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Palmitate-induced Endoplasmic Reticulum stress and subsequent C/EBPα Homologous Protein activation attenuates leptin and Insulin-like growth factor 1 expression in the brain

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ABSTRACT

The peptide hormones Insulin-like growth factor-1 (IGF1) and leptin mediate a myriad of biological effects - both in the peripheral and central nervous systems. The transcription of these two hormones is regulated by the transcription factor C/EBPα, which in turn is negatively regulated by the transcription factor C/EBP Homologous Protein (CHOP), a specific marker of endoplasmic reticulum (ER) stress. In the peripheral system, disturbances in leptin and IGF-1 levels are implicated in a variety of metabolic diseases including obesity, diabetes, atherosclerosis and cardiovascular diseases. Current research suggests a positive correlation between consumption of diets rich in saturated free fatty acids (sFFA) and metabolic diseases. Induction of ER stress and subsequent dysregulation in the expression levels of leptin and IGF-1 have been shown to mediate sFFA-induced metabolic diseases in the peripheral system. Palmitic acid (palmitate), the most commonly consumed sFFA, has been shown to be uptaken by the brain, where it may promote neurodegeneration. However, the extent to which palmitate induces ER stress in the brain and attenuates leptin and IGF1 expression has not been determined. We fed C57BL/6J mice a palmitate-enriched diet and determined effects on the expression levels of leptin and IGF1 in the hippocampus and cortex. We further determined the extent to which ER stress and subsequent CHOP activation mediate the palmitate effects on the transcription of leptin and IGF1. We demonstrate that palmitate induces ER stress and decreases leptin and IGF1 expression by inducing the expression of CHOP. The molecular chaperone 4-phenylbutyric acid (4-PBA), an inhibitor of ER stress, precludes the palmitate-evoked down-regulation of leptin and IGF1 expression. Furthermore, the activation of CHOP in response to ER stress is pivotal in the attenuation of leptin and IGF1 expression as knocking-down CHOP in mice or in SH-SY5Y and Neuro-2a (N2a) cells rescues the palmitate-induced mitigation in leptin and IGF1 expression. Our study implicates for the first time ER stress-induced CHOP activation in the brain as a mechanistic link in the palmitate-induced negative regulation of leptin and IGF1, two neurotrophic cytokines that play an indispensable role in the mammalian brain.

1. Introduction

Leptin and Insulin-like growth factor (IGF1) are two neurotrophic cytokines that exert a wide array of pleiotropic effects both in the peripheral system and in the brain. Leptin is primarily expressed and secreted by the adipocytes and the serum levels are commensurate to the mass of the white adipose tissue [1,2]. IGF1 is synthesized primarily by the hepatocytes as well as most of cells of the peripheral tissues [3,4]. The conventional consensus posited that leptin and IGF1 cross the blood-brain-barrier (BBB) and consequently elicit their effects by activating their respective receptors, Ob-Rb (leptin receptor isoform b) and IGF1R that are widely expressed in the brain. However, recent evidence has shown that both leptin [5–11] and IGF1 [10,12,13] are expressed endogenously in the brain and exert their effects in an autocrine/paracrine fashion. Leptin expression in the brain is critical as it serves as a neurotrophic cytokine that facilitates memory formation

Abbreviations: 4-PBA, 4-phenyl butyric acid; 6-OHDA, 6-hydroxydopamine; Aβ, amyloid beta; AD, Alzheimer's disease; ATF3, activating transcription factor 3; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; BACE1, β-site APP cleaving enzyme 1; BSA, bovine serum albumin; eIF2α, eukaryotic initiation factor 2 alpha; C/EBPα, CCAAT/Enhancer Binding Protein alpha; CHOP, C/EBP homologous protein; ChIP, chromatin immunoprecipitation; GADD153, growth arrest and DNA damage-inducible protein; DDIT3, DNA-damage-inducible transcript 3; ER, endoplasmic reticulum; IDE, insulin degrading enzyme; IGF1, insulin-like growth factor 1; IRE1α, inositol requiring enzyme 1 alpha; IGF1R, insulin-like growth factor 1 receptor; NFT, neurofibrillary tangles; Ob-Rb, leptin receptor isoform b; PD, Parkinson's disease; PERK, protein kinase R (PKR) – like endoplasmic reticulum kinase; sFFA, saturated free fatty acids; XBP1, X-box binding protein 1.

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and enhances cognition by augmenting synaptogenesis [14]. Leptin fosters spatial memory formation in the hippocampus and increases neurogenesis in the dentate gyrus of adult mice [14,15]. Leptin increases neuronal survival and attenuates apoptotic neuronal death in response to a multitude of noxious stimuli, and there is growing consensus that leptin functions as an endogenous growth and survival factor in the brain [9]. Leptin also evokes neurogenesis in the dentate gyrus of adult mice and induces the proliferation of adult hippocampal progenitor cultures [16]. IGF1 possesses pleiotropic functions in the brain and serves an indispensable role in neural plasticity, neuronal survival and in fostering hippocampal neurogenesis [17]. Our previous studies have demonstrated that both leptin and IGF1 are expressed endogenously in the cortex and hippocampal areas of the brain and they mutually regulate each other positively at the transcriptional level [10]. We have shown that the transcription factor, CCAAT/Enhancer binding protein α (C/EBPα) regulates leptin and IGF1 expression in the rabbit hippocampus [10].

A multitude of epidemiological studies have suggested that a diet rich in saturated free fatty acids (sFFA) adversely affects cognition [18,19] and is closely associated with cognitive decline [20]. Also, the degree of saturated fat or saturated fatty acid intake in diet commensurately dictates the degree and extent of cognitive decline [21]. Furthermore, a saturated fat-enriched diet has been shown to elicit cognitive impairments in several rodent models [22]. Palmitate is the most abundant sFFA in the diet [23] and the brain [24]. The serum levels of sFFA are inversely correlated with cognitive ability in diabetic and obese individuals [25]. There is consensus that peripheral circulating sFFA, either emanating from diet or from de novo lipogenesis in the liver, cross the blood-brain-barrier and contribute to the pool of sFFA in the brain [26,27]. Endoplasmic reticulum (ER) stress has emerged one of driving factors in mediating neuronal apoptosis, neuronal inflammation, cognitive impairment, and deficits in adult neurogenesis [28,29]. Numerous studies have shown that palmitate induces ER stress in a wide array of peripheral tissues [30]. Sustained ER stress culminates in the increased expression of the transcription factor C/EBP Homologous Protein (CHOP, also called growth arrest and DNA damage induced gene-153, GADD153 or DDIT3) [31,32]. CHOP negatively regulates the transcriptional activity of C/EBPα [33], a transcription factor indispensable for leptin and IGF1 gene expression [7,34]. A multitude of studies have also demonstrated that a diet rich in palmitate or palmitate treatment of cultured cells results in leptin and IGF1 resistance at the signaling level [35,36]. However, the extent to which palmitate evokes ER stress, activates CHOP, and reduces C/EBPα transcriptional activity that regulates leptin and IGF1 expression in the brain has not been determined. In this study, we determined the extent to which palmitate induces ER stress in the brain and decreases leptin and IGF1 expression in both the mouse brain and the neuroblastoma cells.

2. Materials and methods

2.1. Materials

Human SH-SY5Y neuroblastoma cells and mouse Neuro-2a (N2a) neuroblastoma cells were purchased from ATCC (Manassas, VA). All cell culture reagents, with the exception of fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and antibiotic/antimycotic mix (Sigma Aldrich, Saint Louis, MO) were purchased from Invitrogen (Carlsbad, CA). Palmitic acid, Tunicamycin, and 4-phenylbutyric acid were purchased from Sigma Aldrich (St. Louis, MO. The expression vector for bZIP functional deletion mutant of C/EBPα (C/EBPα bZIP$^-$) (catalogued as CMV500 A-C/EBP in Addgene) was a gift from Dr. Charles Vinson (Addgene plasmid # 33352) [37]. The expression plasmid for overexpressing full length native C/EBPα (pcDNA3 Flag C/EBPα) was a gift from Dr. Christopher Vakoc (Addgene plasmid # 66978) [38]. The expression plasmid for overexpressing full length native CHOP (CHOP 6: mCHOP-WT-9E10-pcDNA1) was a gift from Dr. David Ron (Addgene plasmid # 21913). The expression plasmid for overexpressing the leucine zipper domain deleted CHOP mutant (CHOP LZ$^-$) (CHOP 5: mCHOP10 [dLZ] pSRa) was a gift from Dr. David Ron (Addgene plasmid # 21912). The human CHOP and mouse *Chop* double-stranded siRNA sequences and their respective scrambled non-silencing control siRNA were purchased from Santa Cruz Biotechnology (Dallas, TX) and their target sequences are enumerated in Table 3. Human *CHOP* shRNA (set of 5 different shRNA) and Mouse *Chop* shRNA (set of 5 different shRNA) encoded in pLKO.1 lentiviral vector were purchased from Open Biosystems (GE Dharmacon, Lafayette, CO) and their respective target sequences are enumerated in Table 4.

2.2. Cell culture and treatments

Human neuroblastoma SH-SY5Y cells and N2a cells were grown in Dulbecco's modified Eagle's medium: Ham's F12 with Glutamax (1:1; v/v), 10% fetal bovine serum, and 1% antibiotic/antimycotic mix. Cells were maintained at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO_2. To knock-down CHOP expression in SH-SY5Y cells and N2a cells by siRNA approach, cells were transfected in suspension (reverse transfection) using "PolyFect Transfection Reagent" (Qiagen Inc., Valencia, CA). Briefly, the siRNAs stock solution (10 μM) was prepared by dissolving 3 nmol of siRNAs in 330 μL of RNAse free water. The 10 μM siRNA stock solution was further diluted 1:100 using transfection reagent and transfection medium following to yield a final concentration of 100 nM. 40 μL of the 10 μM siRNA stock solution was added to a tube containing 120 μL of PolyFect transfection reagent and 1.2 mL serum-free, antibiotic-free DMEM and mixed well followed by the solution being incubated for 20 min at room temperature. The cells in suspension were added to this tube containing the siRNA and the transfection reagent mixture in the tube and the volume was made up to 8 mL with normal DMEM. The cells were plated in a 100 mm dish to achieve 50% confluence. After 12 h the medium was aspirated and replaced with 10 mL normal DMEM for additional 24 h before being subjected to respective treatments. To knock-down CHOP expression in SH-SY5Y cells and N2a cells by lentiviral shRNA approach, cells were transfected in suspension (reverse transfection) using "PolyFect Transfection Reagent". Briefly, 5 μg of the respective lentiviral shRNA plasmid was added to a tube containing 150 μL of serum-free, antibiotic-free DMEM and mixed well. To the tube, 15 μL of transfection reagent was added and the solution was incubated for 10 min at room temperature. The cells in suspension were added to the media containing the shRNA and the volume was made up to 4 mL with normal DMEM. The cells were plated in a 100 mm dish to achieve 50% confluence. After 12 h the medium was aspirated and replaced with 10 mL normal DMEM for additional 24 h before being subjected to respective treatments.

SH-SY5Y cells and N2a cells were treated with different concentrations of BSA (bovine serum albumin) – conjugated palmitic acid as follows. Briefly, palmitic acid stock solution of 250 mM was prepared in 100% ethanol (100 mg in 1.56 mL ethanol). A 5 mM BSA stock solution was prepared by dissolving 1 g of fatty acid-free BSA in 3 mL MilliQ water (18 MΩ). Both, the palmitic acid and BSA stock solution were sterile filtered using a 0.2 μm filter. The requisite amounts of palmitic acid and BSA were added to sterile serum-free medium to yield the designated terminal palmitic acid concentrations with the ratio of palmitic acid and BSA being 6:1. The respective media were incubated for 1.5 h to conjugate the palmitic acid to the BSA. The cells were treated with the designated concentration of palmitic acid conjugated to BSA for 24 h.

2.3. Mouse experiments

Mice harboring a homozygous targeting deletion mutation to the Ddit3 gene (*Chop*$^{-/-}$ mice) were procured from The Jackson Laboratory [B6.129S(Cg)-Ddit3$^{tm2.1Dron}$/J, Stock # 005530] (Bar Harbor, ME). The corresponding C57BL/6J control mice were also procured from The