Published in final edited form as:

Cell Signal. 2012 February ; 24(2): 484–492. doi:10.1016/j.cellsig.2011.09.029.

Endoplasmic reticulum stress-induced CHOP activation mediates the down-regulation of leptin in human neuroblastoma SH-SY5Y cells treated with the oxysterol 27-hydroxycholesterol

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Abstract

Epidemiological studies have suggested an inverse relationship between the adipocytokine leptin and the onset of Alzheimer's disease (AD), and leptin supplementation decreases amyloid- β (A β) production and tau phosphorylation (p-tau), two major biochemical events that play a key role in the pathogenesis of AD. We have previously shown that the cholesterol oxidized product 27-hydroxycholesterol (27-OHC) inhibits leptin expression, an effect that correlated with increased levels of A β and p-tau. We have also shown that 27-OHC induces endoplasmic reticulum (ER) stress, a cellular response that is implicated in AD and confers leptin resistance. However the extent to which ER stress is involved in 27-OHC-induced attenuation in leptin expression has not been determined. In this study we determined the involvement of ER stress in the 27-OHC-induced attenuation of leptin expression in SH-SY5Y human neuroblastoma cells. We demonstrate that 27-OHC-induced ER stress attenuates leptin expression by activating C/EBP Homologous Protein (CHOP) which negatively regulates C/EBP α , a transcription factor required for leptin expression. The molecular chaperone 4-phenylbutyric acid (4-PBA) precludes 27-OHC-evoked ER stress and down-regulation of leptin. Furthermore, we demonstrate that the activation of the transcription factor CHOP in response to ER stress is pivotal in the attenuation of leptin expression as knocking-down CHOP alleviates the attenuation in leptin expression. Our study implicates ER stress as the mechanistic link in the 27-OHC-induced negative regulation of leptin, a hormone that has potential therapeutic effects in AD by reducing A β and phosphorylated tau accumulation.

Keywords

Alzheimer's disease; C/EBP Homologous Protein; C/EBP α ; Endoplasmic reticulum; Leptin; 27-hydroxycholesterol

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Disclosure statement: The authors confirm that there are no conflicts of interest. All authors have approved the final article

1. Introduction

Alzheimer's disease (AD) is neuropathologically characterized by the accumulation of β -amyloid (A β) peptide as extracellular plaques and the deposition of hyperphosphorylated tau in intracellular neurofibrillary tangles (NFTs). We and others have shown that the adipocytokine leptin is produced endogenously in the brain [1–5] and can attenuate A β production and tau hyperphosphorylation *in vivo* [6–8] and *in vitro* [3,4, 9–11]. Epidemiological studies have demonstrated that higher circulating leptin levels are associated with lower risk of dementia including AD [12], and lower circulating levels of leptin have been reported in AD patients [13]. Multiple lines of evidence suggest the involvement of ER stress in AD pathology [14–16] and in β -amyloid-induced cell death [17–19]. The endoplasmic reticulum (ER) is a cellular organelle involved in various functions including protein folding, maintenance of Ca²⁺ homeostasis and cholesterol synthesis. Stress to the endoplasmic reticulum triggers a cascade of events that leads to increased expression of the transcription factor C/EBP Homologous Protein (CHOP, also called growth arrest and DNA damage induced gene-153, GADD153 or DDIT3) [20–23] via the activation of the PERK/eIF2 α /ATF4 and ATF6 ER stress signaling pathways [24–26]. There is evidence that the ER stress induces leptin resistance which culminates in deficient leptin signaling [27–28]. Leptin expression in the brain is contingent on mTORC1 signaling pathway [3] and requires the activation of the transcription factor CCAAT enhancer binding protein α (C/EBP α) [5]. Evidence suggests that CHOP inhibits the DNA binding activity of C/EBP α and therefore acts as a negative regulator of C/EBP α -mediated transcription [29,30]. CHOP belongs to the same family of transcription factors as C/EBP α . Notably, CHOP shares significant homology with C/EBP α and this homology renders CHOP and C/EBP α conducive to form heterodimers [29]. However the CHOP-C/EBP α heterodimers are unable to bind to their cognate elements on the promoters of target genes and elicit changes in gene expression [29]. Therefore, CHOP acts as a negative regulator of C/EBP α mediated transcription by sequestering C/EBP α and rendering it effete in binding to the cognate DNA elements in the promoter regions of target genes.

We have recently demonstrated that the cholesterol metabolite 27-hydroxycholesterol (27-OHC) decreases leptin expression levels in the rabbit hippocampus [4]. Previous studies from our laboratory have implicated 27-OHC in increased A β 42 production and tau hyperphosphorylation in hippocampal organotypic slices [4,31]. Furthermore, we showed that leptin treatment attenuates the increase in A β 42 production and tau phosphorylation induced by 27-OHC [4]. We have also shown that 27-OHC induces ER stress and activates CHOP in ARPE-19 cells [32]. As ER stress activates CHOP and given that CHOP negatively regulates C/EBP α , a transcription factor required for leptin expression, we hypothesized that ER stress negatively regulates leptin expression via CHOP induction. We also speculated that 27-OHC attenuates leptin expression by precipitating ER stress and activating CHOP. In this study we determined the potential of ER stress induced by 27-OHC to activate CHOP and subsequently down-regulate C/EBP α -mediated leptin expression in human neuroblastoma SH-SY5Y cells.

2. Materials and Methods

2.1. Reagents

27-OHC was purchased from Medical Isotopes (Pelham, NH). 4-phenylbutyric acid (4-PBA), molecular chaperone known to oppose ER stress was purchased from Sigma Aldrich (Saint Louis, MO). 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). Human SH-SY5Y neuroblastoma cells were purchased from ATCC (Manassas, VA). C/EBP α -luciferase

reporter construct was purchased from SA Biosciences (Frederick, MD) while the human leptin-luciferase promoter construct was obtained from SwitchGear Genomics (Menlo Park, CA).

2.2. Cell Culture and Treatments

Human neuroblastoma SH-SY5Y 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% CO₂. After having reached 80% confluence, cells were incubated with vehicle (control), 10μM 27-OHC, 1mM 4-PBA, and 1mM 4-PBA + 10μM 27-OHC for 24 h at 37°C in cell medium.

siRNA for CHOP and the respective scrambled non-silencing control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The following human CHOP double-stranded siRNA sequences (5' → 3' orientation) were used (A): Sense GAAGGCUUGGAGUAGACAAAtt, Antisense UUGUCUACUCCAAGCCUUCtt; (B): Sense GGAAAGGUCUCAGCUUGUAtt, Antisense UACAAGCUGAGACCUUUCtt; (C): Sense GUCUCAGCUUGUAUUAUAGAtt, Antisense UCUAUUAACAAGCUGAGACtt. The transfection of siRNA was performed in the cells with siRNA transfection reagent (Santa Cruz Biotechnology) and siRNA transfection medium (Santa Cruz Biotechnology) according to the manufacturer's recommendation. The siRNAs stock solution (10μM) was prepared by dissolving 3 nmol of siRNAs in 330μL of RNase free water. The 10μM siRNA stock solution was further diluted 1:50 using transfection reagent and transfection medium following manufacturer's protocol to yield a final concentration of 200nM. The cells were transfected for 16 hours followed by 24 hour incubation in normal media before being subjected to respective treatments.

To overexpress C/EBPα in SH-SY5Y neuroblastoma cell line, cells grown to 70% confluence were transfected with Adenoviral Vector containing C/EBPα expression cassette (Ad-CMV/C/EBPα) driven by the CMV promoter, custom designed by Vector Labs (Philadelphia, PA). The Ad-CMV-GFP vector system (empty vector) was used as a control vector as well as to determine transfection efficiency. Cells were grown to 80% confluence in 6-well plates and transfected with 2×10⁵ viral particles (PFU)/ml of media with either Ad-CMV-C/EBPα expression cassette or the Ad-CMV-GFP empty vector. The cells were transfected for 16 hours followed by 24 hour incubation in normal media before being subjected to respective treatments.

2.3. Western blot analysis

Whole cell, cytosolic and nuclear homogenates were prepared as the following. Treated SH-SY5Y cells were washed with PBS and trypsinized to collect the cells and centrifuged at 5000g. The pellet was washed again with PBS and homogenized in MPER (mammalian protein extraction reagent) (Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitors for whole cell homogenates or homogenized in NE-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitors for cytosolic and nuclear homogenates. Protein concentrations from the whole cell, cytosolic, and nuclear homogenates were determined with BCA protein assay. Proteins (10μg) were separated on SDS-PAGE gels followed by transfer to a polyvinylidene difluoride membrane (BioRad, Hercules, CA) and incubation with the following monoclonal antibodies: anti-leptin rabbit antibody (1:1000; ABR Affinity Bioreagents, Boston, MA), anti GRP78 mouse antibody (1:500; Cell Signaling, Boston, MA), anti GRP94 rabbit antibody (1:500; Cell Signaling, Boston, MA), anti-phospho Thr⁹⁸⁰ PERK mouse antibody (1:100; Cell Signaling, Boston, MA), anti-phospho Ser⁷²⁴ IRE1α

mouse antibody (1:100; Abcam, Cambridge, MA), anti-ATF6 rabbit antibody (1:100; Abcam, Cambridge, MA), anti-CHOP mouse antibody (1:100, Cell Signaling, Boston, MA). β -actin was used as a gel loading control for whole cell and cytosolic homogenates, whereas Lamin A/C was used as a gel loading control for nuclear homogenates. The blots were developed with enhanced chemiluminescence (Immun-star HRP chemiluminescent kit, Bio-Rad, Hercules, CA). Bands were visualized on a polyvinylidene difluoride membrane and analyzed by LabWorks 4.5 software on a UVP Bioimaging System (Upland, CA). Quantification of results was performed by densitometry and the results analyzed as total integrated densitometric values (arbitrary units).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Leptin levels were quantified using a quantitative sandwich ELISA kit (R & D systems, Minneapolis, MN) as per the manufacturer's protocol. Treated human SH-SY5Y neuroblastoma cells were trypsinized, collected and homogenized in MPER protein extraction reagent supplemented with protease and phosphatase inhibitors. Protein concentrations from the respective cellular homogenates were determined with BCA protein assay. The cellular homogenates belonging to different treatments were further diluted in PBS to yield a protein concentration of 1mg/ml. 1 μ L of the tissue homogenate from each treatment group normalized to 1mg/ml protein concentration was further diluted 1:100 in the assay diluent buffer provided with the kit. A total of 100 μ L of this diluted homogenate was added to each well of the ELISA plate for the assay. The optical density of each well was determined using a microplate reader set at 450nm. The concentrations obtained were multiplied by a factor of 100 to account for the 100-fold dilution. The leptin levels were measured in triplicate for each of four separate cell culture experiments. The final results are expressed as ng of leptin /ml of tissue homogenate.

2.5. Co-Immunoprecipitation

Co-Immunoprecipitation (Co-IP) from cell homogenate was performed for C/EBP α and CHOP by using "Catch and Release" immunoprecipitation kit from Millipore (Bedford, MA) according to the manufacturer's protocol. Briefly, 3 $\times$ 10⁶ cells were homogenized in MPER (mammalian Protein Extraction Reagent, Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitors. The homogenate containing the equivalent to 500 μ g of total protein content was incubated separately with 2 μ g of anti-C/EBP α mouse antibody (1:500; Active Motif, Carlsbad, CA) and 2 μ g of anti-CHOP mouse antibody (1:100, Cell Signaling, Boston, MA) overnight in the spin columns followed by elution using a non-denatured elution buffer provided with the kit. 5 μ L of the eluate from anti-C/EBP α antibody precipitated protein-antibody complex was resolved on a SDS-PAGE gel followed by transfer onto a polyvinylidene difluoride membrane (BioRad, Hercules, CA) and incubation with CHOP antibody followed by development with enhanced chemiluminescence (Immun-star HRP chemiluminescent kit, Bio-Rad, Hercules, CA). Analogously, 5 μ L of the eluate from anti-CHOP antibody precipitated protein-antibody complex was resolved on a SDS-PAGE gel followed by transfer onto a polyvinylidene difluoride membrane (BioRad, Hercules, CA) and incubation with C/EBP α antibody followed by development with enhanced chemiluminescence (Immun-star HRP chemiluminescent kit, Bio-Rad, Hercules, CA). Bands were visualized on a polyvinylidene difluoride membrane and analyzed by LabWorks 4.5 software on a UVP Bioimaging System (Upland, CA).

2.6. Quantitative real time RT-PCR analysis

Total RNA was isolated and extracted from treated cells using the 5 prime "PerfectPure RNA tissue kit" (5 Prime, Inc., Gaithersburg, MD). RNA estimation was performed using "Quant-iT RNA Assay Kit" using a Qubit fluorometer according to the manufacturer's

protocol (Invitrogen, Carlsbad, CA). cDNA was obtained by reverse transcribing 1 µg of extracted RNA using an iScript cDNA synthesis kit[®] (BioRad, Hercules, CA). The oligomeric primers (Sigma, St Louis, MO) used to amplify the leptin mRNA are enumerated in Table 1. The cDNA amplification was performed using an iQ SYBR Green Supermix kit following the manufacturer's instructions (BioRad, Hercules, CA). The amplification was performed using an iCycler iQ Multicolor Real Time PCR Detection System (BioRad, Hercules, CA). The expression of specific leptin transcripts amplified was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.7. Luciferase Reporter Assays

Constructs encoding C/EBPα reporter and leptin promoter conjugated to the firefly luciferase gene were used in the study. Human neuroblastoma SH-SY5Y cells were plated in 96-well plates at a density of 2×10^4 cells/well. The cells were transfected when 80% confluent with 0.25 µg of either C/EBPα-firefly luciferase reporter construct or leptin-firefly luciferase promoter construct. Respective non-inducible reporter constructs containing constitutively expressing *Renilla* luciferase were used as negative internal controls. Constitutively expressing GFP constructs were used as positive control to determine transfection efficiency. Cells were incubated for 24 hours with Opti-MEM serum free medium (Invitrogen, Carlsbad, CA) containing the reporter constructs dissolved in transfection reagent. After 24 hours the medium was changed and the cells were incubated in normal DMEM/F12 medium containing 10% FBS and cells were treated with the different treatment regimens. The cells were treated in triplicate and harvested 24 hours later and subjected to dual-luciferase assay. The dual-luciferase assay was performed using a “Dual-Luciferase Reporter Assay System” from Promega (Madison, WI). The luminescence recorded is expressed as Relative Luminescence Units (RLU) and normalized to per mg protein. Unit value was assigned to control and the magnitude of differences among the samples is expressed relative to the unit value of control cells

2.8. Electrophoretic Mobility Shift Assay (EMSA)

The Electrophoretic Mobility Shift Assay (EMSA) was performed using a kit from Active Motif (Carlsbad, CA) following manufacturer's protocol. Nuclear extract was prepared using NE-PER protein extraction reagent following the manufacturer's instructions (Thermo Scientific, Rockford, IL). The human leptin promoter region spanning 4000 nucleotides upstream of the transcription initiation site in leptin gene was scanned for C/EBPα binding consensus sequences using the “TFsearch” online program that searches highly correlated sequence fragments against TFMATRIX transcription factor binding site profile database in ‘TRANSFAC’ databases [33,34]. The human leptin promoter contains a C/EBPα consensus motif 1424 nucleotides upstream of the transcription start site. The 5'-biotin labeled and unlabeled oligonucleotide probes that correspond to the C/EBPα binding site in the leptin promoter region (–1434 to –1405 of the leptin promoter) (Table 1) were purchased from Sigma Aldrich (St Louis, MO). 10 µg of nuclear proteins were incubated with either 20 femto moles of biotin labeled oligonucleotide probe or 4 pico moles of unlabelled oligonucleotide. To exhibit specificity of the oligonucleotide probes, unlabelled oligonucleotide probe was used as a specific competitor for binding reactions at a concentration of 200 fold of the concentration of the biotin labeled probe. 1 µg of Poly d(I-C) was used as a non-specific competitor for binding reactions. The resulting binding reaction mix was loaded and resolved on a 5% TBE gel (BioRad, Hercules, CA) followed by transfer onto a nylon membrane. The bands were visualized using the HRP-Streptavidin – Chemiluminescent reaction mix provided with the kit on a UVP Bioimaging System (Upland, CA).

2.9. Chromatin Immunoprecipitation (ChIP) Analysis

ChIP analysis was performed to evaluate the extent of C/EBP α binding to the DNA elements in the leptin promoter region using “SimpleChIP™ Enzymatic Chromatic IP kit” from Cell Signaling (Boston, MA). Briefly, cells from each treatment group (3×10^6 cells) were washed with PBS, trypsinized, centrifuged at 5000g. The pellet was further washed with PBS and cross-linked using 37% formaldehyde for 15 min followed by the addition of glycine solution to cease the cross-linking reaction. The pellet was washed with $4 \times$ volumes of $1 \times$ PBS and centrifuged at ~ 220 g for 5 min. The pellet was resuspended and incubated for 10 min in 5ml of cell lysis buffer containing DTT and protease inhibitor provided with the kit and phosphatase inhibitors were added separately. The cells were Dounce homogenized and sonicated to shear the DNA. The homogenates were centrifuged at 1000g and the pellet was resuspended in a buffer containing DTT (provided with the kit). 5% of micrococcal nuclease (provided with the kit) was added to each tube to digest DNA to a length ranging approximately from 150–900 bp for 20 min at room temperature followed by stopping the digest by the addition of 100 μ L of 0.5M EDTA. The homogenates were now centrifuged at 15000g for 2 min and the pellet was resuspended and incubated for 10 min in 1ml of ChIP buffer containing protease and phosphatase inhibitors. The lysates were sonicated to disrupt the nuclear membrane and centrifuged at 15000g for 15 min. The cross-linked chromatin from each sample was apportioned into three equal parts. One third of the cross-linked chromatin was set aside as “input”. One third of the cross-linked chromatin from each sample was incubated with 5 μ g of anti-C/EBP α mouse antibody (1:500; Active Motif, Carlsbad, CA), while the remaining one third of the cross-linked chromatin from each sample was incubated with 5 μ g of normal Rabbit IgG to serve as negative control. The cross-linked chromatin samples were incubated overnight at 4°C with their respective antibodies. The DNA-protein complexes were collected with Protein G agarose beads and washed to remove non-specific antibody binding. The DNA from the DNA-protein complexes from all the samples including the input and negative control was reverse cross-linked by incubation with 2 μ L of Proteinase K for 2 hours at 65°C. The crude DNA extract was eluted and then washed several times with wash buffer containing ethanol (provided with the kit) followed by purification with the use of DNA spin columns provided by the manufacturer. The pure DNA was eluted out of the DNA spin columns using 50 μ L of the DNA elution buffer provided in the kit. 1 μ L of the purified DNA was used for DNA concentration analysis using the “Quant iT™ dsDNA Assay kit from Invitrogen (Eugene, OR). The DNA fragment size was determined by electrophoresis on a 1.2% agarose FlashGel[®] system (Lonza, Rockland, ME). The relative abundance of the C/EBP α antibody precipitated chromatin containing the C/EBP α binding site in the leptin promoter region was determined by qPCR using an iQ SYBR Green Supermix kit following the manufacturer's instructions (BioRad, Hercules, CA) and sequence specific primers (Table 1). The amplification was performed using an iCycler iQ Multicolor Real Time PCR Detection System (BioRad, Hercules, CA). The fold enrichment of the bound C/EBP α in the leptin promoter region was calculated using the $\Delta\Delta C_t$ method [35] which normalizes ChIP C_t values of each sample to the % input and background.

2.10. Statistical analysis

The significance of differences among the samples was assessed by One Way Analysis of Variance (One Way ANOVA) followed by Tukey's post-hoc test, except for the analysis of two treatment groups where an unpaired t-test was used. Statistical analysis was performed with GraphPad Prism software 4.01. Quantitative data for Western blotting analysis, ChIP analysis, and luciferase assays are presented as mean values $\pm$ S.E.M with unit value assigned to control and the magnitude of differences among the samples being expressed relative to the unit value of control. Quantitative data for ELISA analysis is presented as mean values $\pm$ S.E.M of the absolute values determined by the assay. Quantitative data for

RT-PCR analysis are presented as mean values $\pm$ S.E.M, with reported values being the product of absolute value of the ratio of leptin mRNA to GAPDH mRNA multiplied by 1000000.

3. Results

3.1. 27-OHC-induced ER stress decreases leptin expression, an effect alleviated with 4-PBA

Treatment with 27-OHC (10 μ M) evoked ER stress and increased the ER stress markers GRP78, GRP94, p-PERK, and pIRE1 α (Fig 1a). Furthermore 27-OHC caused the release and translocation to the nucleus of ATF6 (Fig 1b), a classical hallmark of ER stress which may result in the increased transcription of the transcription factor CHOP. We next investigated the effects of 27-OHC treatment on leptin expression. We found that 27-OHC induces a 2.5 fold reduction in leptin protein levels as determined by Western blotting (Fig 1c–d) and ELISA immunoassay (Fig 1e). 27-OHC also elicited a 3-fold attenuation in leptin mRNA (Fig 1f). This data is in accordance with our previously published study demonstrating a reduction in leptin expression levels with 27-OHC in rabbit hippocampal organotypic slices [4]. This data also further provides correlative evidence of ER stress involvement in the 27-OHC-induced mitigation of leptin expression. To further implicate ER stress in 27-OHC-induced mitigation in leptin expression, 27-OHC-treated SH- SY5Y cells were pretreated with the molecular chaperone 4-phenylbutyric acid (4-PBA) which is known to ameliorate ER stress by stabilizing protein conformation and facilitate ER folding capacity [36]. Pretreatment with 1mM 4-PBA for 12h completely precluded the 27-OHC-induced attenuation in leptin protein levels and mRNA (Fig 1c–f).

3.2. 27-OHC-induced ER stress decreases leptin expression via the induction of CHOP

ER stress results in the increased expression of CHOP via the PERK-eIF2 α -ATF4 signaling pathway [24,25]. CHOP expression is also induced by the ATF6 branch of ER stress signaling pathway [23,26]. CHOP acts as a negative regulator of C/EBP α mediated transcription by inhibiting the binding of C/EBP α to the cognate elements in the promoter regions of target genes [29,30]. We have shown previously that leptin expression in the brain is contingent on C/EBP α binding to the leptin promoter [5]. We found that 27-OHC induces ER stress in SH-SY5Y cells and causes the nuclear translocation of ATF6. We therefore investigated the involvement of CHOP in 27-OHC-evoked ER stress-induced mitigation of leptin expression. CHOP expression is usually very low in unstressed cells and CHOP is sequestered in the cytosol. Treatment with 27-OHC elicited a profound increase in CHOP expression levels both in the cytosol and the nucleus, with an increase of 3.9 fold in the nucleus (Fig 2c,d) and 1.7 fold in the cytosol (Fig 2a,b). This suggests that 27-OHC evoked nuclear translocation of CHOP. To implicate CHOP in the 27-OHC-induced mitigation of leptin expression, we silenced the expression of CHOP with RNAi followed by treatment with 27-OHC for 24 hours. siRNA-mediated CHOP silencing resulted in the depletion of CHOP by 78%. CHOP levels were only 22% and 19% in siRNA transfected cells compared to basal CHOP levels in control cells and scrambled CHOP siRNA transfected cells respectively (Fig 2e,f). Silencing CHOP expression completely reversed 27-OHC-induced mitigation in leptin protein levels (Fig 2g–i) and leptin mRNA (Fig 2j). This further implicates the involvement of CHOP in 27-OHC-induced attenuation in leptin expression.

3.3. 27-OHC attenuates leptin transcription by reducing the binding of C/EBP α to the leptin promoter

We next investigated the mechanism involved in the regulation of leptin transcription by CHOP. We have shown previously that the transcription factor C/EBP α is necessary for

leptin expression [5]. Other studies have also demonstrated the critical role of C/EBP α in the leptin expression [37–40]. Multiple lines of evidence suggest that CHOP induces its effects on cell differentiation and cell death by negatively regulating the transcription factor C/EBP α and precluding it from binding to the cognate DNA elements in the promoter regions of target genes [29,30]. We therefore studied the effects of 27-OHC-induced ER stress on C/EBP α binding to the leptin promoter. To this end, we first performed a Co-Immunoprecipitation (Co-IP) analysis to determine the extent of CHOP-C/EBP α interaction. Co-IP analysis revealed a significant increase in association between CHOP and C/EBP α in 27-OHC treated cells (Fig 3a,b) suggesting that CHOP and C/EBP α heterodimerize potentially resulting in decreased C/EBP α mediated transcription of target genes such as leptin. Furthermore, pretreatment of 27-OHC treated cells with 4-PBA precludes this effect as evidenced by a decrease in CHOP-C/EBP α interaction (Fig 3a,b). We next performed an Electrophoretic Mobility Shift Assay (EMSA) to evaluate the extent of binding of C/EBP α to an exogenous consensus sequence corresponding to the C/EBP α binding motif on the leptin promoter in 27-OHC treated samples. We found that 27-OHC-induced ER stress abrogates C/EBP α binding to this oligonucleotide DNA probe that corresponds to C/EBP α consensus motif in the leptin promoter (Fig 3c). We subsequently performed a Chromatin Immunoprecipitation Assay (ChIP) to evaluate the extent of C/EBP α binding to the native cognate C/EBP α binding site in the leptin promoter region. Analogous to EMSA, ChIP analysis revealed that 27-OHC-induced ER stress resulted in a 4-fold decrease in C/EBP α binding to the leptin promoter (Fig 3d).

3.4. siRNA to CHOP reduces 27-OHC-induced attenuation of C/EBP α binding to the leptin promoter

To further elucidate the role of CHOP in mediating the attenuation of C/EBP α binding to the leptin promoter in 27-OHC-induced ER stress, we silenced CHOP expression by RNAi in 27-OHC treated samples. As expected, Co-IP analysis shows that silencing CHOP expression decreases the amount of C/EBP α complexed with CHOP (Fig 4a,b) thereby not sequestering C/EBP α and allowing it to bind to the leptin promoter. Co-IP analysis also shows a significant attenuation in CHOP immunoprecipitation in CHOP-silenced cells, thereby demonstrating specificity of the immunoprecipitation process (Fig 4c). Furthermore, to eliminate the confounding factor that silencing CHOP expression may elicit effects on leptin expression by altering C/EBP α expression rather than sequestration, we determined the effect of silencing CHOP on C/EBP α expression. We found that silencing CHOP expression does not affect C/EBP α expression (Fig 4d). However, silencing CHOP expression completely reverses 27-OHC-induced attenuation in C/EBP α binding to the leptin promoter as demonstrated by EMSA and ChIP analysis (Fig 4e,f). This suggests that CHOP expression is required for 27-OHC-evoked ER stress-induced inhibition of C/EBP α binding to the leptin promoter.

To concur with the observation that decreased or increased binding of C/EBP α to the leptin promoter in response to respective treatments correlates with decreased or increased leptin expression respectively, both in the native and CHOP deficient paradigms, we performed a dual-luciferase assay to determine C/EBP α -mediated leptin promoter activity. Consistent with the decreased binding of C/EBP α to the leptin promoter in 27-OHC-treated cells, we observed a marked attenuation in C/EBP α reporter activity (Fig 4g) and leptin promoter activity (Fig 4h) by dual-luciferase reporter assay. Furthermore, silencing CHOP expression completely precluded this attenuation in C/EBP α reporter activity and leptin promoter activity induced by 27-OHC (Fig 4g,h).

3.5. Overexpression of C/EBP α reverses 27-OHC-induced downregulation of leptin expression levels

To further implicate C/EBP α inhibition by CHOP as a target of ER stress-induced attenuation in leptin expression, we overexpressed C/EBP α by transfecting SH-SY5Y cells with an adenoviral vector (Ad-CMV-C/EBP α) containing C/EBP α cDNA conjugated downstream to a CMV promoter. SH-SY5Y cells transfected with the C/EBP α cDNA conjugated (Ad-CMV-C/EBP α) expression vector exhibit a ~3-fold increase C/EBP α protein levels compared to basal C/EBP α levels in control cells or cells transfected with the empty expression vector (Ad-CMV-GFP) (Fig 5a,b). 27-OHC treatment in Ad-CMV-C/EBP α transfected cells elicited a lesser degree of attenuation in leptin protein levels as determined by Western blotting (Fig 5c,d) and ELISA (Fig 5e) as well as leptin mRNA expression (Fig 5f) compared to cells transfected with the empty vector, suggesting that inhibition of C/EBP α -mediated leptin transcription underlies, at least in part, the ER stress-induced attenuation in leptin expression. Inference can be drawn from our data that 27-OHC-induced ER stress results in increased expression and nuclear translocation of CHOP which subsequently causes decreased C/EBP α binding to the leptin promoter and consequently results in an attenuation of leptin transcription.

4. Discussion

This study was conceived to elucidate the impact of ER stress on leptin expression and examine the role of the ER stress-induced transcription factor CHOP in regulating leptin expression. We demonstrate that 27-OHC, an oxidized metabolite of cholesterol, inhibits leptin expression by activating ER stress and inducing CHOP expression, culminating in a decrease in C/EBP α -mediated leptin transcription. Our results demonstrate for the first time that ER stress negatively regulates leptin expression via the transcription factor CHOP.

Leptin, an adipocytokine, also produced endogenously in the brain [1–5] has been demonstrated to attenuate A β production and tau hyperphosphorylation. We have previously shown that 27-OHC attenuates leptin expression, an effect accompanied by an increase in A β levels and tau phosphorylation [4]. Moreover, in the same study we demonstrated that treatment with leptin reverses the 27-OHC-induced increase in A β and tau phosphorylation [4]. Our data suggests that leptin downregulation by 27-OHC precedes A β and phospho tau accumulation induced by 27-OHC. However, the mechanisms involved in the 27-OHC-induced downregulation of leptin expression remained undetermined.

We and others have shown that transcription factor C/EBP α is necessary for the leptin expression [5, 37–40]. We also have previously shown that 27-OHC-induces ER stress and CHOP upregulation in ARPE-19 cells [32]. CHOP was identified as a protein that exhibits high homology with other C/EBP proteins such as C/EBP α and inhibits C/EBP α in a dominant negative manner [29]. Further studies support CHOP as an endogenous negative regulator of the transcription factor C/EBP α [30]. We therefore speculated that 27-OHC may inhibit C/EBP α -mediated transcription of leptin by evoking ER stress and inducing the expression of CHOP. We first determined the impact of 27-OHC on ER stress and leptin expression levels. 27-OHC treatment resulted in a profound increase in ER stress markers GRP78, GRP94, p-IRE1 α , and p-PERK and significantly attenuated leptin expression levels. Pretreatment of cells with 4-PBA significantly rescued the attenuation of leptin expression triggered by 27-OHC, thus suggesting that 27-OHC mitigates leptin expression by inducing ER stress.

To further elucidate and implicate CHOP as the mechanism by which 27-OHC-induced ER stress results in the down-regulation of leptin expression, we assessed the impact of 27-OHC on leptin expression in a CHOP deficient paradigm. In CHOP silenced cells, 27-OHC failed

to evoke a significant attenuation in leptin expression, thereby implicating CHOP as a downstream effector of 27-OHC-induced ER stress and down-regulation of leptin expression. We next investigated the molecular mechanism involved in the CHOP regulation of leptin expression. In line with our hypothesis, we focused on the effects of CHOP on the transcription factor C/EBP α . We found increased co-immunoprecipitation of CHOP in C/EBP α immunoprecipitated cells treated with 27-OHC. To ensure specificity, we also performed the reverse experiment and found an analogous increase in co-immunoprecipitation of C/EBP α in CHOP immunoprecipitated cells treated with 27-OHC. Having confirmed that 27-OHC induces CHOP, resulting in sequestration of C/EBP α , we next determined the extent of binding of the C/EBP α to the leptin promoter region in cells treated with 27-OHC in the native as well as CHOP deficient paradigms. Consistent with our hypothesis, 27-OHC attenuated C/EBP α binding to the leptin promoter as determined by EMSA and ChIP analyses. To correlate the decreased binding with decrease in C/EBP α -mediated leptin transcription, we performed a dual-luciferase assay to assess C/EBP α reporter activity and leptin promoter activity. 27-OHC-induced decreased binding of C/EBP α to the leptin promoter resulted in an attenuation of leptin transcription as determined by dual-luciferase assays measuring C/EBP α reporter activity and leptin promoter activity. Furthermore, CHOP-silenced cells exhibited no such attenuation of C/EBP α binding to the leptin promoter in response to 27-OHC treatment. This suggests that CHOP is necessary for 27-OHC-induced attenuation in C/EBP α binding to the leptin promoter and subsequent decrease in leptin transcription. Finally, to eliminate the confounding factor that 27-OHC-induced CHOP may evoke attenuation in C/EBP α binding to the leptin promoter by reducing C/EBP α expression, we determined the C/EBP α expression levels in CHOP-silenced cells. We found that CHOP-silencing does not elicit any affect on C/EBP α expression levels. This further suggests that 27-OHC-induced CHOP decreases C/EBP α binding to the leptin promoter and attenuates the subsequent C/EBP α -mediated transcription of leptin by sequestration of C/EBP α and not by reducing the global expression of C/EBP α .

Several studies have implicated ER stress in the pathophysiology of AD [17–19]. Consistent with this, ER stress markers such as GRP78 and p-PERK have been shown to be increased in the temporal cortex and hippocampus of autopsied brains from AD patients [41]. Also of high importance is the recent finding that the levels of 27-OHC are increased in the brains of patients with familial AD bearing Swedish APP 670/671 mutation [42]. ER stress has also been demonstrated to induce leptin resistance [27,28]. Furthermore, epidemiological studies have posited an inverse relationship between leptin levels and onset of dementia [12]. Our study is the first to demonstrate that 27-OHC-induced ER stress mitigates leptin expression via the activation of CHOP. Taking into consideration that leptin mitigates A β -production by inhibiting BACE1 [4,6] and attenuates tau phosphorylation [3,4,7, 9–11], it is plausible that ER stress contributes to AD pathology by lowering leptin expression.

In summary, we demonstrate in this study that 27-OHC-induced ER stress activates CHOP, which negatively regulates C/EBP α -mediated transcription of leptin expression (Fig 6). As ER stress is a potential etiological factor in various conditions including AD and given that leptin regulates A β production and tau phosphorylation, our results are of high relevance for understanding the pathophysiology and searching for therapeutic targets for AD. We provide here an insight into the role and putative mechanisms through which ER stress may exert its pathological effects. Further studies are warranted to examine the effect of ER stress-induced down-regulation in leptin expression in different animal models as well as in the context of diseases such as AD, PD, or diabetes, which are all associated with ER stress [43].

Acknowledgments

This work was supported by a Grant from the NIH (NIEHS, R01ES014826) to OG.

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Highlights

1. The cholesterol metabolite 27-hydroxycholesterol induces endoplasmic reticulum stress
2. Endoplasmic reticulum stress activates CHOP and down-regulates C/EBP α expression
3. Down-regulation of C/EBP α negatively regulates the adipocytokine leptin
4. siRNA to CHOP and C/EBP α over-expression preclude down-regulation of leptin

Figure 1. 27-OHC induces ER stress and attenuates leptin expression

(a) 27-OHC (10 μ M) induces ER stress as demonstrated by a marked increase in ER stress markers GRP78, GRP94, p-IRE1 α , and p-PERK in whole cell homogenates. (b) Western blotting demonstrates that 27-OHC-induced ER stress increases the levels of the transcription factor ATF6 in the nucleus. (c) Western blotting, (d) densitometric analysis, (e) ELISA immunoassay and (f) Real time RT-PCR analysis demonstrate that 27-OHC (10 μ M) attenuates leptin protein expression while pretreatment with the molecular chaperone 4-PBA precludes the attenuation in leptin expression induced by 27-OHC. Data is expressed as Mean + S.E.M and includes determinations made in four separate cell culture experiments (n=4). *p<0.05, **p<0.01 and ***p<0.001 versus control, ††p<0.01 and †††p<0.001 versus 27-OHC.

Figure 2. siRNA to CHOP significantly alleviates the 27-OHC-induced reduction in leptin expression

27-OHC-induced ER stress significantly increases CHOP levels in the cytosol (a,b) and the nucleus (c,d) as determined by Western blotting and densitometric analysis. (e) Western blotting and (f) densitometric analysis confirm the silencing of CHOP expression in the whole cell extracts of SH-SY5Y cells transfected with CHOP siRNA. (g) Western blotting, (h) densitometric analysis, (i) ELISA immunoassay, and (j) Real time RT-PCR analysis demonstrate that silencing CHOP expression reduces the 27-OHC-induced attenuation in leptin protein expression. Data is expressed as Mean + S.E.M and includes determinations made in four separate cell culture experiments (n=4). *p<0.05, **p<0.01 and ***p<0.001 versus control, ††p<0.01 and †††p<0.001 versus 27-OHC.

Figure 3. 27-OHC induced increase in CHOP expression reduces binding of C/EBPα to the leptin promoter region and subsequently attenuates leptin transcription

(a,b) 27-OHC results in increased co-immunoprecipitation of CHOP in C/EBPα immunoprecipitated cells and C/EBPα in CHOP immunoprecipitated cells while pretreatment with 4-PBA precludes this effect. (c) EMSA analysis shows that 27-OHC significantly reduces the binding of C/EBPα to the exogenous sequence corresponding to the leptin promoter region, an effect that is reversed by 4-PBA. (d) ChIP analysis demonstrates that 27-OHC significantly decreases binding of C/EBPα to the leptin promoter region and 4-PBA obviates this effect. Data is expressed as Mean + S.E.M and includes determinations made in four separate cell culture experiments (n=4). ***p<0.001 versus control, † p<0.05 versus 27-OHC

Figure 4. siRNA to CHOP reduces 27-OHC-induced attenuation of C/EBPα binding to the leptin promoter

(a,b) Silencing CHOP expression completely precludes the 27-OHC-induced increase in co-immunoprecipitation of CHOP in C/EBPα immunoprecipitated cells and C/EBPα in CHOP immunoprecipitated cells. (c) Immunoprecipitation (IP) analysis of CHOP demonstrates a marked attenuation in CHOP IP using CHOP antibody in CHOP-silenced cells. (d) Representative Western blot shows that silencing CHOP expression does not affect C/EBPα levels in the whole cell extracts. (e) EMSA and (f) ChIP analyses demonstrate that silencing CHOP expression prevents the 27-OHC-induced attenuation of C/EBPα binding to the leptin promoter. (g,h) Luciferase assay shows that 27-OHC significantly decreases C/EBPα transcriptional activity and attenuates leptin promoter activity. Data is expressed as Mean + S.E.M and includes determinations made in four separate cell culture experiments (n=4). ***p<0.001 versus control, ††p<0.01 and †††p<0.001 versus 27-OHC.

Figure 5. C/EBPα overexpression precludes the 27-OHC-induced attenuation in leptin expression

(a) Western blotting and (b) densitometric analysis demonstrate and confirm the overexpression of C/EBPα in the whole cell homogenates of SH-SY5Y cells transfected with the Ad-CMV-C/EBPα expression vector. (c) Western blotting, (d) densitometric analysis, (e) ELISA immunoassay, and (f) real time RT-PCR analyses demonstrate that C/EBPα overexpression precludes the attenuation in leptin expression induced by 27-OHC. Data is expressed as Mean + S.E.M and includes determinations made in four separate cell culture experiments (n=4). **p<0.01 and ***p<0.001 versus control, † p<0.05, ††p<0.01 and ††† p<0.001 versus 27-OHC.

Figure 6. Schematic depiction of the molecular events involved in the ER stress and 27-OHC-induced attenuation in leptin expression

27-OHC-induced ER stress (1) results in the induction of CHOP expression (2). Increase in expression of CHOP results in augmentation of CHOP binding to the transcription factor C/EBPα (3). At the basal state, the transcription factor C/EBPα forms homodimers that bind to C/EBPα binding sites in the leptin promoter and positively regulate leptin transcription (4). Upon CHOP activation, the CHOP-C/EBPα heterodimer binds to distinct elements in the leptin promoter, but fails to induce leptin expression thereby negatively regulating leptin transcription (5). The molecular chaperone 4-PBA precludes the 27-OHC-evoked ER stress-induced increase in CHOP expression and subsequent attenuation in leptin transcription (6). Silencing CHOP expression also rescues the attenuation in leptin transcription-induced by 27-OHC (7). Overexpression of the transcription factor C/EBPα significantly ameliorates the reduction in leptin transcription induced by 27-OHC (8).

Table 1

Primers designed and used for RT-PCR, EMSA, and ChIP analyses

Gene	Primer	GenBank Accession Number	Sequence	Assay
Leptin	Forward	NM000230	5'-gaagaccacatccacacag-3'	RT-PCR
Leptin	Reverse	NM000230	5'-agctcagccagaccatcta-3'	RT-PCR
Leptin		NG007450	5'-tagcttttgggcattaccaaacccggcagt -3'	EMSA
Leptin	Forward	NG007450	5'-tgcacatggcctctctttt-3'	ChIP
Leptin	Reverse	NG007450	5'-actacagcctgggcaacaag-3'	ChIP