Original Paper

Effects of Leptin on $\text{Na}^+/\text{Ca}^{2+}$ Exchanger in PC12 Cells

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Key Words

PC12 cells • Calcium • Leptin • $\text{Na}^+/\text{Ca}^{2+}$ -exchanger

Abstract

Background/Aims: Alzheimer's disease (AD) is known to be related to alterations in neuronal intracellular calcium activity ($[\text{Ca}^{2+}]_i$). The present study revealed the distinct role of leptin in $\text{Na}^+/\text{Ca}^{2+}$ -exchanger activity. **Methods:** $[\text{Ca}^{2+}]_i$ was determined utilizing Fura-2 fluorescence. The activity of NCX was measured by removal of extracellular Na^+ in the presence of external Ca^{2+} . $\text{Na}^+/\text{Ca}^{2+}$ -exchanger activity was further quantified from whole cell currents following removal of extracellular Na^+ . $\text{Na}^+/\text{Ca}^{2+}$ -exchanger isoform NCX1 transcript levels and protein abundance were quantified by RT-PCR and Western blotting, respectively. **Results:** Exposure of PC12 cells to 30 μM amyloid ($\text{A}\beta_{42}$) increased $[\text{Ca}^{2+}]_i$, an effect significantly blunted by 6 hours incubation with leptin before $\text{A}\beta_{42}$ treatment. Moreover, leptin treatment significantly increased $\text{Na}^+/\text{Ca}^{2+}$ -exchanger mediated Ca^{2+} transport and current, NCX1 transcript level as well as NCX1 membrane protein abundance. **Conclusion:** We show that leptin blunts $\text{A}\beta_{42}$ -evoked $[\text{Ca}^{2+}]_i$ increase by increasing expression and activity of $\text{Na}^+/\text{Ca}^{2+}$ -exchanger NCX1.

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Introduction

Dysregulation of intracellular Ca^{2+} signaling has been observed as an early event prior to the presence of clinical symptoms of Alzheimer's disease (AD) and is believed to be a crucial factor contributing to the pathogenesis [1-3]. Systemic calcium concentration oscillations accompany nearly all the whole brain pathology process that is observed in AD patients, including synaptic dysfunction, mitochondrial dysfunction, $\text{A}\beta$ production and Tau phosphorylation [4]. Intracellular Ca^{2+} concentration is regulated by a variety of systems

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and mechanisms such as Ca^{2+} release from intracellular stores with subsequent stimulation of store operated calcium entry (SOCE) [5, 6] and $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCX) [7-10]. NCX is a transporter that can move sodium across the membrane in exchange for calcium [11-13]. The carrier extrudes Ca^{2+} at high intracellular Ca^{2+} concentration and at hyper-polarized cell membrane potential, whereas at high intracellular Na^+ concentration and depolarized cell membrane the carrier is reversed thus mediating Ca^{2+} entry [14, 15]. Given the ubiquitous involvement of Ca^{2+} signaling in AD, NCX may play a pivotal role in AD pathogenesis.

Alzheimer's disease (AD), the most prevalent dementia in old age [16], is characterized by two major hallmarks: the deposition and accumulation of β -amyloid ($\text{A}\beta$) peptide in extracellular plaques, the deposition of hyperphosphorylated tau in intracellular neurofibrillary tangles (NFTs) [17]. $\text{A}\beta_{42}$ oligomers promote Ca^{2+} overload and neuron cell death in aged rat hippocampal neurons [18]. Leptin, an adipocytokine produced endogenously in the brain [19-22], is decreased in AD patients [23]. Upon binding to its receptor [24], leptin leads to the activation of several intracellular signaling pathways [25] including JAK-STAT [26, 27]. JAK signaling is a candidate involved in the regulation of Ca^{2+} signalling [14, 28, 29]. It is reported that pharmacological inhibition of JAK3 decreases SOCE and NCX in MCF-7 Breast Cancer Cells [12]. Moreover, Leptin downregulates epithelial Na^+ channel (ENaC) activity [30], which in turn causes high extracellular Na^+ concentration. Sets of evidence imply that leptin might contribute to $\text{Na}^+/\text{Ca}^{2+}$ exchange. However, the distinct functions of leptin on NCX are still elusive and need further investigation.

The present study explored whether leptin modified $\text{Na}^+/\text{Ca}^{2+}$ exchange in PC12 cells. Utilizing whole cell patch clamp recording and Fura-2 fluorescence, the present observations reveal that leptin is a powerful regulator of NCX.

Materials and Methods

Cell culture

Highly differentiated neuron-like PC12 cell line (Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences, Shanghai, China) was cultured in DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin under standard conditions. Cells grown on the coverslips were treated with leptin (125 ng/ml) (Sigma Aldrich, USA) in the presence or absence of JAK2 inhibitor TG101348 (1 μM) (Axon Medchem, The Netherlands) or NCX inhibitor KB-R7943 (10 μM) (Sigma Aldrich, USA) for 6 h.

Western blotting

Total protein was prepared as follows. The 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-NCX1 (1:1000, Santa Cruz Biotech). 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). The membranes were also probed with GAPDH (1:1000, cell signaling) antibody as a loading control. Densitometric analysis was performed using quantity One software (Abbiotec, United States).

Measurement of intracellular Ca^{2+}

Fluorescence measurements were carried out with an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany). The cells were loaded with Fura-2/AM (2 μM , Biodee, China) for 30 min at 37°C. Cells were excited alternatively at 340 or 380 nm and the light was deflected by a dichroic mirror into either the objective (Fluar 40 $\times$ /1.30 oil, Zeiss, Germany) or a camera. The emitted fluorescence intensity was recorded at 505 nm and data acquisition was accomplished by using specialized computer software (Metafluor, USA). The corresponding ratios (F_{340}/F_{380}) were used to obtain intracellular Ca^{2+} concentrations.

The following equation was used: $[\text{Ca}^{2+}]_{\text{free}} = K_d \times ((R - R_{\text{min}})/(R_{\text{max}} - R)) \times S_f$ (K_d – dissociation constant of Fura-2; R – ratio of emission intensity, exciting at 340 nm, to emission intensity, exciting at 380 nm; R_{min} – ratio at zero free Ca^{2+} ; R_{max} – ratio at saturating Ca^{2+} ; S_f – instrumental constant). As a measure for the increase of cytosolic Ca^{2+} activity, the slope and peak of the changes in the intracellular Ca^{2+} concentration were calculated for each experiment.

Intracellular Ca^{2+} was examined before and after addition of $\text{A}\beta_{42}$ (2 M) (Genscript, China) to the standard solution. The changes in cytosolic Ca^{2+} activity upon removal of extracellular Na^+ were taken as evidence for $\text{Na}^+/\text{Ca}^{2+}$ exchange. N-methyl-D-glucamine (NMDG) was used to replace Na^+ . The Na^+ - standard and Na^+ - free solution contained either 5 mM or 40 mM KCl. To measure $\text{Na}^+/\text{Ca}^{2+}$ exchange the standard solution contained (in mM/l): 130 or 90 NaCl, 5 or 40 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, 5 glucose, pH 7.4. Na^+ - free solution contained (in mM/l): 130 or 90 NMDG, 5 or 40 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, 5 glucose, pH=7.4. For calibration purposes ionomycin (10 μM , Sigma-Aldrich, USA) was applied at the end of each experiment (Data not shown).

Patch clamp

The patch clamp experiments were performed at room temperature in voltage-clamp, fast-whole-cell mode. The cells were continuously superfused through a flow system inserted into the dish. The bath was grounded via a bridge filled with NaCl Ringer solution. Borosilicate glass pipettes (1-3 MOhm tip resistance; GC 150 TF-10, Clark Medical Instruments, USA) manufactured by a microprocessor-driven DMZ puller (Zeitz, Germany) were used in combination with a MS314 electrical micromanipulator (Piezopatch, China). The currents were recorded by an EPC-9 amplifier (Heka, China) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, USA). Voltage clamp steps were applied every 2 s to potentials between -100 and +50 mV from a holding potential of 0 mV. The currents were recorded with an acquisition frequency of 10 kHz and 3 kHz low-pass filtered.

To measure $\text{Na}^+/\text{Ca}^{2+}$ exchanger-mediated currents, a Na^+ -based pipette solution was used (in mM): 120 NaCl, 40 KCl, 20 TEA-Cl, 2 MgCl_2 , 2 Mg-ATP, 8 glucose, 10 HEPES (pH 7.2/CsOH) and 1 μM free Ca^{2+} . The external solution contained (in mM): 130 NaCl, 20 TEA-Cl, 2 MgCl_2 , 10 glucose, 10 HEPES (pH 7.2/CsOH) and 500 μM EGTA. $\text{Na}^+/\text{Ca}^{2+}$ exchange currents were elicited by switching to a bath solution that contained (in mM): 130 LiCl, 20 TEA-Cl, 2 MgCl_2 , 2 CaCl_2 , 10 glucose and 10 HEPES (pH 7.2/CsOH). The KCl content of the bath solutions was either 0 or 40 mM.

Quantification of mRNA expression

The total RNA was extracted from PC12 cells in TriFast (Peqlab, USA) 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, USA). 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 (Biyuntian, China) 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:

Tbp (TATA box-binding protein): forward (5'-3'): ACT CCT GCC ACA CCA GCC, reverse (5'-3'): GGT CAA GTT TAC AGC CAA GAT TCA

NCX1 [31]: forward (5'-3'): ACC ACC AAG ACT ACA GTGCG, reverse (5'-3'): TTG GAA GCT GGT CTG TCTCC

NCX2 [32]: forward (5'-3'): cga gca ctt ctt cgt gag, reverse (5'-3'): ctc cag ctc tcc aca agcat

NCX3 [33]: forward (5'-3'): GCC ATA CAC AAGAG, reverse (5'-3'): CTC TTG TGT ATGGC.

The 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, USA) 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 [34].

Statistics

Data are provided as means $\pm$ 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 accordance with previous observations [18], addition of $\text{A}\beta_{42}$ led to a rapid increase in $[\text{Ca}^{2+}]_i$ (Fig. 1A, B). In a first series of experiments, cells were incubated with leptin (50 ng/ml, 125 ng/ml) for 6 h. Acute application of $\text{A}\beta_{42}$ (2 μM) resulted in a blunted increase in $[\text{Ca}^{2+}]_i$ in leptin treated (125 ng/ml) cells compared with untreated cells (Fig. 1A, B).

Given that enhanced Ca^{2+} extrusion could have resulted in blunted increase of Ca^{2+} , we checked expression levels of $\text{Na}^+/\text{Ca}^{2+}$ exchangers NCX1-3 in PC12 cells in the presence or absence of leptin. As shown in Fig. 2 and Fig. 3, NCX1 was indeed expressed in PC12 cells. A 6 hours treatment with leptin significantly increased the NCX1 transcript level and protein abundance.

Fig. 1. Effect of Leptin on $\text{A}\beta_{42}$ -induced increase of cytosolic Ca^{2+} concentration in PC 12 cells. (A) Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded control and leptin(50 ng/ml, 125 ng/ml) 6h incubated PC12 cells before and after acute addition of $\text{A}\beta_{42}$ (2 μM). (B-C) Mean ($\pm$ SEM, $n = 60$ -62-76 cells) of the peak value (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in Fura-2/AM loaded untreated and leptin(50ng/ml, 125 ng/ml) treated cells before and after acute addition of $\text{A}\beta_{42}$ (2 μM). **($p < 0.01$), ***($p < 0.001$) indicate statistically significant difference.

Fig. 2. Effect of Leptin on NCX1 protein abundance in PC12 cells. (A) Original western blot showing the protein abundance of NCX1 as well as respective GAPDH in PC12 cells without or with Leptin (125 ng/ml) treatment for 6 h. (B) Arithmetic means $\pm$ SEM ($n = 3$ independent experiments) of NCX1 protein abundance in PC12 cells without(black bar) or with Leptin(125 ng/ml) treatment for 6 h. ** ($p < 0.01$), *** ($p < 0.001$) indicate statistically significant difference.

Fig. 3. Effect of Leptin on the transcriptional levels of NCX1 in PC12 cells. Arithmetic means $\pm$ SEM ($n = 3$ independent experiments) of mRNA levels of NCX1 in PC12 cells without(black bar) or with Leptin(125 ng/ml) treatment for 6 h. ** ($p < 0.01$), *** ($p < 0.001$) indicate statistically significant difference.

Fig. 4. Effect of Leptin on activity of $\text{Na}^+/\text{Ca}^{2+}$ exchangers in PC 12 cells. (A) Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded cells prior to and following removal of external Na^+ (0 Na^+) in the presence of 40 mM K^+ . The cells were untreated (white circles) or treated for 6 hours with leptin (125 ng/ml) in the absence (black circles) or the presence (grey circles) of KB-R7943 ($10 \mu\text{M}$) prior to the experiment. (B, C) Mean ($\pm \text{SEM}$, $n = 60\text{--}70\text{--}75$ cells) of the peak value (B) and slope (C) of the change in intracellular Ca^{2+} concentrations in Fura-2/AM loaded untreated (white bars) and leptin (125 ng/ml) treated cells in the absence (black bars) or the presence (grey bars) of KB-R7943 ($10 \mu\text{M}$) following removal of external Na^+ in the presence of 40 mM K^+ . ** ($p < 0.01$), *** ($p < 0.001$) indicates significant difference.

Fig. 5. Effect of Leptin on $\text{Na}^+/\text{Ca}^{2+}$ exchanger currents in PC12 cells. (A) Whole cell currents in PC12 cells recorded at -80 mV during the switch between external solutions that contained 40 mM K^+ and either 130 mM Na^+ and no Ca^{2+} ($130 \text{ Na}^+ 0 \text{ Ca}^{2+}$) or 2 mM Ca^{2+} and no Na^+ ($0 \text{ Na}^+ 2 \text{ Ca}^{2+}$). The internal solution stimulated Na^+ -overload and Ca^{2+} plateau levels ($1 \mu\text{M}$ free Ca^{2+} , 120 mM Na^+ , 40 mM K^+). Cesium and TEA $^+$ were present in the solutions to block K^+ channel currents. The cells were not treated (white bars) and leptin (125 ng/ml) treated (black bars) for 6 h prior to the experiment. (B) Mean ($\pm \text{SEM}$, $n = 9\text{--}20$ cells) current density changes (ΔI , pA/pF) in PC12 cells at -80 mV induced by the switch between external solutions containing 40 mM K^+ and either 130 Na^+ , 0 Ca^{2+} and 0 Na^+ , 2 Ca^{2+} . The cells were not treated (white bars) and leptin (125 ng/ml) treated (black bars) for 6 h. ** ($p < 0.01$), *** ($p < 0.001$) indicates significant difference.

To measure $\text{Na}^+/\text{Ca}^{2+}$ exchangers activity, Fura-2 fluorescence has been used for the determination of $[\text{Ca}^{2+}]_i$. As illustrated in Fig. 4, leptin significantly augmented the $[\text{Ca}^{2+}]_i$ following removal of extracellular Na^+ in PC12 cells. The up-regulation of NCX activity by leptin was blocked when cells were incubated together with NCX blocker KB-R7943 ($10 \mu\text{M}$) (Fig. 4B).

In an additional series of experiments, whole cell currents were recorded in PC12 cells elicited by removal of extracellular Na^+ and addition of extracellular Ca^{2+} ($0 \text{ Na}^+ 2 \text{ mM Ca}^{2+}$). As illustrated in Fig. 5, the current generated by removal of extracellular Na^+ was significantly higher in leptin (125 ng/ml) treated cells than in untreated cells.

Leptin leads to the activation of JAK2. Finally, we checked the NCX1 protein abundance in PC12 cells with leptin treatment in the presence of JAK2 inhibitor TG101348 ($1 \mu\text{M}$) for 6 h. As

Fig. 6. JAK2 inhibitor abolished Leptin-induced NCX1 protein abundance in PC12 cells. (A) Original western blot showing the protein abundance of NCX1 and GAPDH in PC12 cells with leptin (125 ng/ml) treatment in the presence of TG101348 (1 μM). (B) Arithmetic means $\pm$ SEM (n = 4 independent experiments) of the protein abundance of NCX1 in PC12 cells with leptin (125 ng/ml) treatment in the presence of TG101348 (1 μM). *** (p < 0.001) indicate statistically significant difference.

illustrated in Fig. 6, TG101348 significantly blunted leptin-induced NCX1 levels in PC12 cells.

Discussion

The present study confirms previous finding [18] that exposure of hippocampal neurons to $\text{A}\beta_{42}$ oligomers promotes Ca^{2+} overload. Our experiments disclose a novel effect of leptin upon Ca^{2+} signaling in PC12 cells. As shown in the experiments, leptin blunts the increase of cytosolic Ca^{2+} concentrations following $\text{A}\beta_{42}$ treatment and increases the protein level of NCX. Moreover, JAK2 inhibitor abolishes the effect of leptin on the NCX. We provide evidence that NCX activity is regulated by leptin through JAK2 signaling (Fig. 7).

Calcium is associated with many facets of neuronal physiology and plays a pivotal role in the pathogenesis of

neurodegenerative diseases. Despite intense research suggest that disturbances in calcium homeostasis is involved in AD, much more attention was focused on amyloid and tau hyperphosphorylation that constitute the hallmarks of AD [35]. Nevertheless, accumulated evidence have shown that dysregulation of calcium plays a central role in AD pathogenesis. Perturbation of calcium balance has deleterious consequences for cells and in particular for neurons, leading to necrosis and/or apoptosis and subsequently to stroke and neurodegeneration [4, 36, 37]. Moreover, different calcium channel blockers have been reported to be effective in preventing long/short-term memory impairment induced by $\text{A}\beta_{25-35}$ [38]. Individuals with AD have a down regulation of the expression of the Ca^{2+} buffering protein calbindin which aids in restricting the Ca^{2+} amplitude thus regulating Ca^{2+} signalling [39]. In addition, activated $\text{A}\alpha_{42}$, a specific amyloid plaque, carries out Ca^{2+} cellular responses allowing Ca^{2+} influx that induces excitotoxicity [40]. Given the ubiquitous involvement of Ca^{2+} dysregulation in AD, it logically presents a variety of potential therapeutic targets for AD prevention and treatments. $\text{Na}^+/\text{Ca}^{2+}$ exchangers serve to terminate the increases of the cytosolic Ca^{2+} concentration [11]. At least in theory, increased NCX activity contributes to preventing intracellular Ca^{2+} from being overload, thereby alleviating neurotoxicity. Moreover, NCX has been shown to be involved in neurodegeneration of both retinal ganglion cells [41] and spinal cord axons [42].

Fig. 7. Leptin regulates NCX activity via JAK2 signaling.

Leptin, the level of which is significantly decreased in AD patients, improves cognitive disorders and is referred to as a potential cognitive enhancer [43]. Moreover, leptin exerts its neurotrophic effects and neuroprotective activity by slowing down neuronal damage after acute brain injuries as well as during long-term neurodegenerative processes [44]. Additionally, recent study has found that Leptin decreases $\text{A}\beta_{42}$ -induced tau phosphorylation and apoptosis via JAK2 signaling [45]. According to our observations, leptin upregulates the activity of $\text{Na}^+/\text{Ca}^{2+}$ exchangers by increasing transcription and the membrane protein abundance of the carrier, effects of which are abolished by JAK2 inhibitor. The enhanced $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity can in turn facilitate the extrusion of Ca^{2+} and thus blunt the increase of Ca^{2+} concentration following the addition of $\text{A}\beta_{42}$.

In conclusion, the present study shows that leptin stimulates the expression and consequently the activity of $\text{Na}^+/\text{Ca}^{2+}$ exchangers in PC12 cells. Moreover, upregulation of cell viability by leptin is, at least in part, NCX dependent. Given the pivotal role of Ca^{2+} in all respects of cells live, the stimulation of Ca^{2+} extrusion likely plays an important role in the known neuroprotective effect of leptin.

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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