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Original Paper

Leptin-Sensitive JAK2 Activation in the Regulation of Tau Phosphorylation in PC12 Cells

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Key Words
PC12 cells • JAK2 • Leptin •Tau •Aβ

Abstract
Background/Aims: Alzheimer's disease (AD) is characterized by two major hallmarks: the deposition and accumulation of β-amyloid (Aβ) peptide and hyperphosphorylated tau in intracellular neurofibrillary tangles. Sets of evidence show that leptin reduces Aβ production and tau phosphorylation. Herein, we investigated the signaling pathways activated by leptin, to extensively understand its mechanism. ***Methods:*** Western blotting was employed to assess the protein abundance of p-tau and BAX, MTT assay to decipher the cells viability. ***Results:*** Leptin decreased tau phosphorylation, an effect was dependent on the activation of JAK2. ***Conclusion:*** The data suggest that JAK2 is involved in AD-related pathways.

Introduction

Alzheimer's disease (AD) is characterized by two major hallmarks: the deposition and accumulation of β-amyloid (Aβ) peptide in extracellular plaques, the deposition of hyperphosphorylated tau in intracellular neurofibrillary tangles (NFTs) [1].

Leptin, an adipocytokine produced endogenously in the brain [2-5], is decreased in AD patients [6] and has also been shown to reduce Aβ levels *in vitro* and *in vivo* [7-9]. On bindig to the ObRb [10], leptin leads to the activation of several intracellular signaling pathways, including JAK/STATS pathway [11]. JAK/STAT signaling exerts diverse effects on a number of biological processes including immunity, hematopoiesis, inflammation and development [12]. Furthermore, it has been demonstrated that intraneuronal Aβ causes memory impairment by attenuating JAK-STAT signaling in hippocampal neurons [1]. Sets of evidence show that leptin reduces β-amyloid (Aβ) production and tau phosphorylation,

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and various reports demonstrate the interaction between leptin and JAK-STAT pathway [13, 14]. However, there is still no evidence showing the related mechanism. Here, we have reported for the first time the leptin-sensitive JAK2/STAT5 activation in the regulation of tau phosphorylation and Aβ-induced apoptosis in PC12 cells.

In this study we have established an AD model from the adrenal pheochromocytoma PC12 cell line, and show that the hyperphosphorylation of tau protein and apoptosis induced by $A\beta_{25-35}$ are abolished by JAK2, which is leptin sensitive.

Materials and Methods

Cell culture

Highly differentiated neuron-like PC12 cells(Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin under standard conditions. Since $A\beta_{25-35}$ has the functional domain of Aβ required for both neurotrophic and neurotoxic effects [15, 16], the PC12 Cells grown on the coverslips were treated in 30 μM $A\beta_{25-35}$ (Sigma Aldrich, USA) in the presence or absence of leptin(Sigma Aldrich,USA) and/or JAK2 inhibitor TG101348 (Axon Medchem, The Netherlands) for 24h.

Western blotting

Total protein was prepared. Cells were lysed in RIPA buffer (Beyotime, Shanghai, China) with 1% phenylmethylsulfonyl fluoride (Beyotime) and 1% protein phosphatase inhibitor (Beyotime) on ice for 30 min. The samples were centrifuged at 14,000 rpm and 4°C for 20 min. The supernatant was removed and used for Western blotting. Total protein (40-60 μg) was separated by SDS-PAGE, thereafter transferred to PVDF membranes and blocked in 5% non-fat milk/Tris-buffered saline/Tween-20 (TBST) at room temperature for 1 hour. Membranes were probed overnight at 4°C with polyclonal rabbit anti-p-tau(1:1000, cell signaling), ObRb(1:1000, cell signaling), and p-JAK2(1:700, cell signaling). After incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000, Sigma, United States) for 1 h at room temperature, the bands were visualized with enhanced chemiluminescence reagents (Sigma, United States). Membranes were also probed with ACTIN antibody as loading control. Densitometric analysis was performed using quantity One software (Abbiotec, United States).

Cell viability assay

PC12 cells were grown in DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin. The cells were incubated in a stable environment with 5% CO_2 at 37°C in a humidified incubator. The medium was replaced every 24 hours. Cells were grown to about 80% confluence prior to $A\beta_{25-35}$ treatment, and then exposed to $A\beta_{25-35}$ at 30 μM.

To measure the effects of leptin upon $A\beta_{25-35}$-induced apoptosis in PC12 cells, cells were treated with $A\beta_{25-35}$ in the presence of leptin with or without TG101348(1μM). Each group of cells were seeded in 96-well microtiter plates and incubated for 24 hours. At different points, 20μl of MTT was added to each well followed by 4 hours incubation.

The medium was discarded and 150μl of DMSO was added into each well, and incubated for 20min. The OD(optical density) 492nm was measured. The proliferation inhibition rate was calculated as: (1-the OD of the experimental group/the OD of the control group) ×100%. Each experiment was repeated three times.

Quantification of mRNA expression

Total RNA was extracted from PC12 cells in TriFast (Peqlab, United States) according to the manufacturer's instructions. After DNAse digestion reverse transcription of total RNA was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Shanghai). Real-time polymerase chain reaction (RT-PCR) of the respective genes were set up in a total volume of 20 μl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix (Promega, United States) according to the manufacturer's protocol. Cycling conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 15 sec and 68°C for 20 sec. For amplification the following primers were used (5`>3`orientation):

The following primers were used:
Human Tbp (TATA box-binding protein):
forward (5'-3'): ACTCCTGCCACACCAGCC
reverse (5'-3'): GGTCAAGTTTACAGCCAAGATTCA
Human Bax
forward (5'-3'): ATGTGAAGCACCCTTTCCTG
reverse (5'-3'): TAGAACAGCTCTCCGCCATT

Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad, United States) and all experiments were done in duplicate. The house-keeping gene Tbp (TATA binding protein) was amplified to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the ΔCT method as described [17].

Statistics

Data are provided as means ± SEM, n represents the number of independent experiments. All data were tested for significance using unpaired Student t-test or ANOVA. Only results with $p < 0.05$ were considered statistically significant.

Results

In order to identify the effect of leptin upon the $A\beta_{25-35}$-induced cytotoxicity in PC12 cells, cells were pretreated with $A\beta_{25-35}$ in 30μM prior to MTT assay. As illustrated in Fig. 1, exposure of cells to $A\beta_{25-35}$ for 24 hours leaded to a decrease of the cell viability, an effect abolished by leptin(125 ng/ml) treatment.

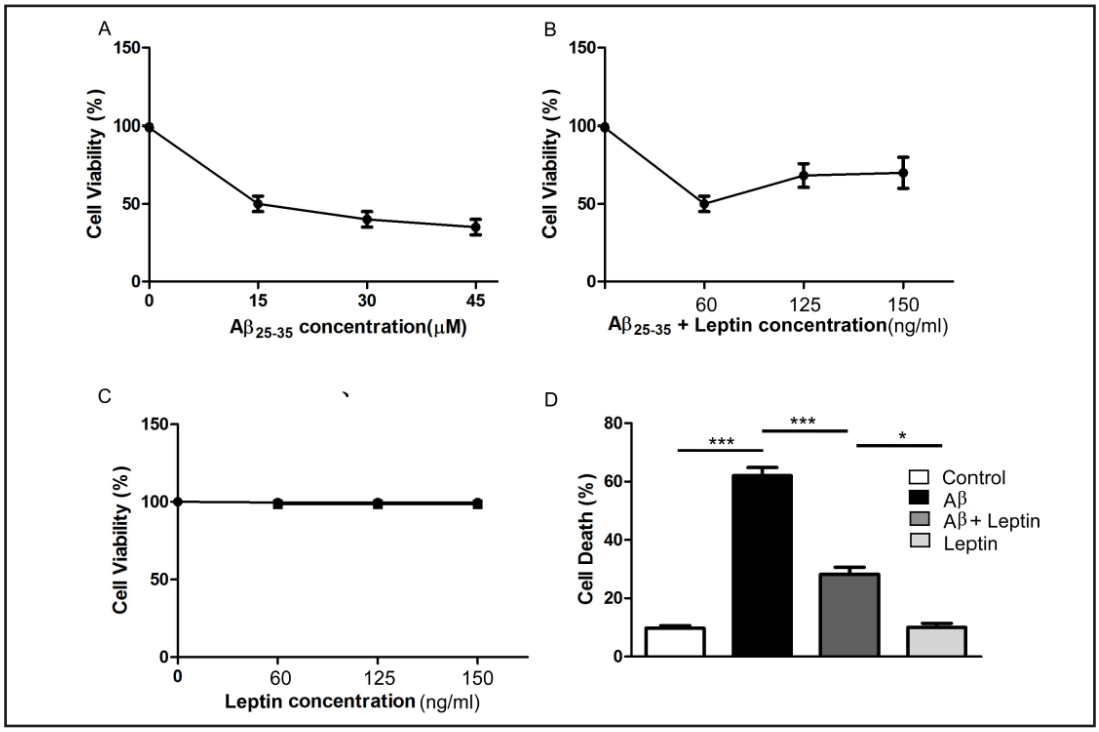

Fig. 1. Effect of Leptin on $A\beta_{25-35}$-induced neurotoxicity in PC12 cells. A: PC12 cells were treated with different concentrations of $A\beta_{25-35}$ for 24h. B: PC12 cells were incubated with different concentrations of Leptin(4nM) in the presence of $A\beta_{25-35}$(30μM) for 24h. C: PC12 cells were incubated with different concentrations of Leptin(4nM) in the absence of $A\beta_{25-35}$(30μM) for 24h. D: MTT analyse was used to determine cell death. PC12 cells were incubated with $A\beta_{25-35}$(30μM) without or with the treatment of Leptin(125 ng/ml) for 24h. Data are means ± SEM (n = 5 independent experiments). ***($p<0.001$) indicate statistically significant difference.

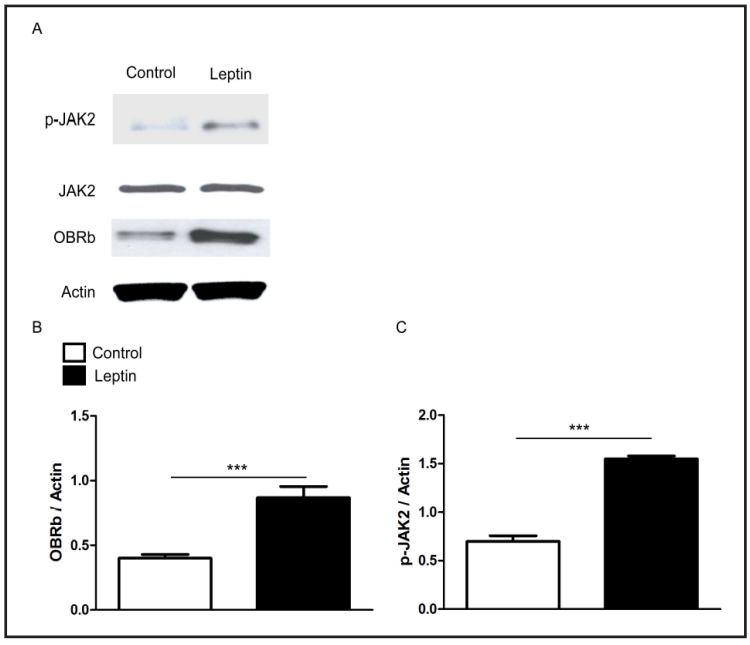

Fig. 2. Effect of leptin on OBR band JAK2 phosphorylation. A: Original western blot showing the protein abundance of OBRb, p-JAK2 and JAK2 as well as respective actin in PC12 cells with Leptin(125 ng/ml) treatment. B: Arithmetic means ± SEM (n = 4 independent experiments) of OBRb in PC12 cells without(white bar) or with Leptin(125 ng/ml)(black bar) treatment. C: Arithmetic means ± SEM (n = 4 independent experiments) of p-JAK2 in PC12 cells without(white bar) or with Leptin(125 ng/ml)(black bar)treatment. ***(p<0.001) indicate statistically significant difference.

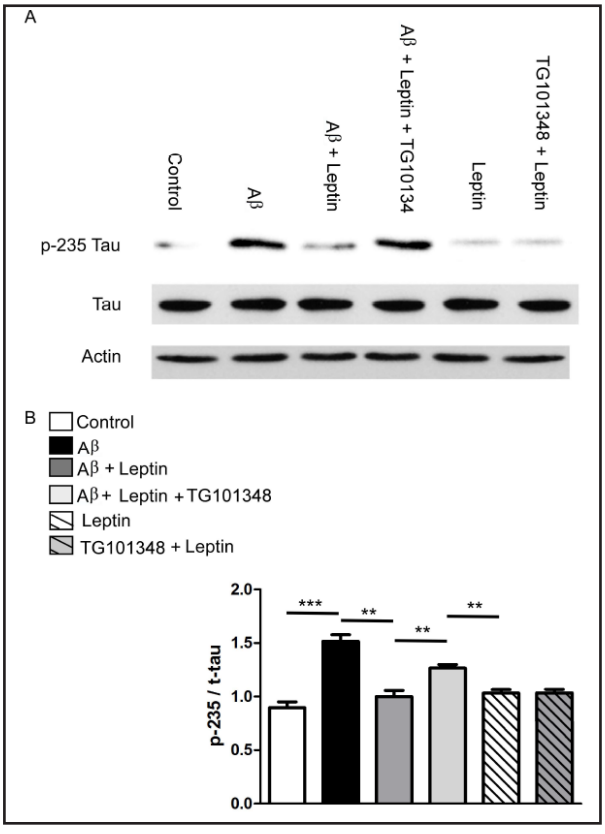

Fig. 3. Inhibition of JAK2 kinase blocked tau phosphorylation in PC12 cells. A: Original western blot showing the protein abundance of p-tau and tau protein as well as respective actin in $A\beta_{25-35}$-pretreated PC12 cells without or with Leptin (125 ng/ml) in the presence or absence of TG101348 (1μM). B: Arithmetic means ± SEM (n = 3 independent experiments) of p-tau in in $A\beta_{25-35}$-pretreated PC12 cells without(black bar) or with Leptin(125 ng/ml) in the presence(light grey) or absence(dark grey) of TG101348. **(p<0.01), ***(p<0.001) indicate statistically significant difference.

In order to confirm the expression of ObRb and the effect of leptin upon JAK2 activation, cells were treated without or with leptin(125 ng/ml) and performed western blotting. As illustrated in Fig. 2, leptin receptor ObRb was expressed in PC12 cells and leptin enhanced the protein abundance of ObRb and p-JAK2.

In order to explore whether the effect of leptin on $A\beta_{25-35}$-mediated tau hyperphosphorylation is sensitive to the JAK2, $A\beta_{25-35}$-pretreated PC12 cells were treated with leptin(125 ng/ml) with or without TG101348(1 μm). As illustrated in Fig. 3, treatment with TG101348(1 μm)

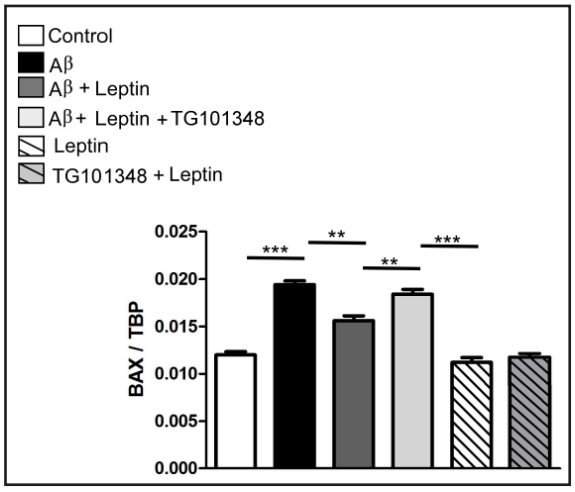

Fig. 4. Inhibition of JAK2 kinase blocked mRNA level of Bax in PC12 cells. Arithmetic means ± SEM (n = 4 independent experiments) of BAX in $A\beta_{25-35}$-pretreated PC12 cells without(black bar) or with Leptin in the presence(light grey) or absence(dark grey) of TG101348. **(p<0.01), ***(p<0.001) indicate statistically significant difference.

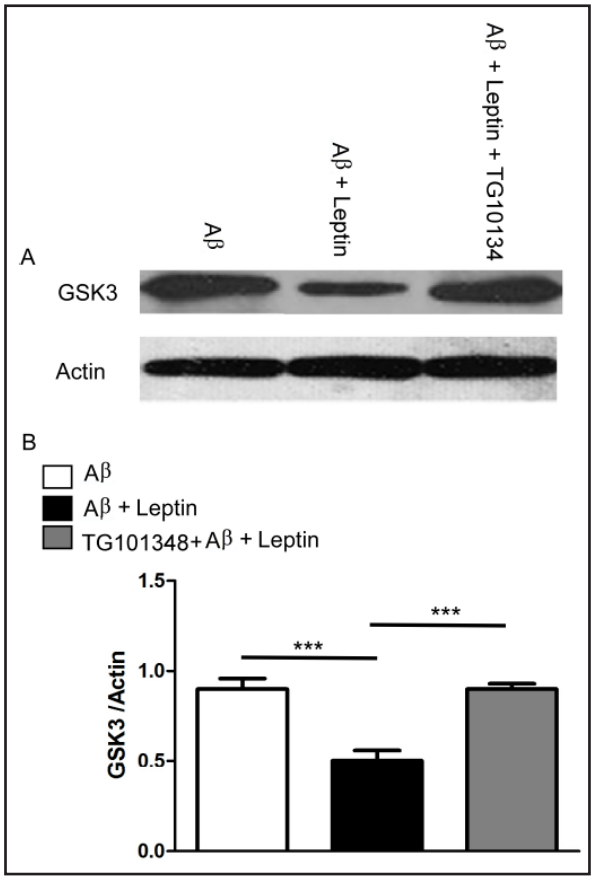

Fig. 5. GSK-3 protein abundance in $A\beta_{25-35}$-pretreated PC12 cells. A: Original western blot showing the protein abundance of GSK-3 and respective actin in $A\beta_{25-35}$-pretreated PC12 cells with Leptin treatment(125ng/ml) in the presence or absence of TG101348(1μM). B: Arithmetic means ± SEM (n = 4 independent experiments) of GSK-3 and respective actin protein abundance without(white bar) and with Leptin treatment(125ng/ml)(dark bar) in the presence of TG101348(1μM)(grey bar) in $A\beta_{25-35}$-pretreated PC12 cells. ***(p<0.001) indicate statistically significant difference.

significantly blunted Leptin-mediated inhibition of $A\beta_{25-35}$ phosphorylation of Tau at Ser252 (Fig. 3).

In order to elucidate the role of leptin in the apoptosis-related gene expression and whether the effect is JAK2 dependent, RT-PCR was applied to detect the transcriptional level of Bax in PC12 cells. As shown in Fig. 4, treatment with TG101348(1 μm) significantly blunted Leptin-mediated inhibition of $A\beta_{25-35}$ upon the mRNA level of Bax.

In order to explore the effect of leptin on GSK3 and whether the effect is sensitive to JAK2 pathway. $A\beta_{25-35}$-pretreated PC12 cells were treated with leptin and/or TG101348. As illustrated in Fig. 5, treatment with TG101348 (1 μm) significantly blunted Leptin-mediated inhibition of GSK3 protein abundance in $A\beta_{25-35}$-pretreated PC12 cells.

Discussion

This study was conceived to assess the role of leptin signaling in the modulation of AD. We demonstrate that $A\beta_{25-35}$ induces a marked upregulation of tau phosphorylation and apoptosis, the effects of which are reversed by treatment with the adipocytokine leptin. Leptin is known to activate JAK [18] and our present experiments indicate that JAK2/STAT5 mediates leptin's effects.

JAK/STAT pathway is a common mediator of astrocyte reactivity, which is a hallmark of neurodegenerative diseases [19].GSK-3 is highly expressed in the brain of AD patient [20], which results in the over-phosphorylation of Tau and the formation of NTF [21]. Reports show that leptin inhibits GSK-3β to prevent tau phosphorylation in neuronal cells [22, 23]. Furthermore, GSK-3β is downstream of JAK2 in H9C2 cells [24]. Here we have demonstrated that leptin inhibits GSK-3β to prevent tau phosphorylation by activating JAK2 in PC12 cells.

Our findings have shown that Leptin modulates Aβ production through JAK2 pathway. Aβ has been reported to induce neuronal tauopathy in APP-V717I×Tau-P301L biogenic mice through activation of GSK-3β [25]. Therefore, in addition to directly inactivating GSK-3β by kinases, the Leptin-JAK pathway may also indirectly inactivate the enzyme via regulating Aβ.

It was reported previously that leptin treatment could protect cells in the developing rodent brain against apoptosis. We found that leptin blunted the effects of $A\beta_{25-35}$ upon cell viability. These findings suggest that leptin exerts a cytoprotective role in AD.

Presently, we have demonstrated that leptin regulates the major AD pathways via distinct JAK2-dependent mechanisms in neuronal cells. Taken together, Leptin, potentially JAK2 activators, provides a novel therapeutic approach to AD treatment.

Disclosure Statement

All authors disclose that they have not any potential conflict of interest (e.g., consultancies, stock ownership, equity interests, patent-licensing arrangements, lack of access to data, or lack of control of the decision to publish).

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