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Effects of leptin on norepinephrine in acute ischemic stroke

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Stroke is a multifactorial disease and a consequence of morbidities of diabetes, obesity, hypertension, and heart diseases. Leptin is a major adipokine that regulates weight balance and energy homeostasis, the level of which has been considered as an indicator of acute ischemic stroke. In the present study, we confirmed the high level of leptin and noradrenaline in stroke patients and mouse models as well as oxygen-glucose deprivation (OGD) primary cerebral neurons. Leptin administration increased noradrenaline concentration and dopamine β-monooxygenase (DBH) but decreased noradrenaline transporter (NET) expression in primary cerebral neurons. Moreover, induced noradrenaline concentration, DBH activity, and inhibited NET were blunted by TG101348 (JAK2 inhibitor). JAK2 silencing also abolished the effects of leptin on noradrenaline metabolism.

1. Introduction

Leptin, an adipokine hormone secreted by adipocytes, plays a key role in the neuroendocrine function and metabolic processes. High circulating leptin levels increase the risk of many diseases including vascular diseases. Stroke, a consequence of morbidities of diabetes, obesity, hypertension, and heart diseases, has also been reported to be associated with a high level of leptin despite some controversial opinions. Some researchers report that high circulating level of leptin contributes to the risk of stroke (Kim et al. 2012; Soderberg et al. 2003, 2004; Gairolla et al. 2017; Liu et al. 2010), but other studies claim that no such association exists (Rajpathak et al. 2011; Prugger et al. 2012).

It is well documented that leptin regulates immune responses (Yan et al. 2018) and potentiates platelet aggregation (Nakata et al. 1999; Corsonello et al. 2003). Sympathetic activity facilitates coagulation and induces thrombotic events such as acute ischemic stroke (AIS) by activating platelets and inhibiting the fibrinolytic system (Yun et al. 2005). There might be a link between leptin and the sympathetic system in stroke. Jochem et al. (2016) reported that there were higher plasma concentrations of noradrenaline after leptin treatment for twenty minutes in rats. However, studies have shown that leptin lowers noradrenergic neurotransmission in the paraventricular nucleus and inhibits hypothalamic noradrenaline (NE) release after stress (Kawakami et al. 2008; Kutlu et al. 2010).

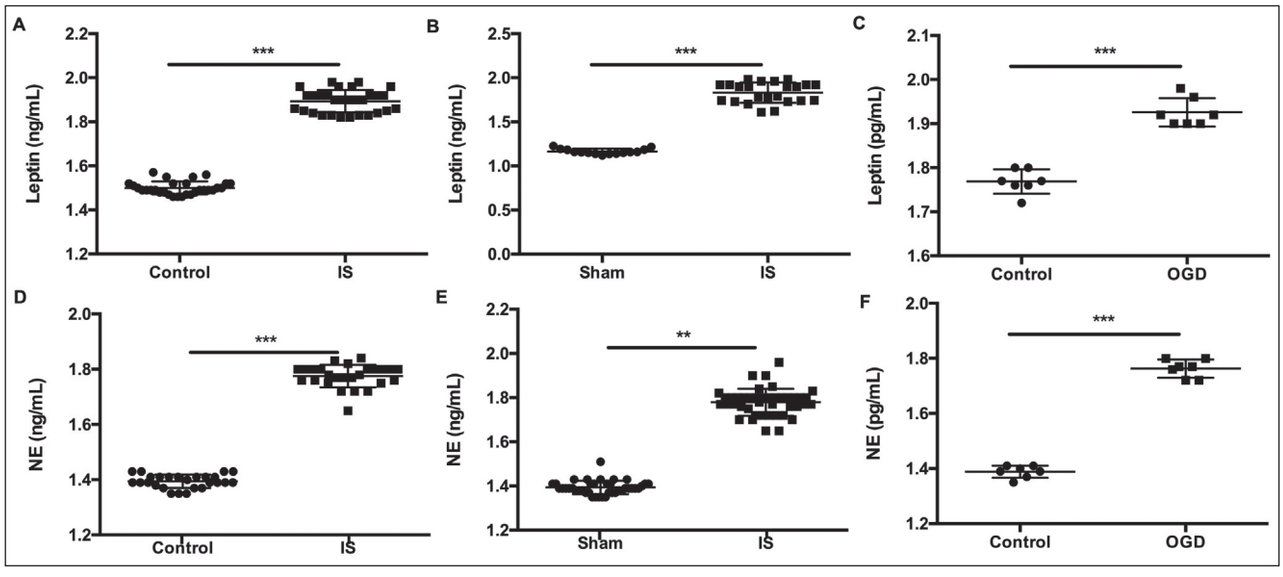

Fig. 1: Enhanced leptin and NE levels in acute ischemic stroke. A: Arithmetic means±SEM (n=40) showing leptin concentration in acute ischemic stroke patients and healthy subjects.B: Arithmetic means±SEM (n=12) showing leptin concentration in acute ischemia stroke mouse models. C: Arithmetic means±SEM (n=5) showing leptin concentration in cerebral neurons after OGD treatment.D: Arithmetic means±SEM (n=40) showing NE concentration in acute ischemia stroke patients and healthy subjects. E: Arithmetic means±SEM (n=12) showing NE concentration in acute ischemic stroke mouse models.F: Arithmetic means±SEM (n=5) showing NE concentration in cerebral neurons after OGD treatment.***($p<0.001$) indicate statistically significant difference.

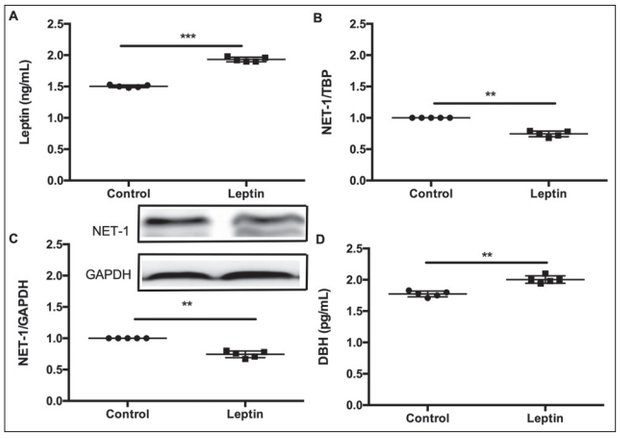

Fig. 2: Leptin increased NE levels in cerebral neurons. A: Arithmetic means±SEM (n=5) showing NE concentration in cerebral neurons after leptin treatment. B: Arithmetic means±SEM (n=5) showing the NET mRNA level in cerebral neurons after leptin treatment.C: Original western blot and arithmetic means±SEM (n=5) showing the level of NET-1 in cerebral neurons with leptin treatment. D: Arithmetic means±SEM (n=5) showing DBH activity in cerebral neurons after leptin treatment.***(p<0.001) indicate statistically significant difference

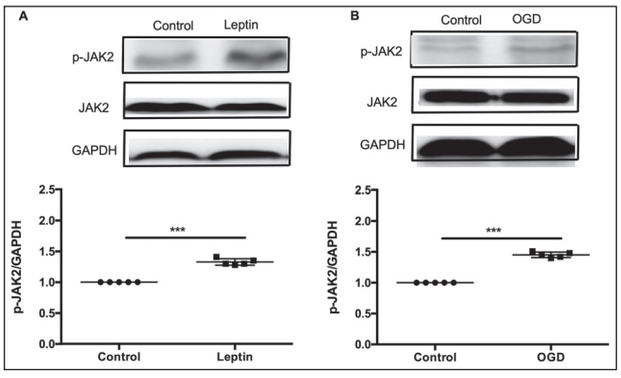

Fig. 3: Leptin and OGD increased JAK2 phosphorylation. A: Original western blot showing the level of p-JAK2 in cerebral neurons with leptin or OGD treatment. B:Arithmetic means±SEM (n=5) showing the level of p-JAK2 in cerebral neurons with leptin or OGD treatment.**(p<0.01), ***(p<0.001) indicate statistically significant difference

Given the role of leptin and the sympathetic system in the pathological aspects of ischemic stroke (IS), it might be beneficial to ascertain the relationship between them in view of IS therapy.
In the present study, we confirmed the elevated levels of leptin and NE and explored the relationship between them. Acute ischemic stroke mouse models and oxygen-glucose deprivation (OGD) were established to mimic the disease. The metabolism of NE including NE synthesis and re-uptake was explored after leptin treatment.

2. Investigations and results

2.1. Increased leptin and NE contents in acute ischemic stroke

Elisa was performed to examine NE and leptin contents in AIS. We collected AIS patient's serum, established AIS mouse models and performed OGD treatment in primary cerebral neurons. As shown in Fig. 1A-C, leptin concentration was significantly elevated in patients and models. Similarly, NE levels were also increased in AIS (Fig. 1D-F).

2.2. Leptin contributed to NE metabolism in primary cerebral neurons

To ascertain the mechanism by which leptin regulates NE concentration, the NE synthesizing enzyme (DBH) and NET were exam-

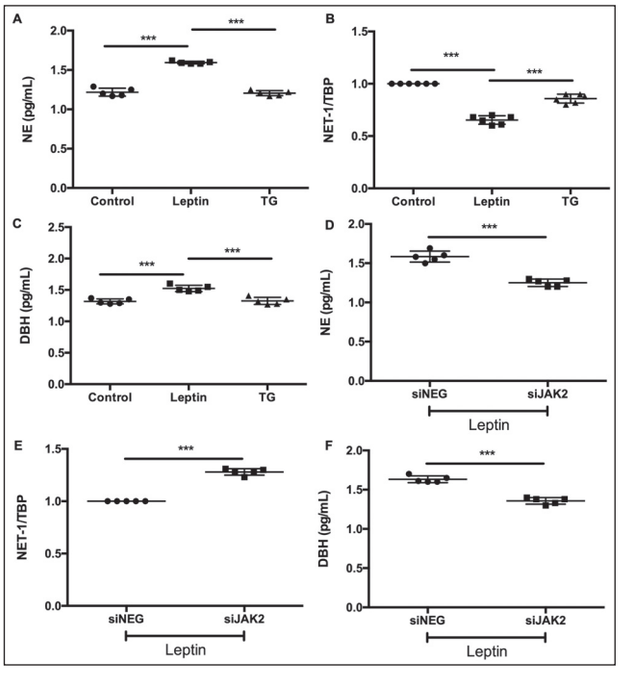

Fig. 4: Effects of leptin on cerebral neurons were JAK2 dependent. A. Arithmetic means±SEM (n=5) showing NE contents in cerebral neurons with leptin treatment in the presence of TG101348 (1 μM) for 12 h. B: Arithmetic means±SEM (n=5) showing NET mRNA level in cerebral neurons with leptin treatment in the presence of TG101348 (1 μM) for 12 h. C: Arithmetic means±SEM (n=5) showing DBH activity in cerebral neurons with leptin treatment in the presence of TG101348 (1 μM) for 12 h. D: Arithmetic means±SEM (n=5) showing NE contents in siNEG and siJAK2 cerebral neurons.E: Arithmetic means±SEM (n=5) showing NET mRNA level in siNEG and siJAK2 cerebral neurons.F: Arithmetic means±SEM (n=5) showing DBH activity in siNEG and siJAK2 cerebral neurons.***(p<0.001) indicate statistically significant difference

ined by Elisa and RT-PCR. As illustrated in Fig. 2A, leptin administration significantly increased NE concentration. Moreover, NET-1 was inhibited by leptin (Fig. 2B, C) both at the protein and the mRNA level. DBH activity was elevated under leptin treatment (Fig. 2D). Therefore, we propose that leptin enhances NE synthesis and inhibits NE re-uptake, resulting in elevated NE levels.

2.3. Effects of leptin are JAK2 dependent

Firstly, western blot showed that both OGD and leptin treatment augmented the phosphorylation of JAK2 (Fig. 3), suggesting the possible involvement of JAK2 in the process. Secondly, leptin-regulated NE metabolism was blunted by TG101348 (1 μM) (Fig. 4A-C): increased NE contents and DBH activity, inhibited NET expression were all blunted by TG. Thirdly, these effects of leptin on NE metabolism were absent in siJAK2 neurons (Fig. 5A-C).

3. Discussion

In the present study, we confirmed the high level of NE and leptins in AIS patients and models, and examined the relationship between leptin and NE. Leptin treatment enhanced DBH activity whereas inhibited NET expression, hence resulting in a high level of NE content in primary cerebral neurons. Notably, both leptin and oxygen-glucose deprivation (OGD) increased the phosphorylation of JAK2, suggesting the possible involvement of JAK2 in IS. To further confirm the role of JAK2 in leptin-regulated NE synthesis and re-uptake, JAK2 was inhibited and silenced. Expectedly, the effects of leptin on NE metabolism were abolished by TG101348 and also absent in siJAK2 cerebral neurons.
Whether the serum leptin level accounts for a high risk of stroke has been debated. Given the importance of sympathetic activity during AIS (Yun et al. 2005), instead of determining the controver-

sial question we examined the effects of leptin on AIS-induced NE levels. Normally NE concentration is much higher in stroke patients than in healthy subjects. Though there are lots of reports about the reason why NE level is increased, the relationship between leptin and NE has been unknown so far. NE is synthesized by DBH, and uptaken via NET. We found that leptin increased DBH activity while inhibits NET expression, indicating a positive role of leptin on the sympathetic system. We conclude that high leptin levels in AIS contribute to the activation of the sympathetic system, hence exacerbating the pathological process of IS. Therefore, leptin is suggested to be a potent target for AIS therapy.

Leptin activates the JAK/STAT signaling pathway by binding to its receptor LEP-R(OB-R). Activation of the JAK2-STAT3 signaling pathway has been reported in cerebral ischemia (Zhang et al. 2018). We confirmed that p-JAK2 levels are much higher in the OGD group which mimics AIS circumstance. Moreover, silencing JAK2 blocked the effects of leptin on NE in cerebral neurons.

In conclusion, the high circulating NE level in IS induces NE synthesis and inhibits NE uptake by activating JAK2.

4. Experimental

4.1. Patient samples

A total of 60 Patients with ischemia stroke between 2016 and 2018 at Changzheng Hospital were chosen randomly for this study. All untreated patients were diagnosed with BC histologically. Written informed consent was obtained from all patients who participated in the study and the study was approved by Jingjiang Hospital Ethics Review Board. Blood Samples were collected from patients, and then serum was separated after centrifugation at 2800 x g for 8 min and stored at -80 °C.

4.2. Focal cerebral ischemia model

Mice (male, 20g) were anesthetized by 1.5 % isoflurane. Briefly, the animals were placed in a stereotaxic frame, a scalp incision was made and the right temporal muscle was dissected. The area between zygomatic arch and squamous bone was thinned by a high-speed drill and cooled with saline. MCA trace was visualized and a thin bone film over MCA was lifted up. Then a Laser-Doppler flowmetry probe was placed 2 mm posterior, 6 mm lateral to the bregma to monitor the regional cerebral blood flow (rCBF). After obtaining a stable epoch of the pre-ischemic rCBF, a piece of $FeCl_3$ saturated filter paper was placed over the intact dura mater along the trace of MCA and the rCBF was continuously monitored (Karatas et al. 2018).

4.3. Cell culture and transfection

Primary cerebral cultures were prepared from C57 mice (4~5 weeks, 18 g) (Beijing Vital River Laboratory Animal Technology Co., Ltd.). Briefly, collected neurons were seeded in a concentration of 1×10^6 cells/cm^2. Cells were cultured for six days in medium, consisting of Eagle's minimum essential medium containing 10 mm sodium bicarbonate, 1 % glucose, 1 mm l-glutamine, 20 mm KCl, 1 mm sodium pyruvate, and 10 % (v/v) heat-inactivated fetal bovine serum (Sigma). After 24 h in culture, the culture medium was replaced with neurobasal medium containing B27 supplements (Invitrogen) in a humidified atmosphere (5 % CO_2, 95 % Oxygen) at 37 °C. Approximately ten hours later, 5 μM cytosine arabinoside was administered to the dishes to prevent the growth of non-neuronal cells (Yan et al. 2016a).

For silencing, neurons were transfected with 5 μl/1000 μl ON-TARGETplus MOUSE JAK2 siRNA (5 μM, Thermo Fisher Scientific, USA) and ON-TARGETplus Non-targeting siRNA (5 μM, Thermo Fisher Scientific) using the cationic lipid DharmaFECT 1 transfection reagent (0.5 μl/1000 μl, Thermo Fisher Scientific) according to the manufacturer's protocol. Cells were collected 24 h after transfection. To confirm silencing efficiency, transcript levels were quantified. As a result, the JAK2 mRNA level were 0.57±0.04 (n=11) in siNEG cells bute 0.11±0.13 (n=11) in si JAK2 cells. Cells were treated with TG101348(1 μM)(JAK2 inhibitor) (Yan 2016b) for 12 h.

4.4. Oxygen-glucose deprivation exposure

Primary cerebral neurons were treated with an experimental medium lacking glucose (EM-G) and containing NaCl (120 mM), KCl (25 mM), $MgSO_4$ (0.62 mM), $CaCl_2$ (1.8 mM), HEPES (10 mM) and placed in a humidified hypoxic chamber. The chamber was then sealed and placed in a thermostat at 37 °C for 4 h.

4.5. Western Blot

Cells were washed twice in ice-cold PBS. RIPA lysis buffer (Cell Signaling, United States) containing phosphatase and protease inhibitor cocktail tablet (Thermo Fisher Scientific, United States) was added to the washed cells. The samples were incubated on ice for 30 min and then centrifuged at 14,000 rpm and 4 °C for 20 min. The supernatant was removed and used for Western blotting. Total protein (40 μg) was separated by SDS-PAGE, transferred to PVDF membranes and blocked in 5 % non-fat milk/Tris-buffered saline/Tween-20 (TBST, PH 7.4) at room temperature for 30 min. Membranes were incubated overnight at 4 ºC with p-JAK2 (Tyr1007/1008) (Abcam, 1:50), JAK2 (Abcam, 1:200), NET-1 /Actin antibodies (Sigma, United States, 1:1000). After incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma, United States, 1:1000) for 2 h at room temperature, the bands were visualized with enhanced chemiluminescence reagents (Sigma, United States). Densitometric analysis was performed using quantity One software.

4.6. qRT-PCR

Total RNA was extracted 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 s, 58 °C for 15 s and 68 °C for 20 s. For amplification the following primers were used (5`>3`orientation): The following primers were used:

GAPDH (TATA box-binding protein)(8):
forward (5'-3'): CGGAGTCAACGGATTTGGTCGTAT
reverse (5'-3'): AGCCTTCTCCATGGTGGTGAAGAC
NET-1
forward (5'-3'): GTTGCTCTGCCCTGTGTTTG
reverse (5'-3'): CAAACACAGGGCAGAGCAAC
DBH
forward (5'-3'): CGAGGAGAGATGGAGAACGC
reverse (5'-3'): GCGTTCTCCATCTCTCCTCG

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. GAPDH was amplified to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the ΔCT method as described.

4.7. ELISA

ELISA assay was applied to detect NE and DBH level. For NE and DBH detection, plasma samples from patients and models as well as neurons were collected. NE and DBH concentration in plasma was determined via ELISA according to the manufacturer´s instructions (Elabscience, Wuhan, China). The spectrophotometry of panels were read at 450 nm and calculated according to the standard curve.

4.8. Statistics

Data are provided as means±SEM, n represents the number of independent experiments. All data were tested for significance using one-way 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.

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Authors' contributions: PT, ZJ, GS-H performed experiments; PS-J and JJ-P analyzed data; LW-Q, CW-S designed the project and drafted the manuscript. All authors corrected and approved the manuscript.

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