FINAL

Send Orders for Reprints to reprints@benthamscience.ae

Current Alzheimer Research, 2015, *12*, 000-000

Insulin Resistance as Common Molecular Denominator Linking Obesity to Alzheimer's Disease

Domenico Nuzzo[a], Pasquale Picone[a], Sara Baldassano[b], Luca Caruana[a], Elisa Messina[a], Antonella Marino Gammazza[c,d], Francesco Cappello[c,d], Flavia Mulè[b] and Marta Di Carlo[a,*]

[a]*Institute of Biomedicine and Molecular Immunology CNR, Palermo, Italy;* [b]*Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Italy;* [c]*Department of Experimental Biomedicine and Clinical Neurosciences, University of Palermo, Palermo, Italy,* [d]*Euro-Mediterranean Institute of Science and Technology, Palermo, Italy*

Marta Di Carlo

Abstract: Alzheimer's disease (AD) is an aging-related multi-factorial disorder to which metabolic factors contribute at what has canonically been considered a centrally mediated process. Although the exact underlying mechanisms are still unknown, obesity is recognized as a risk factor for AD and the condition of insulin resistance seems to be the link between the two pathologies. Using mice with high fat diet (HFD) obesity we dissected the molecular mechanisms shared by the two disorders. Brains of HFD fed mice showed elevated levels of APP and $A\beta_{40}/A\beta_{42}$ together with BACE, GSK3β and Tau proteins involved in APP processing and Aβ accumulation. Immunofluorescence, Thioflavin T staining experiments, confirmed increased Aβ generation, deposition in insoluble fraction and plaques formation in both the hippocampus and the cerebral cortex of HFD mice. Presence of $A\beta_{40}$ and $A\beta_{42}$ in the insoluble fraction was also shown by ELISA assay. Brain insulin resistance was demonstrated by reduced presence of insulin receptor (IRs) and defects in Akt-Foxo3a insulin signaling. We found reduced levels of phospho-Akt and increased levels of Foxo3a in the nuclei of neurons where proapototic genes were activated. Dysregulation of different genes related to insulin resistance, especially those involved in inflammation and adipocytokines synthesis were analyzed by Profiler PCR array. Further, HFD induced oxidative stress, mitochondrial dysfunction and dynamics as demonstrated by expression of biomarkers involved in these processes. Here, we provide evidence that obesity and AD markers besides insulin resistance are associated with inflammation, adipokine dyshomeostasis, oxidative stress and mitochondrial dysfunction, all mechanisms leading to neurodegeneration.

Keywords: Adipokines, Alzheimer's disease, gene expression, inflammation, insulin resistance, mitochondrial dysfunction, obesity.

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is mainly diagnosed by its clinical features including progressive impairment of cognitive function, orientation, and motor activity, language disturbance, difficulty in recognizing or identifying objects and persons. Histopathological hallmarks of AD are the so-called senile plaques and neurofibrillary tangles obtained respectively by deposition of the aggregated β amyloid peptide (Aβ) and hyperphosphorylated Tau protein [1]. Aβ peptide derives from the sequential proteolytic cleavage of amyloid precursor protein (APP) by β-site APP cleaving enzyme 1 (BACE1) and γ−secretase, a multi-subunit protease complex comprised of proteins such as presenilin 1 or 2 (PS1, PS2) [1, 2].

The prevalence of AD is progressively increasing in the world population. Thirty-five million people are now considered to be affected by AD and this number is expected to double in the next few decades [3]. In parallel, an increase in incidence of obesity has been observed during the last years [4]. In countries with a developed status the obesity is currently the most common nutritional disorder, with serious health-impairing consequences, not the least of which cognitive impairment [5]. In fact, some studies have reported that obesity is associated with reduced cognitive performance in humans [6, 7] as well as decreased brain volumes [8] and it is correlated with an increased probability of developing cognitive decline, including AD [9, 10]. According to Rotterdam studies, patients with obesity or other metabolic diseases, such as Type 2 Diabetes (T2D), have almost a two-fold greatest risk of developing AD [11].

Unfortunately, to date the precise mechanisms associating obesity to AD are unclear. In particular, specific molecular mechanism/s linking the pathophysiology of obesity to the brain alterations of AD remains poorly known. Different factors have been implicated, such as increased levels of APP in adipose tissue [12] and Aβ in plasma of obese individuals [13] and the presence of insulin resistance [5]. Indeed, numerous studies have indicated obesity as a factor which predisposes people to T2D and induces insulin resistance [14]. In fact, rates of T2D are more than double

*Address correspondence to this author at the Institute of Biomedicine and Molecular Immunology CNR, via Ugo La Malfa 153, 90146 Palermo, Italy; Tel +390916809538; Fax +390916809548;
E-mail: marta.dicarlo@ibim.cnr.it

1567-2050/15 $58.00+.00

amongst people who are obese. Insulin has a significant role in modulation of synaptic plasticity and learning memory [15, 16]. High concentrations of insulin are present in brain extracts and insulin receptors (IR) are highly expressed in brain regions that support memory formation, indicating that insulin may have a role in maintaining regular cognitive function [16]. Alterations in the concentration of insulin and insulin receptors in the brain have been reported in AD, leading some to consider AD as brain diabetes or ''Type 3 diabetes'' [17]. As such, irregularities in the insulin signaling pathway may contribute to impairment of memory function, comparable to those observed in patients with AD [18]. Moreover, animal and clinical studies have shown that intranasal administration of insulin improves cognitive behavior in mice, perhaps by modulating neuronal communication within the brain [19, 20].

A high fat diet (HFD) is one of main factors that can promote obesity and previous studies have shown that such a diet can produce learning and memory impairments in rodents [21-24]. HFD was previously shown to induce a remarkable brain insulin resistance as well as spatial memory impairment in a normal mouse or a transgenic model of AD [25]. However, whether the state of hyperinsulinemia in rodents can induce alterations in the central nervous system (CNS) remains controversial and observations seem to depend on the experimental approach to induce hyperinsulinemia and the specific tests performed [26-29]. Therefore this study was undertaken in an attempt to understand the underlying mechanisms by which adipose tissue might influence cerebral structures related to learning and memory. With this view, we explored different points of molecular alterations present in the brains of C57BL6 mice fed HFD an animal model of diet-induced obesity and insulin resistance. In particular, we analyzed the effect of high fat diet intervention on brain metabolism, redox balance and mitochondrial homeostasis. In addition, the gene expression changes involved in the brain insulin resistance were analyzed.

2. MATERIAL AND METHODS

2.1. Mice

The experimental procedures employed in the present study were in accordance with the Italian D.L. no. 116 of 27 January 1992 and subsequent variations and the recommendations of the European Economic Community (86/609/ECC). The studies were approved by Ministero della Sanita` (Rome, Italy). Male C57BL/6J (B6) mice, purchased from Harlan Laboratories (San Pietro al Natisone Udine, Italy) at 4 weeks of age, were housed under standard conditions of light (12h light: 12h darkness cycle) and temperature (22–24°C), with free access to water and food. After acclimatization (1 week), the animals were weighed and divided in two groups, which were fed a standard diet (STD) (4RF25, Mucedola, Milan, Italy), or fed for 23 weeks (7 months) a high fat diet (HFD) (PF4051/D, Mucedola) consisting of 23% protein, 38% carbohydrates, and 34% fat (60% caloric fat content), as previously described [30]. Animals were weighed and killed by cervical dislocation. The brains of age-matched animals were immediately exported, weighted and processed for subsequent analysis.

2.2. Glucose, Insulin and Insulin Tolerance Tests

Body weight, blood glucose and insulin levels were determined before sacrifice. Briefly blood glucose levels were measured from nicked tails using a glucometer (GlucoMen LX meter, Menarini, Italy). Quantification of plasma insulin was carried out by mouse enzyme-linked immunosorbent assay (Alpco Diagnostics, Salem, NH USA) according to the manufacturer's instructions. Insulin tolerance test (ITT) was carried out in mice fasted for 6 h with free access to water. Mice were given an i.p. injection of insulin (1.2 U/kg body weight) (Insuman Rapid, Sanofi Aventis, Italy) in 0.9% saline and plasma glucose was measured at different interval up to 120 min (0, 15, 30, 60, 90, 120).

2.3. Total Protein Extraction and Western Blotting

Brain of mice were homogenized in RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 1mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton, 0.1%SDS, and 0.5% deoxycholate) with protease inhibitors (Amersham) and phosphatase inhibitor cocktail II and III (SIGMA). To remove insoluble material tissue lysates were sonicated and centrifuged (14,000 rpm, at 4°C, for 30 min). Proteins (50 μg) were resolved by 10% SDS-PAGE gel and transferred onto nitrocellulose filters for Western blotting using anti-APP (1:1000), anti-Presenilin1 (1:1000), anti-p-ERK (1:1000), anti-heme-Oxygenase (1:500), anti-SOD2 (1:500), anti-Bad-2 (1:500), anti-VDAC1 (1:500), and anti-BACE1 (1:1000), anti-phospho-GSK-3β (Tyr216) (1:500) purchased from Santa Cruz, anti-IR (1:500), anti-GSK3α/β (1:1000), anti-i-NOS (1:500), anti-HSP60 (1:500), anti-FoxO3a (1:1000) and anti-phospho-Foxo3a (Ser253) (1:500) purchased from Cell Signaling, anti-AKT (1:1000), anti-phospho-Akt (Ser473) (1:500) purchased from Invitrogen, anti-phospho-Tau (Thr205) (1:200) purchased from Abcam, anti-β-actin (1:5000) purchased from Sigma. Secondary antibodies conjugated to horseradish peroxidase (1:2000) purchased from Cell Signaling were detected using the NOVEX® ECL HRP chemiluminescence kit (Cat. n° WP20005, Invitrogen) according to the manufacturer's instructions. In some instances, antibodies were stripped from blots with Restore Western Blot Stripping Buffer (Thermo Scientific) for 10 minutes at room temperature, for antibody reprobing. Band intensities were analyzed with a gel documentation system (BioRad), expression was normalized with β-actin expression. The protein levels were expressed as densitometry and percentage of controls.

2.4. Preparation of Soluble and Insoluble Brain Fractions

Brain tissues were homogenated in ~10 ml buffer/gram tissue in low salt buffer (LSB) (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 10% (wt/vol) sucrose, Sigma protease inhibitor cocktail II and III). After centrifugation at 14,000 rpm for 30 min at 4°C, the supernatant was retained as *soluble fraction* and the pellet was resuspended in a detergent buffer (LSB plus 1% sarkosyl, N-Lauroylsarcosine and 0.5M NaCl). After brief sonication and centrifugation at 14,000 rpm for 50 min at room temperature, the supernatant was retained as *detergent insoluble fraction*. The *soluble fraction* and the *detergent insoluble fraction* were utilized for the ELISA assays. An enzyme-linked immunosorbent sandwich

assay (ELISA) (Invitrogen) was performed for quantitative detection of mouse β-Amyloid (1-40) and (1-42) in *soluble* and *detergentin soluble* brain fractions according to manufacturer's instructions.

2.5. Preparation of Cytoplasmic and Nuclear Protein Extracts

Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Cat. n° 78833, Thermo Scientific) according to the manufacturer's instructions using buffers provided.

2.6. Immunomorphological Analyses: Immunohistochemistry and Immunofluorescence

For tissue preparation, brains from STD and HFD fed mice (n=3 per group) were harvested. The mice were sacrificed by cervical dislocation; the brains were removed, weighted and fixed in 4% formalin for 24h. The brains were embedded in paraffin and sectioned coronally (5μm) using a microtome. Brain sections including the hippocampus and cerebral cortex were mounted on superfrost plus tissue slides (Menzel-Gläser, Menzel-Glaser, Braunschweig, Germany) and deparaffinized. The slides were immersed in 0.3% H_2O_2 for 5 min to quench endogenous peroxidase activity, and treated with 10 mM, pH 6.0, 0.05% Tween 20 tri-sodium citrate at 95°C for 8 min for antigen retrieval. Subsequently, immunohistochemistry was performed as previously described [31] using the Histostain-Plus IHC detection Kit (Histostain-plus Kit3rd Gen IHC Detection Kit, Life Technologies, Monza, Italy) according to the manufacturer's instructions. The sections were incubated with anti-APP (1:200) purchased from Santa Cruz and 3–3-diaminobenzidine (DAB chromogen solution, DAKO, Glostrup, Denmark) was used as developer chromogen. Nuclear counter staining was done using haematoxylin (DAKO). Appropriate positive and negative controls, were run concurrently. The slides were mounted with cover slips and images were taken with a Leica DM5000 upright microscope (Leica Microsystems, Heidelberg, Germany) at a magnification of 20X. For immunofluorescence sections were stained using the same primary antibody against APP used for immunohistochemistry (Santa Cruz, dilution 1:100) and with anti-IR (Cell Signaling, dilution 1:100) at 4°C overnight. After washing in PBS, the slides were incubated with anti-rabbit TRITC-conjugate and anti rabbit-FITC-coniugated secondary antibodies (SIGMA). Nuclear staining was performed using Hoechst 33258 (5μg/mL). For Thioflavin T (ThT) staining the brain slides were incubated with a 70 μM work solution for 10 minutes. The samples were analyzed by using a DHL fluorescent microscope (Leica Microsystems, Heidelberg, Germany) at a magnification of 20X. Fluorescence intensity was measured using Leica QFluoro program.

2.7. Quantitative Real-Time qPCR

Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen). Two ng of RNA was used to synthesize the first strand cDNA using RT First-Strand kit (Qiagen). Synthesized cDNAs were amplified using RT2 SYBR Green/ROX qPCR Mastermix (Qiagen) and StepOne Real-Time instrument (Applied Biosystem). Gene expression validation was performed using home-made sequence primers for human APP, Foxo3a, Bim, Fas-Ligand, P27 and β-actin (Table **1**) or using commercial sequence primers listed in Table **2** (SABiosciences). Gene expression was normalized to β-actin. On the basis of the Ct value (threshold cycle-the number of reaction cycles after which fluorescence exceeds the defined threshold) of the examined gene and of the internal control gene the relative expression level of RNA was calculated according to the $2^{-\Delta\Delta Ct}$ approximation method.

2.8. RT2 ProfilerPCR Array

Mouse Insulin resistance (PAMM-156Z) focused pathway arrays (Insulin Resistance PCR Array, SABiosciences) in 96-well plate format were used to assay gene expression changes. Samples were prepared from pooled RNA extracted from STD and HFD mouse brains. Samples were added to the reaction plates following the manufacturer's instructions and and a StepOne Real-Time instrument (Applied Biosystem) was used to perform the array. Analysis was performed using the spreadsheet provided by SABiosciences.

2.9. Statistical Analysis

The results are presented as mean ± SD. Statistical evaluation was conducted by two-way ANOVA, followed by Student's t-test for analysis of significance. Results with a p-value <0.05 were considered statistically significant, $*P<0.05$, $**P<0.02$.

Table 1. Sequences of primers used for real-time PCR analysis.

Gene	Forward	Reverse
APP	5'-CTGACCACTCGACCAGGTTCT-3'	5'-GTCGATAACCCCTCCCCCAGC-3'
FoxO3a	5'- TCTACGAGTGGATGGTGCGTT-3′	5'-CGACTATGCAGTGACAGGTTGTG-3′
Bim	5'-AACCTTCTGATGTAAGTTCT-3'	5'-GTGATTGCCTTCAGGATTAC-3'
Fas L	5'- CAAGTCCAACTCAAGGTCCATGCC-3'	5'-AGAGAGAGCTCAGATACGTTTGAC-3'
P27	5'- TGCGAGTGTCTAACGGGAG-3'	5'- GTTTGACGTCTTCTGAGGCC-3'
β-actin	5'- GTGGGGCGCCCCAGGCACCA-3'	5'- CTCCTTAATGTCACGCACGATTTC-3'

3. RESULTS

3.1. Effects of HFD on Body and Brain Weights

In an effort to better understand the effect of high caloric diet on brain metabolism and risk of developing AD, C57BL6/J mice were fed with a high fat diet or a standard diet for seven months. Body and whole brain weights were measured (n=5 per group) at the time of sacrifice and the brain/body weight ratio was calculated. The HFD animals had a significantly higher weight, body weight relative to controls fed a standard diet. Moreover, the mean brain weights were lower in HFD than standard diet fed animals. However, the calculated mean brain/body weight ratio was significantly reduced in the HFD mice due to the combined effects of obesity and mild brain atrophy (Table **3**). For the expression experiments we chose to utilize STD mice with a brain/body weight ratio > 0.01 (n=3 per group) and HFD mice with a brain/body weight ratio < 0.08 (n= 3 per group).

3.2. AD Markers Increase in Brains from HFD Versus Control Fed Mice

To examine whether HF feeding changed the expression levels of proteins with a well-established link to AD pathology such as APP, BACE, PSN1, total and active GSK3β and phosphorylated Tau, a Western blot of proteins extracted from brains of HFD and standard diet fed animals was performed (Fig. **1A,B**). Brain extracts from HFD mice revealed a significant increase in expression of APP and its cleavage peptides $A\beta_{40}$ and $A\beta_{42}$ compared to control diet fed mice. In agreement with this, an increase of BACE, an enzyme required for processing of APP to produce its pathogenic cleavage product Aβ, was observed, whereas no significant change was observed for PSN1. A substantial increase of GSK3β a protein involved in multiple processes such as Tau hyper-phosphorylation and Aβ production and mitochondrial dysfunction, was also detected. Furthermore, activation of GSK3β corresponded to an increase in Tau phosphorylation. Accordingly with protein levels observed, expression of APP mRNA, analyzed by quantitative real-time PCR (qRT-PCR), was significantly increased in HFD mice (Fig. **1C**). Furthermore, we visualized the presence of APP by immunofluorescence and immunohistochemistry experiments using coronary brain sections in which the hippocampus and cerebral cortex, two regions damaged in AD, were visible (Fig. **1D,E,F**). We observed increased immunoreactivity of APP in both the hippocampus and cortex of HFD mice compared to controls and some Aβ deposits were detected, indicating an increase in APP processing and aggregation of Aβ. Moreover, staining with Thioflavin T, a dye used to visualize the presence or fibrilization of misfolded protein aggregates, or amyloid plaques, demonstrated the presence of insoluble Aβ aggregates (Fig. **1G**). To confirm the presence of insoluble Aβ aggregates or amyloid plaques we utilized an anionic detergent to obtain a soluble and insoluble fraction from STD and HFD brain tissue. Increased levels of $A\beta_{40}$ and $A\beta_{42}$ aggregates were detected by ELISA assay in the deter-

Table 2. List of genes and catalog number of commercial primers used for real-time PCR analysis.

Symbol	Description	RT^2 Catalog
Ifng	Interferon gamma	PPM03121A
Pck1	Phosphoenolpyruvate carboxykinase 1, cytosolic	PPM05113F
Cd3e	CD3 antigen, epsilon polypeptide	PPM04598A
Cxcr3	Chemokine (C-X-C motif) receptor 3	PPM03145F
Adipoq	Adiponectin, C1Q and collagen domain containing	PPM05260A
Retn	Resistin	PPM05076A
Pdx1	Pancreatic and duodenal homeobox 1	PPM04509D
Ccr4	Chemokine (C-C motif) receptor 4	PPM03147A
Lep	Leptin	PPM03504B
Actb	Actin, beta	PPM02945B

Table 3. Brain weights from C57BL/6J (n =5 each) maintained with standard (STD) or high fat diet (HFD) for 7 months. Data represent means ± S.D.

Diet	n	Age (months)	Body (±sd)	p-value	Brain (±sd)	p-value	Weight ratio brain/body	p-value
STD	5	7	27.6 g ± 0.9	< 0.005	0.33 g ± 0.06	< 0.05	0.0123 ± 0.002	< 0.05
HFD	5	7	36.4 ± 2		0.27 ± 0.04		0.0077 ± 0.001	

gent insoluble fraction with respect to soluble fraction (Fig. **1H**).

3.3. High Fat Diet Fed Mice Show Insulin Resistance

The HFD mice had significantly higher fasting plasma glucose levels (Fig. **2A**) and increased insulin (11.8 ± 3.5 ng/ml, n=5 P<0.01) compared to standard diet (0.8 ± 0.2 ng/ml; n=5) fed animals. Moreover, the ITT showed that in HFD mice the levels of blood glucose after intraperitoneal exogenous administration of insulin decreased less in comparison to standard diet fed animals (Fig. **2A**), suggesting the presence of insulin resistance. This is also illustrated by the measurement of the glucose area under the curve (AUC) (Fig. **2B**). Insulin receptors were present on neurons in the brain and their reduced presence and activity was detectable in insulin resistance. Western blotting analysis revealed that brain levels of insulin receptor (IR) were decreased relative to controls (Fig. **2C,D**), confirming cerebral insulin resistance. Furthermore, immunofluorescence with anti-IR showed a reduced presence of these receptors in hippocampus from HFD fed mice compared to controls, confirming the metabolic data (Fig. **2E,F**).

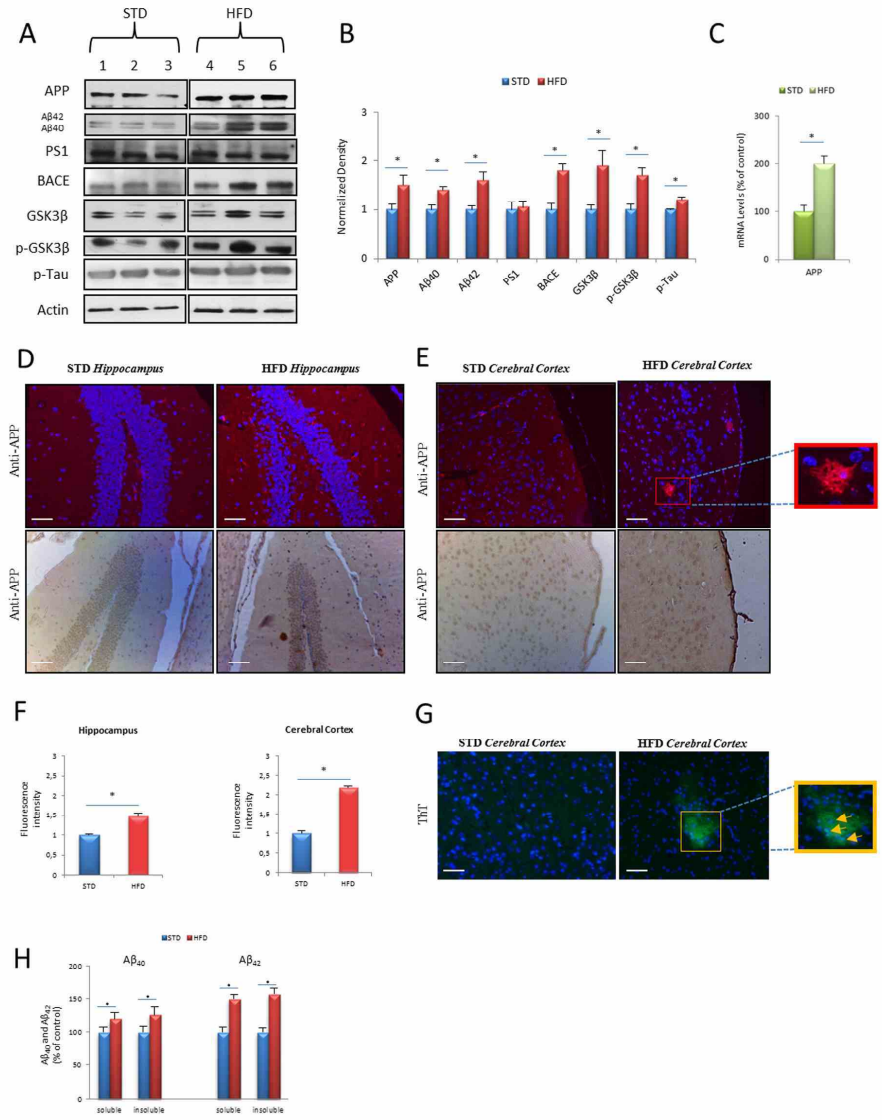


Fig. (1). Brain of diet induced obesity mice accumulates Aβ versus standard diet mice. **A)** Western blot of protein extracted from brain lysates of STD or HFD fed mice were incubated with anti-APP, presenilin 1 (PS1), BACE, GSK3β, phospho-GSK3β, phospho-Tau and β-Actin (loading control) antibodies. **B)** Quantification of immunoreactivity was performed using densitometric analysis. **C)** Effect of HFD on APP transcript levels determined by quantitative real-time PCR. *P < 0.05 versus control group. **D,E)** Immunofluorescence and immunohistochemistry of hippocampal (CA3 area) and cerebral cortex sections (CTX area) of STD or HFD fed mice stained using anti-APP antibody. The outlined area in E are enlarged to the right to show the clearly stained plaque. **F)** Histograms represent the fluorescence intensity of APP in hippocampal and cortex sections of STD and HFD fed mice. **G)** ThT staining of Aβ aggregates on cerebral cortex section. Nuclei were stained with Hoechst 33258 and merged images with ThT staining are shown. Arrows indicate the fragmented nuclei of the cells trapped in the plaque, in the enlarged figure on the right. Representative images from 3 animals per condition are shown. Bar 20 μm. **H)** $A\beta_{40}$ and $A\beta_{42}$ quantification by ELISA in the soluble and detergent insoluble fractions of brain homogenates from STD and HFD fed mice.

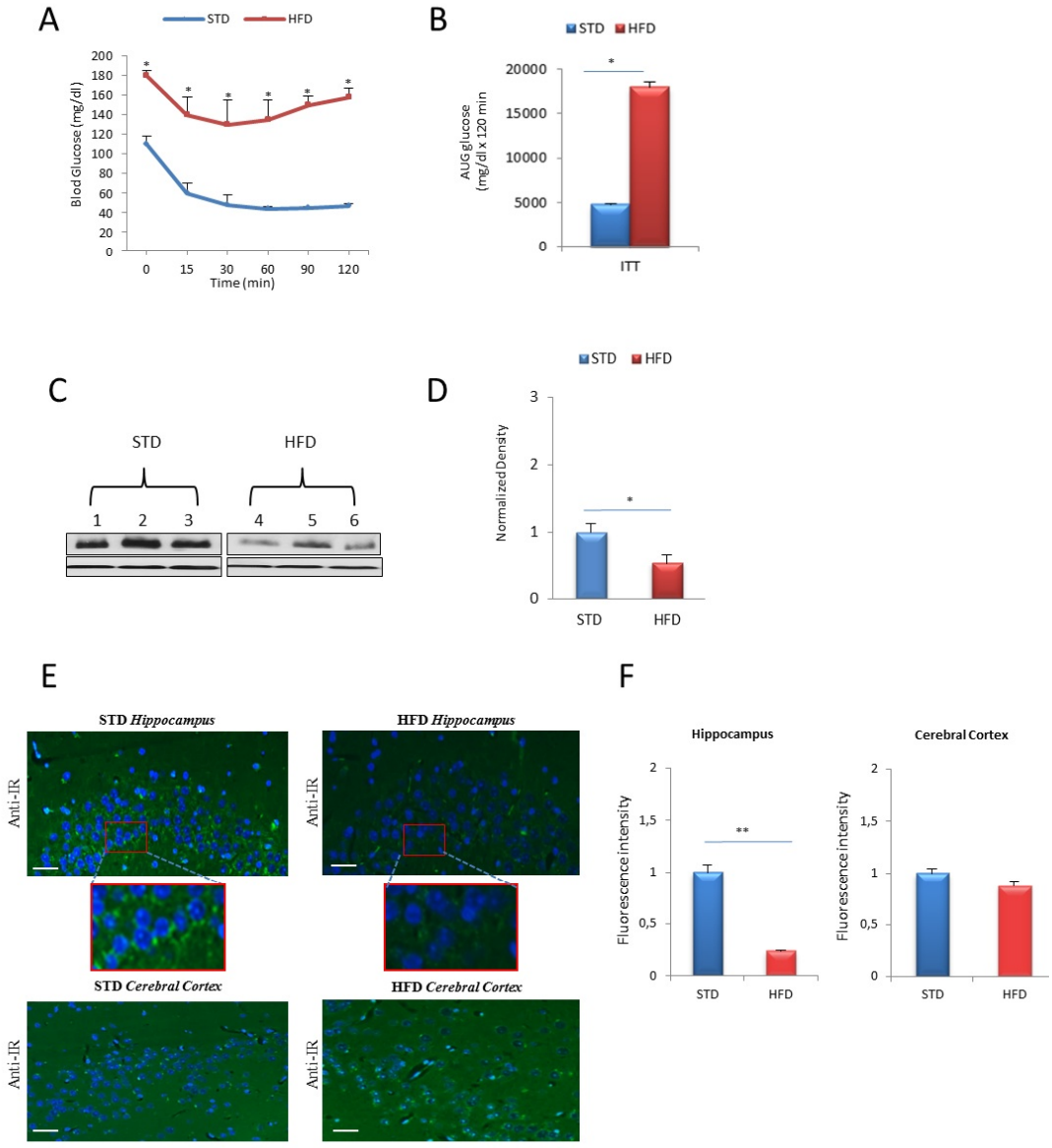


Fig. (2). HFD induces insulin resistance. **A)** Plasma glucose concentrations during insulin tolerance test (ITT) **B)** AUC for blood glucose concentrations during ITT. Data are mean values ± SD (n=5 mice/group). *p ≤ 0.05. **C)** Western blot of brain lysates from STD and HFD fed mice incubated with anti-Insulin receptor (IR) and β-Actin (loading control) antibodies. **D)** Quantification of immunoreactivity was performed using densitometric analysis. **E)** Immunofluorescence of hippocampal and cerebral cortex sections of STD or HFD fed mice stained using anti-Insulin receptor (Anti-IR). The outlined areas are enlarged in the squares. **F)** Histograms represent the fluorescence intensity of IR in hippocampal and cortex sections of STD and HFD fed mice. Representative images from 3 animals per condition are shown. Bar 20 μm.

3.4. High Fat Diet Feeding Affects Insulin Signaling

To assess whether reduced IR presence compromises insulin signaling activation, a Western blot of total proteins extracted from brains of HFD and standard diet fed mice was incubated with antibodies against both the total and phosphorylated forms of Akt and Foxo3a. Decrease in Akt, posho-Akt and posho-Foxo3a levels and increase of Foxo3a were observed in HFD mice, indicating that the insulin signaling was affected (Fig. **3A,C**). Furthermore, we investigated about the subcellular localization of activated or unactivated forms of Akt and Foxo3a. In the nuclei of HFD mice, we observed a decrease in both Akt and phospho-Akt levels and a significant increase of Foxo3a (Fig. **3B,D,E**). In contrast, we found an increase in both phospho-Akt and Foxo3a and a decrease of phospho-Foxo3a in the cytoplasm. Moreover, by using quantitative Real Time PCR we confirmed that in the nuclei Foxo3a activates the expression of apoptotic genes such as FasL, Bim and p27. HFD fed mice showed an increase of mRNA levels of both Foxo3a and Bim, Fas-L and p27, compared to control mice (Fig. **3F**). Our results, as schematically shown in (Fig. **3G**), indicate that in STD condition phosphorylated Akt promotes Foxo3a export from the nucleus to the cytoplasm inhibiting its transcriptional activity. In contrast, in HFD condition, a decrease in phospho-Akt reduces Foxo3a phosphorylation and promotes its translocation to the nucleus where it activates transcription of its target genes (Fig. **3G**).

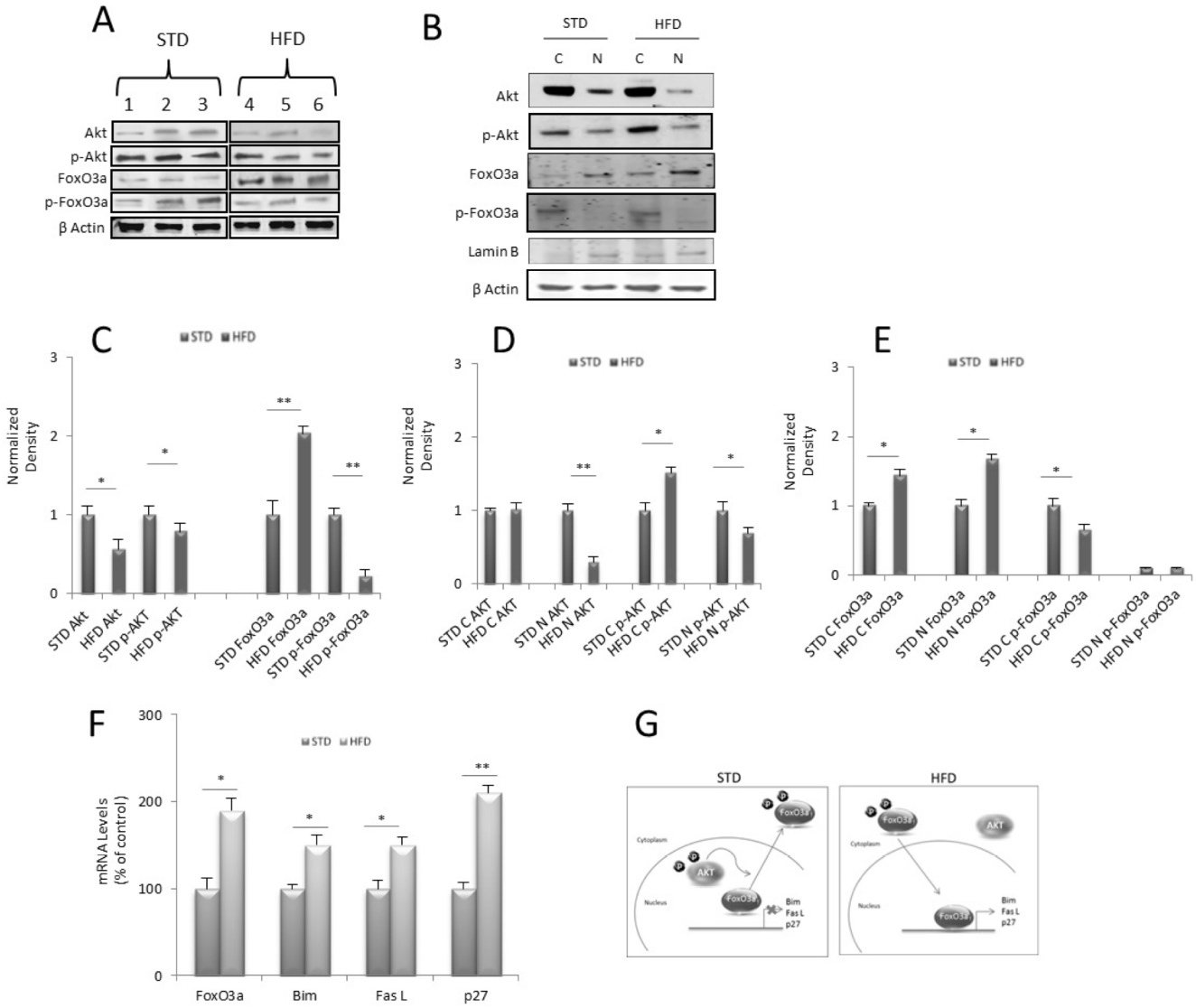


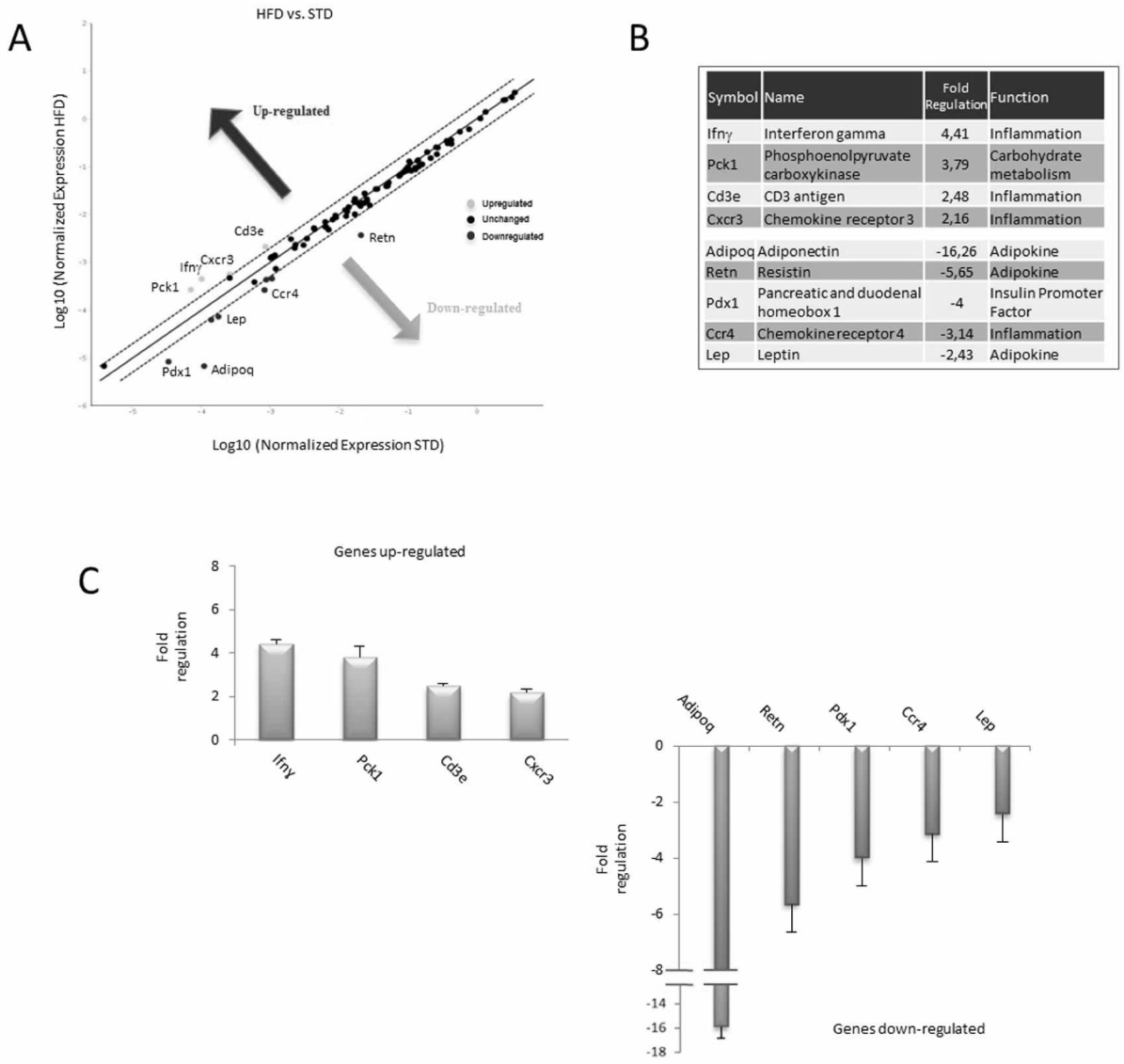
Fig. (3). HFD affects Akt/Foxo3a pathway. **A)** Western blot of brain lysates from STD and HFD fed mice incubated with anti-Akt, phospho-Akt (p-Akt), Foxo3a, phospho-Foxo3a (p-Foxo3a) and β-Actin (loading control) antibodies. **B)** Western blot of brain lysates from STD and HFD fed mice and subjected to subcellular fractionation in cytoplasmic **(C)** and nuclear (N) fractions and incubated with anti-Akt, phospho-Akt (p-Akt), Foxo3a, phospho-Foxo3a (p-Foxo3a), Lamin B (nuclear loading control) and β-Actin (cytoplasmic loading control) antibodies. **C,D,E)** Quantification of immunoreactivity was performed using densitometric analysis. **F)** Effect of HFD on Foxo3a, Bim, FasL, P27 transcript levels determined by quantitative real-time PCR. n= 3 per group. *P < 0.05,**P < 0.02 versus control group. **G)** Schematic representation of p27, Bim and FasL gene activation after high fat diet induced Foxo3a nuclear translocation.

3.5. HFD Affects Expression of Resistance Insulin Related Genes

To assess the expression changes in genes involved in insulin resistance in the brain we utilized a Profiler PCR array. Of the 84 genes, most of them were significantly differentially expressed. These genes and the relative fold changes respect to the control are listed in Supplementary file 1 (Fig. **S1**). Among these, twelve were up- or down-regulated by more than 2 fold in HFD fed mice compared to the control (Fig. **4A**). In addition, we validated the expression of some of the most highly expressed genes that were associated with inflammation (Interferon γ, CD3 antigen, Chemokine Receptor 3, Chemokine receptor 4), adipokine signaling pathway (Adiponectin, Resistin, Leptin), and metabolism (Phosphoenolpyruvate carboxykinase, Pancreatic and duodenal homeobox 1), by quantitative real-time PCR (Fig. **4B,C**). As shown, the observed changes were always of the same order of magnitude as those obtained by PCR Arrays.

3.6. HFD Triggers Oxidative Stress and Mitochondrial Dysfunction

To evaluate whether there was a condition of oxidative stress in the brain of HFD fed mice, protein markers of stress such as phospho-ERK (p-ERK), inducible nitric oxide synthase (i-NOS), and antioxidant proteins such as superoxide dismutase 2 (SOD-2) and heme-oxigenase (H-Oxy), were analyzed by Western blotting. We found that the levels of p-ERK and i-NOS were significantly increased, whereas the



Symbol	Name	Fold Regulation	Function
Ifnγ	Interferon gamma	4,41	Inflammation
Pck1	Phosphoenolpyruvate carboxykinase	3,79	Carbohydrate metabolism
Cd3e	CD3 antigen	2,48	Inflammation
Cxcr3	Chemokine receptor 3	2,16	Inflammation
Adipoq	Adiponectin	-16,26	Adipokine
Retn	Resistin	-5,65	Adipokine
Pdx1	Pancreatic and duodenal homeobox 1	-4	Insulin Promoter Factor
Ccr4	Chemokine receptor 4	-3,14	Inflammation
Lep	Leptin	-2,43	Adipokine

Fig. (4). PCR Array analysis of insulin resistance related genes. Brains from STD and HFD fed mice were used to prepare the mRNA for the PCR Array analysis. **A)** Scatter Plot of relative expression levels for each gene in the two samples (HFD *vs* STD). The figure shows a log transformation plot of the relative expression level of each gene ($2^{-\Delta\Delta Ct}$) between STD mouse (x-axis) and HFD mouse (y-axis). The gray lines indicate a two-fold change in gene expression threshold. **B)** Table of quantitative real time PCR analysis (± SD) of the chosen genes. **C)** Histogram of selected genes with a greater than 2-fold change in expression chosen to validate PCR Array. n= 3 per group.

levels of SOD-2 and H-Oxy were decreased, indicating that a condition of oxidative stress was present (Fig. **5A,B**). Similarly, we determined alterations of some proteins that are markers of mitochondrial dysfunction. By Western blotting in brain homogenates from HFD or STD fed mice we detected an increase of the levels of Bad-2 and voltage-dependent anion channel (VDAC), mitochondrial matrix proteins, and a decrease of the levels of HSP60 indicating a condition of mitochondrial dysfunction (Fig. **6A,B**). This condition was confirmed by analysis of fission and fusion markers, mechanism involved in mitochondrial dynamics. By Real Time PCR an increase in mRNA levels of Drp1 and Fis1 and a decrease in mRNA levels of OPA1 and Mfn1, compared to control mice was revealed (Fig. **6C**), indicating that mitochondrial dynamics were perturbed in HFD fed mice.

4. DISCUSSION

Obesity is often associated with the consumption of a diet high in saturated fats, and several clinical/epidemiological studies have suggested this metabolic disease as a risk factor for the development of AD in later life [32, 33]. There is growing evidence to support the contribution of metabolic factors to this pathology, which have previously been considered limited to the central nervous system. However, the molecular mechanisms underlying this comorbidity, and the influence of high fat diet on cerebral structures, have not been well established. By use of a diet-induced obesity model, some of the molecules and pathogenic mechanisms shared by AD and this metabolic disorder were identified.

Increased body weight and reduced cerebral weight was found in HFD mice. Loss in brain mass and volume is a fea-

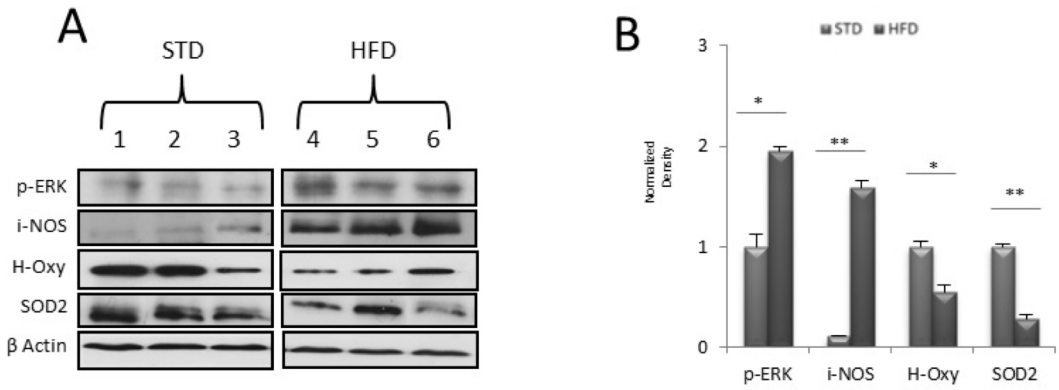


Fig. (5). Stress related proteins are modulated in high fat compared to standard diet fed mice. **A)** Western blot of protein brain lysates from STD and HFD fed mice was incubated with anti-phospho-ERK (p-ERK), i-NOS, H-Oxy, SOD2 antibodies.Uniformity of gel loading was confirmed with β-Actin as standard. **B)** Quantification of immunoreactivity was performed using densitometric analysis. n= 3 per group. *P < 0.05, **P < 0.02 versus control group.

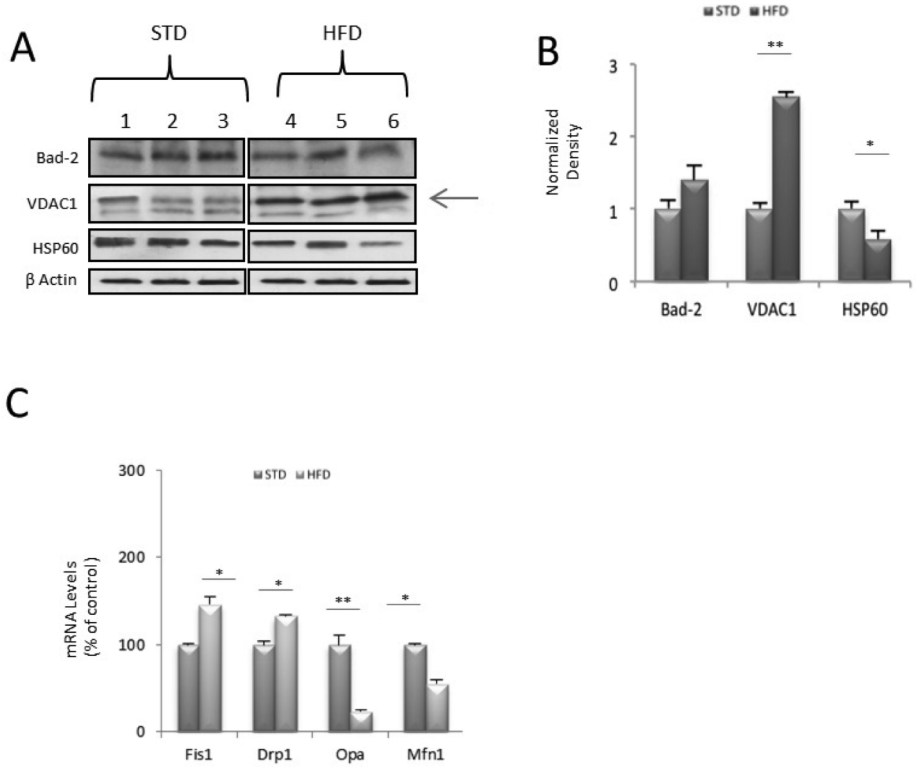


Fig. (6). HFD impairs mitochondrial dynamics and induces mitochondrial dysfunction. **A)** Western blot of brain tissue lysates from STD and HFD fed mice probed with specific antibodies against Bad-2, VDAC, HSP60 mitochondrial proteins and β-Actin (general loading control). Arrow indicates bands of interest being present no specific bands. **B)** Quantification of immunoreactivity was performed using densitometric analysis. **C)** Effect of high fat versus standard diet on Fis1, Drp1, Opa and Mnf1 transcript levels determined by quantitative real-time PCR. n= 3 per group. *P < 0.05, **P < 0.02 versus control group.

ture of AD and neuroimaging studies have indicated that disturbances in insulin metabolism, such as insulin resistance in obesity, T2D and AD, are linked to significant reduction of grey matter volume [34]. Moreover, studies using different diet compositions have demonstrated as high fat diet modulates levels of solubilized Aβ together with brain mass, density and volume [35]. Consistent with this result, we found that APP and certain proteins related to APP processing, including BACE1, are up-regulated in the brain of HDF fed mice. The increased presence of these proteins was associated with the increased production of Aβ40 and Aβ42. This explains the presence of insoluble Aβ fibrillar aggregates and amyloid plaques in hippocampal and cerebral cortex HFD brain sections, suggesting that neuronal network activity could be disturbed. Moreover, we found that activated GSK3β had induced Tau phosphorylation, a step necessary for its aggregation in the form of paired helical filaments and neurofibrillary tangles is a hallmark, with amyloid plaques, of AD.

There is evidence that elevated APP levels are present both in brain and adipose tissue of HFD fed animals supporting the hypothesis that APP expression could be coordinately regulated between brain and fat tissue [36]. Furthermore, a study, using diet-induced insulin resistance in Tg2576 mouse model of AD, has demonstrated that insulin resistance promoted an increase of Aβ peptide levels and early deposits, indicating that manipulation of diet could be a risk for AD [37]. Hence, monitoring of APP expression and processing in peripheral tissues could provide a checkpoint both for AD and obesity development and progression. Increase of APP gene expression may be correlated with insulin resistance and pro-inflammatory and adipocytokine gene expression [38, 39]. Insulin resistance, defined as a lower than expected response to a given dose of insulin, is a pathological condition characteristic of metabolic disorders as T2D, obesity and pathology related to aging [36]. Systemic insulin resistance refers to the state in which high levels of blood insulin (hyperinsulinemia) are associated with hyperglycemia. We found that peripheral and central insulin resistance coexists in HFD fed mice. In fact, HFD fed animals showed high fasting plasma glucose levels, increased insulin and reduced sensitivity to exogenous insulin, suggesting a state of peripheral insulin resistance, as previously shown [40]. At the molecular level, insulin resistance corresponds to a reduced number of IRs and impairment in insulin signaling, with reduced activation of specific pathways involved in metabolism and growth. High levels of IRs are present in healthy hippocampus, in contrast to the reduced levels that have been reported in patients with AD [17, 38]. In addition, studies using cultured neurons have demonstrated that IRs are redistributed or decreased in number under Aβ stimulation [41-43]. Our finding indicates that in the brains of HFD fed mice, the levels of IR are reduced, leading to compromised-insulin signaling, as demonstrated by the analysis of Akt and Foxo3a expression and activation. When HFD is chronically ingested, Akt is down phosphorylated and Foxo3a translocates from the cytoplasm to the nucleus where it activates its proapototic target genes. Thus, impairment of insulin signaling induced by HFD can lead to neurodegeneration. Similarly, caloric restriction mediates, in TG2576 mice, activation of the insulin signaling pathway, leading to phosphorylation and exclusion of Foxo3a from the nucleus, and prevention of AD [44]. Furthermore, we cannot exclude that the consumption of a high fat diet has affected regulation of peripheral lipid metabolism, another factor which may be involved in brain insulin resistance. It has been hypothesized that peripheral insulin resistance may potentially cause brain insulin resistance via a liver-brain axis of neurodegeneration as a result of the trafficking of toxic lipids such as ceramides across the blood brain barrier (BBB) [36].

Further evidence that obesity-induced insulin resistance may be a risk factor for AD onset and development derives from analysis of a specific array in which genes that are deregulated in this state were identified. Changes in levels of expression of genes involved in inflammation, innate immunity, insulin and adipokine signaling were detected. The highest differences in the profile of expression relative to controls were obtained for some of the genes involved in inflammation (up-regulated) and adipocytokine signaling (down-regulated), mechanisms present both in obesity and AD. Inflammation is part of the body's mechanism of defense against many challenges, and similar inflammatory processes are thought to occur in the brain and peripheral tissues [45, 46]. The presence of inflammatory markers such as cytokines/ chemokines and adipokines has been found in the AD brain and has been correlated with changes in the permeability of the BBB [47]. Generally, cytokines increase activity and expression of secretases, contributing to Aβ deposition and pathogenic changes in AD [48]. In particular, interferon γ, which produces pleiotropic effects in the brain, has been found to increase microglial activation and amyloid plaque formation in experimental models [49]. Thus, we cannot exclude that increased APP expression, processing and Aβ production could be correlated with the inflammatory changes induced by the high fat diet.

Leptin, adiponectin and resistin messengers are present in the brain, and their expression is dysregulated in our mouse model of diet-induced obesity. This modulation of adipocytokine messengers suggests that their presence in the brain is not exclusively related to peripheral production, and we cannot exclude a specific role in the central nervous system. Adipose tissue produces and releases into the systemic circulation adipocytokines which have metabolic, endocrine and anti-inflammatory functions. They can cross the BBB, and can therefore exert a neuroprotective role on cognition-related structures [50]. Consequently, dysfunction of adipocytokines related to obesity may increase the risk of AD. Leptin, beside its physiological action, shows beneficial effects on memory and consequent cognitive improvement when directly administrated to the hippocampal region [51]. In rodents, leptin plays a role in APP metabolism, by modulating secretase activity, Aβ peptide formation and clearance [52]. Leptin also seems to promote deactivation of GSK3β [53], and reduced plasma levels of leptin have been associated with an increased risk of AD development [54]. In agreement with this result, we found reduced expression of leptin, activated GSK3β and phosphorylated Tau in the brains of HFD fed mice. Adiponectin is significantly down-regulated in the brain of HFD fed mice where probably it is less able to exert its pro- anti-inflammatory action [55]. Two adiponectin receptors are present in hippocampal neurons, AdipoR1-2, and their neuroprotective role is mediated by activation of adenosine monophosphate-activated protein kinase (AMPK) [56]. Furthermore, adiponectin has been demonstrated to reduce Aβ-induced neurotoxicity, likely via inhibition of NF-κB. Our results are in agreement with the finding of decreased levels of circulating leptin and adiponectin in mild cognitive impairment and AD patients [57]. A protective effect of resistin against Aβ-induced neurotoxicity has been demonstrated in transgenic mice. Resistin attenuates levels of ROS, NO, protein carbonyls, improves mitochondrial function and prevents apoptosis by decreasing cytochrome c release [58]. Our data suggests that lower levels of adipocytokines may be the cause of the failure of their neuroprotective functioning, further evidence for the association between obesity and dementia.

Mitochondrial dysfunction and increased oxidative stress are both associated with obesity and AD. Upon high fat diet molecules involved in oxidative stress such as phosphorylated ERK, i-NOS and H-Oxy are up-regulated with respect to the control. This is in agreement with the common knowl-

edge that inflammatory signals can induce oxidative imbalance and ROS production that can be mediated by stimulation of molecules such as i-NOS, which produces high levels of nitricoxide, facilitating the formation of other reactive oxygen and nitrogen species [59]. Similarly, markers of mitochondrial dysfunction change their expression in HFD. VDAC protein performs several functions in the mitochondrion, including maintenance of the mitochondrial permeability transition pore (PTP), regulating its shape and structure, and in apoptosis signaling [60]. Bcl-2 family members, such as Bad, regulate VDAC, leading to apoptogenic release of cytochrome c. Recent research has revealed that VDAC1 is increased in AD postmortem brains and in AD-affected brain tissues from AβPP transgenic mice. Additionally, other evidence have suggested that a progressive increase in interactions between VDAC1 and Aβ and phosphorylated Tau correlate with disease progression [61]. In contrast, a decrease of HSP60 levels was detected. This mitochondrial chaperonin is a ubiquitous molecule with multiple roles in health and disease [62, 63] recently found to be involved in insulin resistance [62]. HSP60 appears to be modulated by leptin in the hypothalamus of obese and diabetic mice and decreased levels of leptin were found in accordance with the reduced presence of HSP60 [64]. Decreased levels of leptin, as mentioned above, were found correlated with the reduced presence of Hsp60. Thus, the neuroprotective role of leptin via HSP60 is compromised in our model. An increase in mitochondrial fission (Fis1, Drp1) and decrease in fusion genes (OPA1, Mfn1) were found in HDF mice. Disordered mitochondrial dynamics are a feature of mitochondrial dysfunction and contribute to the pathogenesis of neurodegenerative diseases [65, 66]. Fission and fusion regulate fundamental cellular processes such as calcium homeostasis, ATP generation and ROS production. Unbalanced fusion leads to mitochondrial elongation and unbalanced fission leads to excessive mitochondrial fragmentation, both of which impair mitochondrial function. Moreover, recent studies have demonstrated that obesity and excess energy intake shift the balance of mitochondrial dynamics, contributing to mitochondrial dysfunction and metabolic deterioration all conditions leading to insulin resistance [67].

CONCLUSION

This study supports the idea that the metabolic dysfunction leads to both in peripheral and central tissues, insulin resistance, a condition which contributes to inflammation, adypokine dysfunction, oxidative stress and mitochondrial dysfunction. These mechanisms affect each other through up- and down-regulation of specific molecules. In the brain, an insulin resistance status, may contribute to an increase in Aβ peptide levels and deposits, leading to neurodegeneration (Fig. **7**). Our findings suggest that preventing the cellular and metabolic dysfunctions common to both AD and obesity may not only attenuate pathological events in peripheral organs, but also those in the brain.

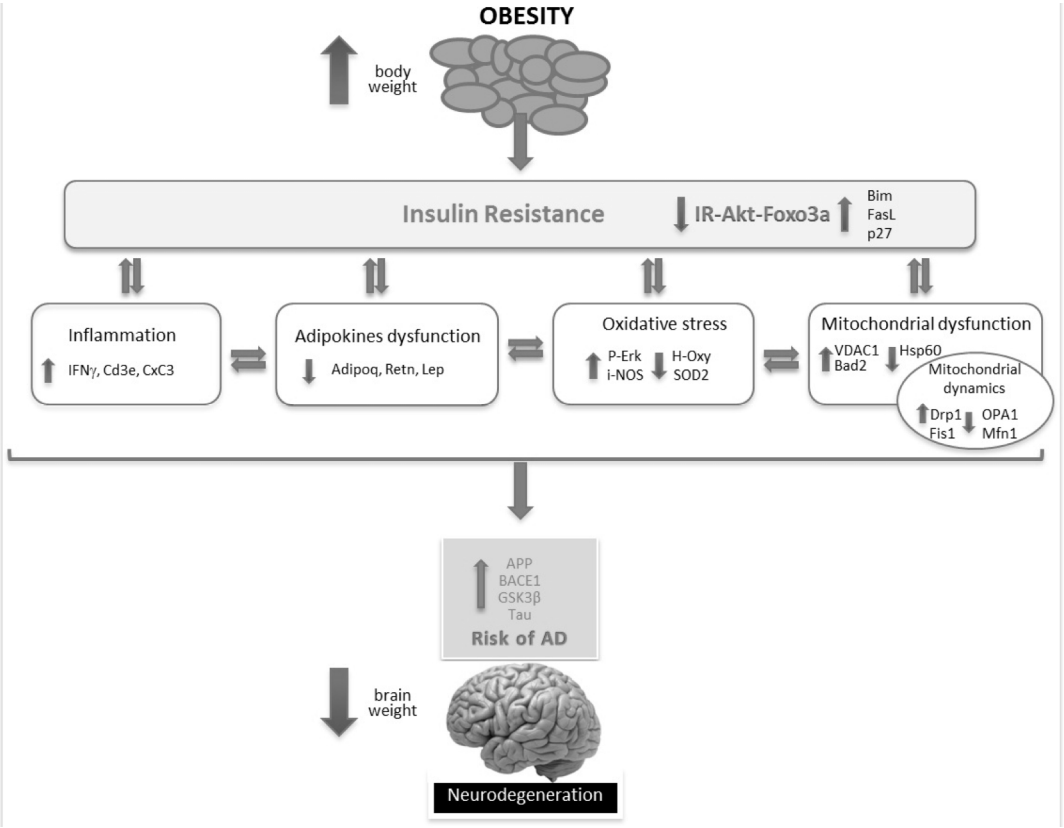


Fig. (7). A model of mechanisms that contribute and lead to peripheral insulin resistance in obesity and to brain insulin resistance in neurodegeneration. Inflammation, adypokine dysfunction, oxidative stress and mitochondrial dysfunction are interrelated through up- and down-regulation of specific molecules.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors deeply thank Jessica Walters for the English revision of the manuscript. This work was funded with the Italian National Operational Program for Research and Competitiveness 2007-2013 grant (Project name: "Di.Me.Sa -Valorization of typical products of Mediterranean diet and their use for healthy and nutraceutical purposes"; project code: PON02_00667 – PON02_00451_3361785European Regional Development Fund; Project name: "Cyber Brain"; project code: PONa3_00210, European Regional Development Fund). The Euro-Mediterranean Institute of Science and Technology (FC) and University of Palermo (FC and AM) funds supported this work. Part of this work was carried out using instruments provided by the Euro-Mediterranean Institute of Science and Technology.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

REFERENCES

[1] Selkoe DJ. The genetics and molecular pathology of Alzheimer's disease: roles of amyloid and the presenilins. Neurol Clin 18: 903-922 (2000).

[2] Cole SL, Vassar R. The Alzheimer's disease beta-secretase enzyme, BACE1. Mol Neurodegener 2: 1-25 (2007).

[3] Alzheimer's Association. Alzheimer's disease facts and figures. Alzheimer's Dement e47-e92 (2014).

[4] González Jiménez E. Genes and obesity: A cause and effect relationship. Endocrinolog Nutr 58: 492-496 (2011).

[5] Hildreth KL, Van Pelt RE, Schwartz RS. Obesity, insulin resistance and Alzheimer's disease. Obesity 20: 1549-57 (2012).

[6] Beydoun MA, Beydoun HA, Wang Y. Obesity and central obesity as risk factors for incident dementia and its subtypes: a systematic review and meta-analysis. Obesity Rev 9: 204-218 (2008).

[7] Fitzpatrick AL, Kuller LH, Lopez OL, Diehr P, O'Meara ES, Longstreth WJ Jr., *et al.* Midlife and late-life obesity and the risk of dementia: cardiovascular health study. Arch Neurol 66: 336-342 (2009).

[8] Gunstad J, Paul RH, Cohen RA, Tate DF, Spitznagel MB, Grieve S, *et al.* Relationship between body mass index and brain volume in healthy adults. Int J Neurosci 118: 1582-1593 (2008).

[9] Cohen RA. Obesity-associated cognitive decline: excess weight affects more than the waistline. Neuroepidemiology 34: 230-231 (2010).

[10] Gunstad J, Lhotsky A, Wendell CR, Ferrucci L, Zonderman AB. Longitudinal examination of obesity and cognitive function: results from the Baltimore longitudinal study of aging. Neuroepidemiology 34: 222-229 (2010).

[11] Ott Ott A, Stolk RP, van Harskamp F, Pols HAP, Hofman A, Breteler MMB. Diabetes mellitus and the risk of dementia: the Rotterdam Study. Neurology 53:1937-1942 (1999).

[12] Lee YH, Tharp WG, Maple RL, Nair S, Permana PA, Pratley RE. Amyloid precursor protein expression is up-regulated in adipocytes in obesity. Obesity 16: 1493-1500 (2008).

[13] Lee YH, Martin JM, Maple RL, Tharp WG, Pratley RE. Plasma amyloid-beta peptide levels correlate with adipocyte amyloid precursor protein gene expression in obese individuals. Neuroendocrinology 90: 383-390 (2009).

[14] Kahn BB, Flier JF. Obesity and insulin resistance. J Clin Invest 106: 473-481 (2000).

[15] Watson GS, Craft S. Modulation of memory by insulin and glucose: neuropsychological observations in Alzheimer's disease. Eur J Pharmacol 490: 97-113 (2004).

[16] Ghasemi R, Haeri A, Dargahi L, Mohamed Z, Ahmadiani A. Insulin in the brain: sources, localization and functions. Mol Neurobiol 47: 145-171 (2013).

[17] Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R *et al.* Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease – is this type 3 diabetes. J Alzheimers Dis 7: 63-80 (2005).

[18] Watson GS, Craft S. The role of insulin resistance in the pathogenesis of Alzheimer's disease: implications for treatment. CNS Drugs 17: 27-45 (2003).

[19] Marks DR, Tucker K, Cavallin MA, Mast TG, Fadool DA. Awake intranasal insulin delivery modifies protein complexes and alters memory, anxiety, and olfactory behaviors. J Neurosci 29: 6734-6751 (2009).

[20] Craft S, Baker LD, Montine TJ, Minoshima S, Watson GS, Claxton A, *et al.* Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. Arch Neurol 69: 29-38 (2012).

[21] Molteni R, Barnard RJ, Ying Z, Roberts CK, Gomez-Pinilla F. A high-fat, refined sugar diet reduces hippocampal brain-derived neurotrophic factor, neuronal plasticity, and learning. Neuroscience 112: 803-814 (2002).

[22] Stranahan AM, Mattson MP. Bidirectional metabolic regulation of neurocognitive function. Neurobiol Learn Mem 96: 507-516 (2011).

[23] Heyward FD, Waltonb RG, Carle MS, Coleman MA, Garvey WT, Sweatt JD. Adult mice maintained on a high-fat diet exhibit object location memory deficits and reduced hippocampal SIRT1 gene expression. Neurobiol Learn Mem 98: 25-32 (2012).

[24] Valladolid-Acebes I, Stucchi P, Cano V, Fernández-Alfonso MS, Merino B, Gil-Ortega M, *et al.* High-fat diets impair spatial learning in the radial-arm maze in mice. Neurobiol Learn Mem 95: 80-85 (2011).

[25] Moroz N1, Tong M, Longato L, Xu H, de la Monte SM. Limited Alzheimer-type neurodegeneration in experimental obesity and type 2 diabetes mellitus. J Alzheimers Dis 15: 29-44 (2008).

[26] Arvanitidis AP, Corbett D, Colbourne F. A high fat diet does not exacerbate CA1 injury and cognitive deficits following global ischemia in rats. Brain Res 1252: 192-200 (2009).

[27] Camargo N, Brouwers JF, Loos M, Gutmann DH, Smit AB, Verhijen MH. High-fat diet ameliorates neurological deficits caused by defective astrocyte lipid metabolism. FASEB J 10: 4302-15 (2012).

[28] Heyward FD, Walton RG, Carle MS, Coleman MA, Garvey WT, Sweatt JD. Adult mice maintained on a high-fat diet exhibit object location memory deficits and reduced hippocampal SIRT1 gene expression. Neurobiol Learn Mem 98: 25-32 (2012).

[29] Ramos-Rodriguez JJ, Molina-Gil S, Ortiz-Barajas O, Jimenez-Palomares M, Perdomo G, Cozar-Castellano I, *et al.* Central proliferation and neurogenesis is impaired in type 2 diabetes and prediabetes animal models. PLoS One 9(2): e89229 (2014).

[30] Baldassano S, Amato A, Cappello F, Rappa F, Mulè F. Glucagon-like peptide-2 and mouse intestinal adaptation to a high-fat diet. J Endocrinol 217: 11-20 (2013).

[31] Marino Gammazza A, Colangeli R, Orban G, Pierucci M, Di Gennaro G, Bello ML, *et al.* Hsp60 response in experimental and human temporal lobe epilepsy. Sci Rep 5: 9434 (2015).

[32] Eskelinen MH, Ngandu T, Helkala EL, Tuomilehto J, Nissinen A, Soininen H, *et al.* Fat intake at midlife and cognitive impairment later in life: a population-based CAIDE study. Int J Geriatr Psychiatry 23: 741-747 (2008).

[33] Kalmijn S, Launer LJ, Ott A, Witteman JC, Hofman A, Breteler MM. Dietary fat intake and the risk of incident dementia in the Rotterdam Study. Ann Neurol 42: 776-782 (1997).

[34] Nickl-Jockschat T, Kleiman A, Schulz JB, Schneider F, Laird AR, Fox PT, *et al.* Neuroanatomic changes and their association with cognitive decline in mild cognitive impairment: a metaanalysis. Brain Struct Funct 217: 115-25 (2012).

[35] Pedrini S, Thomas C, Brautigam H, Schmeidler J, Ho L, Fraser P, *et al.* Dietary composition modulates brain mass and solubilizable Abeta levels in a mouse model of aggressive Alzheimer's amyloid pathology. Mol Neurodegener 21: 4: 40 (2009).

[36] de la Monte SM, Tong M. Brain metabolic dysfunction at the core of Alzheimer's disease. Biochem Pharmacol 88: 548-59 (2014).

[37] Ho L, Qin W, Pompl PN, Xiang Z, Wang J, Zhao Z, *et al.* Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. FASEB J 18: 902-904 (2004).

[38] Letra L, Santana I, Seiça R. Obesity as a risk factor for Alzheimer's disease: the role of adipocytokines. Metab Brain Dis 29: 563-568 (2014).

[39] Puig KL, Floden AM, Adhikari R, Golovko MY, Combs CK. Amyloid precursor protein and proinflammatory changes are regulated in brain and adipose tissue in a murine model of high fat diet-induced obesity. PLoS One 7: e30378 (2012).

[40] Baldassano S, Rappa F, Amato A, Cappello F, Mulè F. GLP-2 as beneficial factor in the glucose homeostasis in mice fed a high fat diet. J Cell Physiol Accepted for publication, (2015).

[41] Zhao WQ, De Felice FG, Fernandez S, Chen H, Lambert H, Quon MJ, *et al*. Amyloid beta oligomers induce impairment of neuronal insulin receptors. FASEB J 22: 246-260 (2008).

[42] De Felice FG, Vieira MNN, Bomfim TR, Decker H, Velasco PT, Lambert MP, *et al.* Protection of synapses against Alzheimer's-linked toxins: insulin signaling prevents the pathogenic binding of Abeta oligomers. Proc Natl Acad Sci USA 106: 1971-1976 (2009).

[43] Picone P, Giacomazza D, Vetri V, Carrotta R, Militello V, San Biagio PL, *et al*. Insulin-activated Akt rescues Aβ oxidative stress-induced cell death by orchestrating molecular trafficking. Aging Cell 10: 832-43 (2011).

[44] Qin W, Zhao W, Ho L, Wang J, Walsh K, Gandy S, *et al*. Regulation of forkhead transcription factor FoxO3a contributes to calorie restriction-induced prevention of Alzheimer's disease-type amyloid neuropathology and spatial memory deterioration. Ann NY Acad Sci 1147: 335-47 (2008).

[45] Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. Annu Rev Immunol 29: 415-445 (2011).

[46] Ferreira ST, Clarke JR, Bomfim TR, De Felice FG. Inflammation, defective insulin signaling, and neuronal dysfunction in Alzheimer's disease. Alzheimers Dement 10: S76-S83 (2014).

[47] Takeda S, Sato N, Ikimura K, Nishino H, Rakugi H, Morishita R. Increased blood-brain barrier vulnerability to systemic inflammation in an Alzheimer disease mouse model. Neurobiol Aging 34: 2064-2070 (2013).

[48] Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. Cell 140: 918-934 (2010).

[49] Mastrangelo MA, Sudol KL, Narrow WC, Bowers WJ. Interferon-γ differentially affects Alzheimer's disease pathologies and induces neurogenesis in triple transgenic-AD mice. J Am Pathol 175: 2076-2088 (2009).

[50] Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 89: 2548-2556 (2004).

[51] Wayner MJ, Armstrong DL, Phelix CF, Oomura Y. Orexin-A (Hypocretin-1) and leptin enhance LTP in the dentate gyrus of rats *in vivo*. Peptides 25: 991-996 (2004).

[52] Fewlass DC, Noboa K, Pi-Sunyer FX, Johnston JM, Yan SD, Tezapsidis N. Obesity-related leptin regulates Alzheimer's Abeta. FASEB J 18: 1870-1878 (2004).

[53] Greco SJ, Sarkar S, Casadesus G, Zhu X, Smith MA, Ashford JW, *et al.* Leptin inhibits glycogen synthase kinase-3beta to prevent tau phosphorylation in neuronal cells. Neurosci Lett 455:19-194 (2009).

[54] Lieb W, Beiser AS, Vasan RS, Tan ZS, Au R, Harris TB, *et al*. Association of plasma leptin levels with incident Alzheimer disease and MRI measures of brain aging. JAMA 302: 2565-2572 (2009).

[55] Gustafson DR. Adiposity hormones and dementia. J Neurol Sci 299: 30-34 (2010).

[56] Erol A. An integrated and unifying hypotesis for the metabolic basis of sporadic Alzheimer's disease. J Alzhemers Dis 13: 241-253 (2008).

[57] Teixeira AL, Diniz BS, Campos AC, Miranda AS, Rocha NP, Talib LL, *et al.* Decreased levels of circulating adiponectin in mild cognitive impairment and Alzheimer's disease. Neuromol Med 15: 115-121 (2013).

[58] Liu J, Chi N, Chen H, Zhang J, Bian Y, Cui G, *et al*. Resistin protection against endogenous Aβ neuronal cytotoxicity from mitochondrial pathway. Brain Res 1523: 77-84 (2013).

[59] Brown GC. Nitric oxide and neuronal death. Nitric Oxide 23: 153-165 (2010).

[60] Reddy PH. Amyloid beta-induced glycogen synthase kinase 3β phosphorylated VDAC1 in Alzheimer's disease: implications for synaptic dysfunction and neuronal damage. Biochim Biophys Acta 1832: 1913-1921 (2013).

[61] Manczak M, Reddy PH. Abnormal interaction of VDAC1 with amyloid beta and phosphorylated tau causes mitochondrial dysfunction in Alzheimer's disease. Hum Mol Genet 21: 5131-5146 (2012).

[62] Marino Gammazza A, Rizzo M, Citarrella R, Rappa F, Campanella C, Bucchieri F, *et al.* Elevated blood Hsp60, its structural similarities and cross-reactivity with thyroid molecules, and its presence on the plasma membrane of oncocytes point to the chaperonin as an immunopathogenic factor in Hashimoto's thyroiditis. Cell Stress Chaperones. 19(3): 343-53 (2014).

[63] Cappello F, Marino Gammazza A, Palumbo Piccionello A, Campanella C, Pace A, Conway de Macario E, *et al.* Hsp60 chaperonopathies and chaperonotherapy: targets and agents. Expert Opin Ther Targets 18(2): 185-208 (2014).

[64] Kleinridders A, Lauritzen HPMM, Ussar S, Christensen JH, Mori MA, Bross P, *et al*. Leptin regulation of Hsp60 impacts hypothalamic insulin signaling. J Clin Invest 123: 4667-4680 (2013).

[65] Wang X, Su B, Siedlak SL, Moreira PI, Fujioka H, Wang Y, *et al*. Amyloid-β overproduction causes abnormal mitochondrial dynamics via differential modulation of mitochondrial fission/fusion proteins. Proc Natl Acad Sci USA 49: 19318-23 (2008).

[66] Wang X, Su B, Lee HG, Li X, Perry G, Smith MA, *et al.* Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. J Neurosci 29: 9090-9103 (2009).

[67] Jheng HF, Tsai PJ, Guo SM, Kuo LH, Chang CS, Su IJ, *et al.* Mitochondrial fission contributes to mitochondrial dysfunction and insulin resistance in skeletal muscle. Mol Cell Biol 32: 309-19 (2012).

Received: ???????????????? **Revised:** ???????????????? **Accepted:** ????????????????