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Original Paper

Leptin Regulates Tau Phosphorylation through Wnt Signaling Pathway in PC12 Cells

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Key Words
Alzheimer's disease • PC12 cells • Wnt • Tau • Leptin

Abstract
Background/Aims: Leptin, an adipocytokine produced endogenously in the brain, is decreased in Alzheimer's disease(AD) and has also been shown to reduce Aβ levels *in vitro* and *in vivo*. Sets of evidence show that leptin reduces Aβ production and tau phosphorylation in neuronal cells and transgenic mice models of AD. Herein, we investigated the signaling pathway activated by leptin, to better understand its mechanism of action. ***Methods:*** *W*estern blotting was performed to assess the levels of phosphor-tau and Bax, RT-PCR to check the mRNA level of Bax. ***Results:*** Leptin treatment significantly blunted Aβ-evoked tau phosphorylation and Bax levels, effects of which could be reversed by antagonist of Wnt signaling. ***Conclusion:*** The data indicate that Leptin may provide a novel therapeutic approach to AD treatment via wnt signaling.

Introduction

The most common neurodegenerative dementia is Alzheimer's disease (AD), which is characterized by two major pathological hallmarks: the deposition and accumulation of β-amyloid (Aβ) peptide in extracellular plaques, the deposition of hyper-phosphorylated tau in intracellular neurofibrillary tangles (NFTs) [1]. Compared to Aβ plaques, NFT with the brain correlates better with neurodegeneration and cognitive decline [2].

Leptin is a 16-kDa peptide hormone and inflammatory cytokine involved in regulating food intake, metabolism, body fat, energy expenditure and neuroendocrine function [3]. Moreover,

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a decline of leptin levels is common in AD [4]. Additionally, leptin reduces Aβ levels *in vitro* and *in vivo* [5-7]. Sets of evidence show that leptin reduces Aβ production and tau phosphorylation in neuronal cells and transgenic mice models of AD [8, 9]. Fewlass et al. has found the leptin's ability to modify Aβ levels *in vitro* and *in vivo* [10]. Interestingly, leptin suppresses Aβ degradation in astrocytes and increases apoE-dependent Aβ uptake *in vitro* [10]. Indeed, leptin signaling is involved in ApoE gene expression and would exert this effect on the removal of Aβ aggregates [11]. Therefore, leptin modulates bidirectional Aβ kinesis reducing its extracellular levels [12].

Moreover, leptin up-regulates Wnt in breast cancer cells [13]. Wnt/ β-catenin signaling pathway is involved not only in the formation of the embryonic dorsoventral axis, but also in a number of other developmental events that depend on establishment of cell polarity or determination of cell fate [14]. Glycogen synthase kinase 3(GSK3), a key component of the β-catenin destruction complex, phosphorylates β-catenin at Ser33, Ser37, and Thr41, leading to its degradation by the proteasome [15, 16]. Of note, GSK-3 is highly expressed in the brain of AD patients [17], which results in the over-phosphorylation of Tau and the formation of NTF [18].

We have previously described that Wnt/β-catenin signaling pathway is involved in the neurotoxic effects of $A\beta_{25-35}$ [19]. Herein, we investigated whether leptin regulated tau phosphorylation via Wnt signaling pathway in $A\beta_{25-35}$-pretreated PC12 cells.

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.

It is reported that addition of purified Aβ and of its active fragment $A\beta_{25-35}$ can induce apoptosis in a variety of mammalian cell type *in vitro* [20]. Therefor, Cells grown on the coverslips were pretreated in 30 μM $A\beta_{25-35}$ (Sigma Aldrich,USA) in the presence or absence of leptin(Sigma Aldrich,USA) and/or Wnt antagonist DKK1(Sigma Aldrich,USA) for 24h.

Western blotting

To examine the protein abundance, cells were lysed in RIPA buffer (Beyotime, Shanghai, China) containing 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. Loading buffer (Beyotime, Shanghai, China) was added to the protein lysate, which then were incubated at 95ºC for 5 min.

Total proteins (40-60 μg) were separated by electroporation on 10% SDS-PAGE. For immunoblotting proteins were electro-transferred onto PVDF membranes and blocked with 5% nonfat milk in triethanolamine-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) at room temperature for 1 h. Then, the membrane was incubated with polyclonal rabbit anti-p-tauSer235(1:1000, cell signaling), Bax(1:1000, cell signaling) at 4°C overnight. All antibodies were diluted in TBS-T with 5% BSA. After washing with TBST the blots were incubated with secondary anti rabbit secondary antibody (1:2000, Sigma, United States) for 1 h at room temperature. After washing antibody binding was visualized with enhanced chemiluminescence reagents(Beyotime, Shanghai, China). Membranes were also probed with Actin antibody as loading control. Densitometric analysis was performed using quantity One software (Abbiotec, United States).

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, United States). 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, Munich, Germany) 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 [21].

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 followed by post hoc Bonferroni test was applied when multiple comparisons between different groups were made. Only results with $p < 0.05$ were considered statistically significant.

Results

In order to explore whether the effect of leptin on $A\beta_{25-35}$-mediated tau phosphorylation is Wnt-dependent, $A\beta_{25-35}$-pretreated PC12 cells were treated with leptin(125 ng/ml) in the presence or absence of DKK1(100 ng/ml). As illustrated in Fig. 1, leptin significantly alleviated Leptin-mediated inhibition of $A\beta_{25-35}$ phosphorylation of Tau at Ser235.

The BAX gene was the first identified pro-apoptotic member of the Bcl-2 protein family. Western Blot was utilized to quantity the expression of Bax protein in PC12 cells. As shown in Fig. 2, the expression level of Bax protein in control group was less than that in $A\beta_{25-35}$ group, and leptin blocked $A\beta_{25-35}$-induced increase of Bax expression. In addition, DKK1(100 ng/ml)

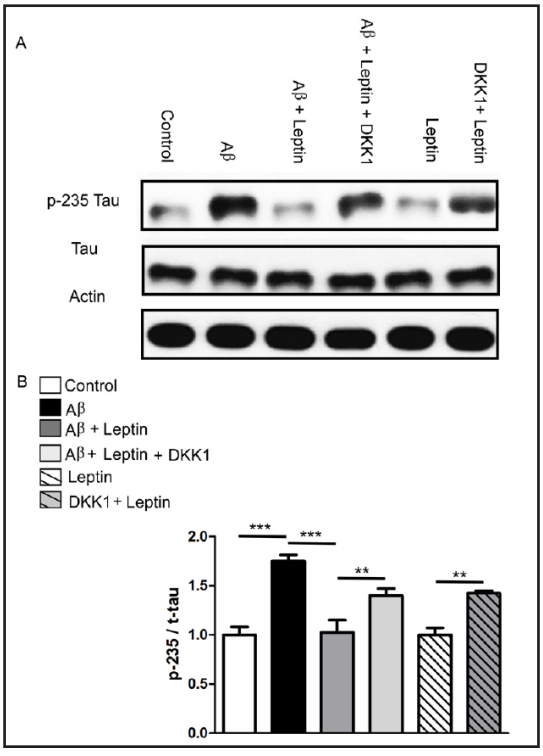

Fig. 1. Leptin abolished $A\beta_{25-35}$-induced tau phosphorylation by Wnt signaling in PC12 cells. (A) Original western blot showing the levels of phosphor-tau and total tau protein as well as respective actin in $A\beta_{25-35}$-pretreated PC12 cells without or with Leptin (125 ng/ml) in the presence or absence of DKK1 (100 ng/ml). (B) Arithmetic means ± SEM (n = 3 independent experiments) of p-tau in in $A\beta_{25-35}$-pretreated PC12 cells without or with Leptin(125 ng/ml) in the presence or absence of DKK1 (100 ng/ml). **(p<0.01), ***(p<0.001) indicate statistically significant difference.

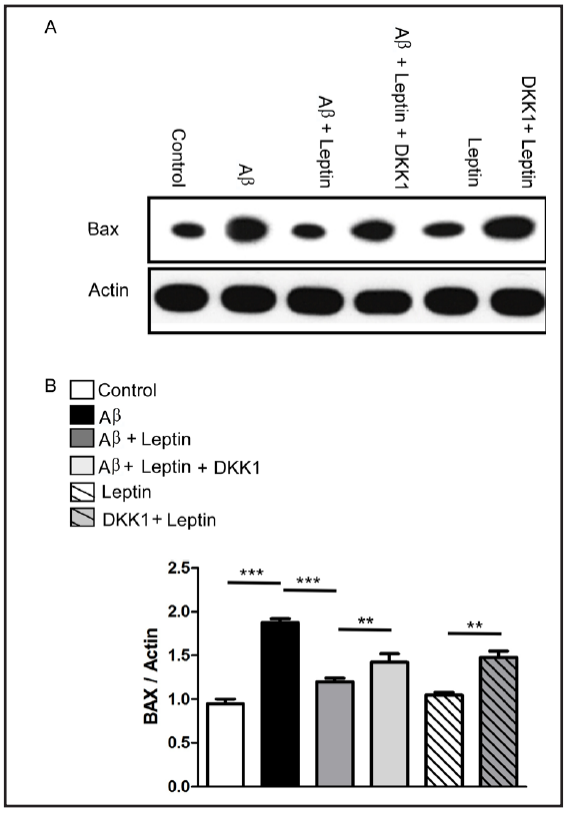

Fig. 2. Leptin regulated $A\beta_{25-35}$-evoked Bax expression via Wnt in PC12 cells. (A) Original western blot showing the protein abundance of Bax as well as respective actin in $A\beta_{25-35}$-pretreated PC12 cells without or with Leptin(125 ng/ml) in the presence or absence of DKK1 (100 ng/ml). (B) Arithmetic means ± SEM (n = 4 independent experiments) of bax in $A\beta_{25-35}$-pretreated PC12 cells without or with Leptin in the presence or absence of DKK1 (100 ng/ml). **(p<0.01), ***(p<0.001) indicate statistically significant difference.

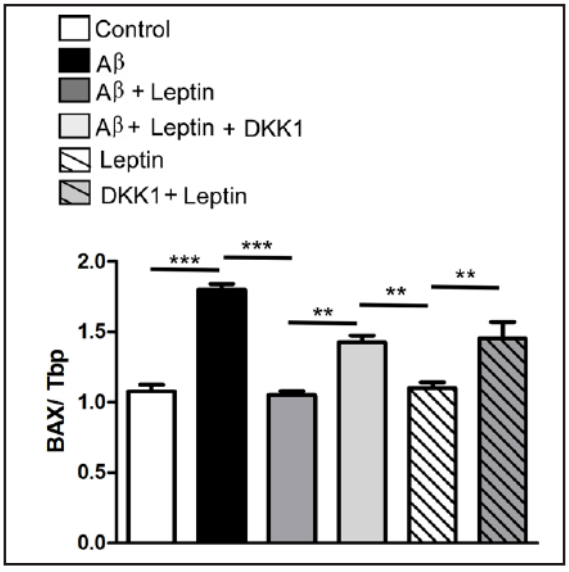

Fig. 3. Leptin effected $A\beta_{25-35}$-evoked Bax transcriptional expression through Wnt in PC12 cells. Arithmetic means ± SEM (n = 4 independent experiments) of Bax mRNA level in $A\beta_{25-35}$-pretreated PC12 cells without or with Leptin in the presence or absence of DKK1 (100 ng/ml). **(p<0.01), ***(p<0.001) indicate statistically significant difference.

significantly blunted Leptin-regulated inhibition of $A\beta_{25-35}$ upon the protein abundance of Bax.

In order to confirm whether Wnt/β-catenin signaling pathway associates with the mRNA levels of Bax, qRT-PCR was utilized. As illustrated in Fig. 3, leptin(125 ng/ml) alleviated $A\beta_{25-35}$-mediated increase of Bax, the effect of which was affected by DKK1.

GSK3 is a key component of the β-catenin destruction complex. To further understand the role of leptin, $A\beta_{25-35}$-pretreated PC12 cells were treated with leptin in the absence and presence of DKK1, then Western blot was applied. As illustrated in Fig. 4, treatment with DKK1(100 ng/ml) blunted Leptin-mediated inhibition of GSK3 protein abundance.

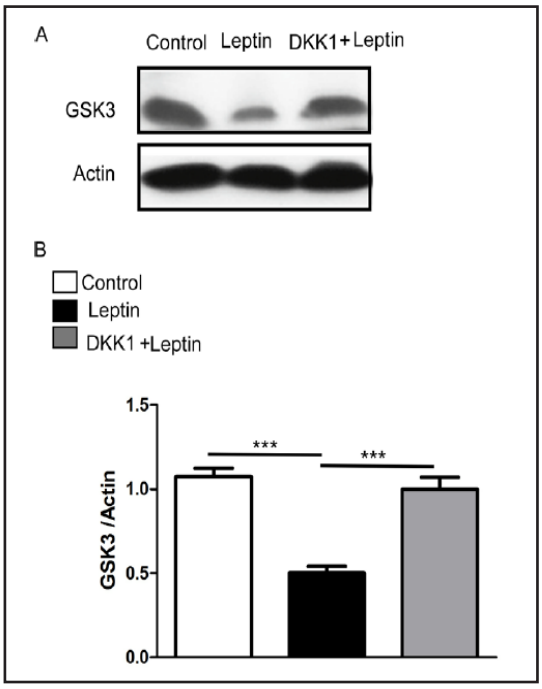

Fig. 4. GSK-3 protein abundance in $A\beta_{25-35}$-pretreated PC12 cells. (A) Original western blot showing the protein abundance of GSK-3 and respective actin in untreated and Leptin treatment(125ng/ml) PC12 cells in the presence of DKK1 (100 ng/ml) in $A\beta_{25-35}$-pretreated PC12 cells. (B) Arithmetic 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 DKK1 (100 ng/ml)(grey bar) in in $A\beta_{25-35}$-pretreated PC12 cells. ***($p<0.001$) indicate statistically significant difference.

Discussion

This study was conceived to assess the role of leptin signaling in the modulation of AD and explore the related mechanism. Leptin is known to activate Wnt in cancer cells [22], and we have previously demonstrated that Wnt/β-catenin signaling pathway blunted $A\beta_{25-35}$-mediated inhibition upon Bcl-2/Bax ratio in PC12 cells by affecting GSK3 [19, 23]. Our present findings indicate that Wnt mediates leptin's effect upon tau phosphorylation and apoptosis.

Leptin, the level of which is significantly decreased in AD patients, improves cognitive disorders and referred to as a potential cognitive enhancer [24]. Reports have shown that Leptin reduces Aβ levels both *in vitro* and *in vivo*, and inhibits tau phosphorylation in neuronal cultures [7]. Moreover, leptin is known to activate Wnt via Jak2 signaling pathway in cancer cells [22], but it is still elusive as to how leptin modulates Wnt signaling in neuronal cells. Sets of evidence point to a possible role for Wnt in AD[25-27]. Precisely, De Ferrari et al. found that Aβ-dependent neurotoxicity induced a loss of function of Wnt signaling components [25].We showed that leptin abrogated Aβ-induced tau phosphorylation and apoptosis-related protein, an effect of which is Wnt dependent.

GSK3β, a downstream effector of Wnt signaling pathway, is highly expressed in the brain of AD patients [17] and results in the over-phosphorylation of Tau and the formation of NTF [18]. It is known that Aβ promotes GSK3 activation [28]. We found that inhibition of Wnt reversed leptin-reduced GSK-3β expression in PC12 cells. These findings suggest that Leptin, and potentially Wnt activators, may provide a novel therapeutic approach to AD treatment.

Disclosure Statement

The authors state that they have no conflict of interest.

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