo* situation of AD pathogenesis, nevertheless the literature information coupled with our results suggest that tight regulation of ceramide production may be an important therapeutic approach to modulating BACE1 level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Changes in ceramide levels in primary rat neurons

Primary rat astrocytes were cultured with BSA (B) or palmitate (P) or palmitate plus L-CS (P+LCS) (LCS: SPT inhibitor) for 12hr. The conditioned media (CM-B, CM-P, or CM-P+LCS, respectively) were subsequently used to treat primary rat neurons for 12 or 24hr. Ceramide was detected by LC/MS/MS and normalized to CM-B (ctrl) (n=3) (A, B). (C) Primary rat neurons were treated with CM-B, CM-P, or pre and co-treated with L-CS (CM-P-LCS) for 12hr. Ceramide was detected by LC/MS/MS and normalized to CM-B (ctrl) (n=3). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. A line indicates comparison between the two bars connected by the line.

Fig. 2. Effect of CM on the SMase level in the neurons

(A, B) The conditioned media, CM-B, CM-P or CM-P+LCS, were used to treat neurons for 1, 6, 12 and 24hr and their mRNA levels were measured by real-time PCR. The mRNA of A-SMase (A) and N-SMase (B) were normalized to actin, then normalized to CM-B (ctrl) at each time point (n=3). A-SMase (C) and N-SMase (D) activities in neurons cultured in CM-B (ctrl), CM-P or CM-P+LCS for 12hr (n=3). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. A line indicates comparison between the two bars connected by the line.

Fig. 3. Effect of inhibiting SMase activities on the ceramide levels in the neurons

Ceramide levels of primary rat neurons cultured in CM-B (ctrl), CM-P, CM-P plus desipramine (A-SMase inhibitor, CM-P-A), CM-P plus GW4869 (N-SMase inhibitor, CM-P-N), or CM-P plus desipramine and GW4869 (CM-P-AN) for 12hr. Ceramide was detected by LC/MS/MS (n=3). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. A line indicates comparison between the two bars connected by the line.

Fig. 4. TNFα and IL-1β levels of astrocytes upon PA treatment

Astrocytes were treated with BSA (B), palmitate (P) or palmitate plus L-CS (P+LCS) for indicated time. (A) mRNA levels of TNFα and IL-1β were detected by real-time PCR (n=3). (B) Protein fold-change of TNFα and IL-1β. The protein levels of cytokines in the supernatant were measured by ELISA (n=3). *: p<0.05, **: p<0.01, ***: p<0.001. A line indicates comparison between the two bars connected by the line.

Fig. 5. Neutralizing TNF α and IL-1 β decrease SMase activity

A-SMase (A) or N-SMase (B) activities in neurons upon treatment with CM-B (ctrl), CM-P, CM-P-T (TNF α in CM-P media was neutralized with TNF α antibody), CM-P-I (IL-1 β in CM-P media was neutralized with IL-1 β antibody), or CM-P-TI (TNF α and IL-1 β were neutralized in CM-P media) treatment for 12hr (n=3). *: p<0.05, **: p<0.01, ***: p<0.001. A line indicates comparison between the two bars connected by the line.

Fig. 6. Effect of CM-P on BACE1 level in the neurons

Primary rat neurons were cultured with CM-B (ctrl) or CM-P for 6, 12 and 24hr. (A) Representative western blot result of BACE1 protein. (B) Quantification of western blot results (n=3). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. A line indicates comparison between the two bars connected by the line.

Fig. 7. Effect of inhibiting SMase on the BACE1 level in neurons upon CM treatment
 (A) Representative western blot result of BACE1 protein levels in primary rat neurons cultured in CM-B (ctrl), CM-P, CM-P plus desipramine (A-SMase inhibitor, CM-P-A), CM-P plus GW4869 (N-SMase inhibitor, CM-P-N), or CM-P plus desipramine and GW4869 (CM-P-AN) for 12hr (n=3). (B) Quantification of western blot results (n=3). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. A line indicates comparison between the two bars connected by the line.

Fig. 8. Neutralizing TNF α and IL-1 β decrease BACE1 in neurons

(A) BACE1 protein levels in neurons upon treatment with CM-B (ctrl), CM-P, CM-P-T (TNF α in CM-P media was neutralized with TNF α antibody), CM-P-I (IL-1 β in CM-P media was neutralized with IL-1 β antibody), or CM-P-TI (TNF α and IL-1 β in CM-P media were neutralized) treatment for 12hr (n=3). (B) Quantification of western blot results (n=3). *: p<0.05, **: p<0.01, ***: p<0.001. A line indicates comparison between the two bars connected by the line.

Fig. 9. Proposed cellular mechanism by which palmitic acid metabolism induces amyloidogenesis in primary neurons mediated by astrocytes

The astrocytes in the brain metabolize PA to generate ceramides through the *de novo* ceramide synthesis pathway by SPT, which initiates the release of soluble molecules, i.e. TNFα and IL-1β, from the astrocytes. In turn, these soluble molecules activate the SMase-ceramide pathway in the neurons to increase ceramide levels. Upregulated ceramide increase BACE1 level and enhance Aβ production (Ko and Puglielli, 2009; Puglielli et al., 2003). The increased extracellular Aβ level may act on both the astrocytes and the neurons to enhance the intracellular ceramide levels. This ceramide-Aβ-ceramide cascade creates a continuing cycle to further induce cell death (Jana and Pahan, 2010; Li et al., 2009).