

Enhanced Hexokinase 2 Expression and Lactate Production Promote The Apoptosis of Dopaminergic Neurons in Parkinson's Disease

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Research

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Abstract

Background: Parkinson's disease (PD) is characterized by impaired mitochondrial function and decreased ATP levels. Glycolysis is upregulated and lactate production is enhanced in PD. Since lactate promotes apoptosis and α -synuclein accumulation in neurons, we hypothesized that the increased lactate resulted from upregulated glycolysis is involved in the apoptosis of dopaminergic neurons in PD.

Methods: We examined the expression of hexokinase 2 (HK2) and lactate dehydrogenase (LDH), the key enzymes in glycolysis, and lactate levels in the substantia nigra pars compacta (SNpc) of MPTP-induced mouse model of PD and in MPP⁺-treated SH-SY5Y cells. We investigated the role of HK2, lactate and AMPK pathway in the apoptosis of dopaminergic neurons by intervened with 3-Brpa, the HK2 inhibitor, in in vivo and in vitro systems.

Results: We found that the expression of HK2 and LDHA, and lactate levels were markedly increased in brain SNpc of MPTP-treated mouse and in MPP⁺-treated SH-SY5Y cells. Meanwhile, the apoptosis of dopaminergic neurons in the mouse model and the apoptosis of the SH-SY5Y in vitro system were increased. Intriguingly, using HK2 inhibitor or siRNA can decrease the lactate levels and suppressed the apoptosis of dopaminergic neurons both in vivo and in vitro. Mechanistically, lactate increased the activity of adenosine monophosphate activated protein kinase (AMPK), and suppressed the phosphorylation of serine/threonine kinase 1 (Akt) and mammalian target of rapamycin (mTOR).

Conclusion Inhibition of HK2 ameliorate the apoptosis of dopaminergic neurons through downregulating the lactate production and AMPK/ Akt/ mTOR pathway activation in PD.

Background

Parkinson's disease (PD) is one of the most common neurodegenerative diseases affecting nearly 6 million people around the world [1]. Aging, genetic alterations and environmental factors contribute to the development of PD. PD is characterized by motor symptoms including tremors, bradykinesia and movement disorders, and non-motor symptoms including cognitive decline [2]. The pathologic features of PD are α -synuclein accumulation and apoptosis of dopaminergic neurons [3].

Glycolysis refers to the process by which glucose is broken down into pyruvate in the cytoplasm under aerobic conditions. It is the preferred metabolic pathway for highly proliferating cells such as cancer cells to get ATP faster and is called the "Warburg effect" [4]. It has been reported that the expression of HIF-1 α (Hypoxia-inducible factor 1 α), a master activator of glycolysis, and HK2 were increased in the SN of sporadic PD patients compared to control subjects [5]. Recently, it was reported that the glycolysis was enhanced in mid-brain of PD patients using 12FDG-PET [6]. In addition, the glycolysis of peripheral monocytes was found increased in early-onset PD [7]. However, the role of enhanced glycolysis in PD pathogenesis remains largely unknown.

As the product of glycolysis, lactate is an important bioenergetic metabolite. Recently, abnormally elevated lactate levels have been reported in cerebrospinal fluid of late-onset PD patients [8]. Lactate levels were also increased in aging brains and Alzheimer disease (AD) [9, 10]. Appropriate lactate level in dopaminergic neurons can be used as energy substrate. However, increased lactate accumulation impaired adult hippocampal neurogenesis in AD [11]. In addition, lactate accumulation was found to be associated with apoptosis of cerebellar granule cells in the early phase in AD [12]. Moreover, lactate promotes α -synuclein accumulation in dopaminergic neuroblastoma cells line [13]. Therefore, we hypothesized that increased lactate production resulted from upregulated glycolysis may contribute to the apoptosis of dopaminergic neurons in PD.

In this study, we measured the expression of HK2 and LDHA in MPTP-induced mouse model and MPP⁺-treated SH-SY5Y cell model of PD. Western blot and immunostaining results revealed that HK2 and LDHA were enhanced in dopaminergic neurons in in vitro and in vivo systems, which promoted the lactate accumulation and apoptosis of dopaminergic neurons by activating AMPK/Akt/mTOR pathway. We also demonstrated that inhibiting the HK2 alleviated the lactate accumulation and apoptosis of dopaminergic neurons and ameliorate the motor dysfunction in animals in PD.

Materials And Methods

Animals

C57BL/6 mice were purchased from Beijing HFK Bioscience (Beijing, China). All the animal work presented has been approved by the Institutional Animal Care and Use Committee of the Tongji Medical College and followed NIH guidelines for the care and use of laboratory animals. All mice were housed in a specific pathogen-free animal facility at the Tongji Medical College on a 12/12 h light/dark cycle.

In vivo mouse model of MPTP

C57BL/6 mice (8–10 weeks old) were treated with 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP, 2.5mg/kg, Sigma) by intra-peritoneal injection for 5 days. Animals motor behavior were analyzed 1 week after the last injection. Mice were decapitated and the brain SNpc region were removed for WB, qPCR, immunofluorescence analysis or metabolomics related test. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Tongji Medical College following NIH guidelines.

Pole test and Rotaroad test

Behavior tests were evaluated 7 days after MPTP injection. Mice were trained to climb the rotaroad and pole 2 days before the test. For rotaroad test, the angular speed (RPM) is 20 RPM. Animals with impaired motor coordination fall off quickly and the endurance time of each animal was calculated. For pole test, a 50 cm length, 1 cm tip diameter of the vertical wooden with a cork in the top was used. Animals were placed on the cork, and the time for the mouse to climb from the cork to the low end was calculated.

Cell Culture and Treatment

Human SH-SY5Y cells were cultured in 1:1 mixture of Dulbecco's Modified Eagle Medium and F12 medium (DMEM/F12; Gibco, Thermo Fisher Scientific, Suzhou, China) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Grand Island, USA). Cells were treated with MPP⁺ iodide (MPP⁺, 2mmol, Sigma, USA) at a final concentration of 2 mmol for 48h. Specific siRNA sequences, targeting human *HK2* gene (*HK2* siRNA: ACGACAGCATCATTGTAA), were transfected in SH-SY5Y cells by using the lipofectamine 3000 reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. A non-targeting siRNA sequence (Thermo Fisher Scientific, USA) was used as a negative control. SH-SY5Y was exposed to MPP⁺ in 6-8h after siRNA transfection. 5nm HK2 inhibitor 3-Bromopyruvic acid (3-Brpa, MedChemExpress, USA) or 2mM MPP⁺ were used for treatment. SH-SY5Y cells were exposed to 3-Brpa together with MPP⁺. Cells were harvested after MPP⁺ treatment for 48h. 15mM L-(+)-lactic acid (L-lac, sigma, USA) treated SH-SY5Y was collected after 24h incubation in the incubator. Cells had been plated 24 h earlier at a concentration of 1×10^6 cells per well.

Cell Viability Assay

Cell viability was detected using the CCK8 assay system (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, USA). Briefly, SH-SY5Y cells transfected *HK2* siRNA, negative control siRNA or intervened with 3-Brpa were treated with or without 2 mmol MPP⁺ for 48 h. Medium was discarded after incubation for 48 h and replaced with 100 μ l of medium containing 10 μ l of CCK8 reagent per well and incubated at 37°C for 3 h. The absorbance at 450 nm was measured using an ELX800 microplate reader (Bio-Tek, Winooski, VT, United States). The values of absorbance were calculated as percentage of the control group.

Flow Cytometry Assay

Cell apoptosis was analyzed using the Annexin V-FITC/PI-Percp apoptosis detection kit (Vazyme, Jiangsu, China). Briefly, SH-SY5Y cells were treated and incubated in incubator. Then, both attached and detached cells were harvested and resuspended with 200 μ l of binding buffer, 5 μ l Annexin V-FITC and 5 μ l PI-Percp were added to the mixtures respectively. After staining for 15 min in the dark, flow cytometry was performed with FACS Calibur (Becton, Dickinson and Company, USA). Cells that were negative for PI and positive for Annexin V were identified as early apoptotic cells, and cells that were positive for PI and Annexin V were identified as late apoptotic cells.

Quantitative PCR

Total RNA was extracted from circulating leukocytes using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using HiScript III RT SuperMix for qPCR (+g DNA wiper) (Vazyme, Jiangsu, China) according to the manufacturer's instructions. Quantitative PCR was performed using ChamQ SYBR Color qPCR Master Mix (Without ROX)

(Vazyme, Jiangsu, China) as the instructions in Biorad CFXTM Real-Time PCR detection system. The primer sequences for real-time PCR are listed in Table S1.

Protein extraction, and western immunoblot

Cell lysates were prepared using the radio-immunoprecipitation assay (RIPA) buffer (Servicebio, Wuhan, China) containing a protease inhibitor cocktail (Roche, IN, USA). Western blot analysis of target proteins was conducted as described using appropriate primary antibodies, followed by probing to the corresponding conjugated secondary antibody. The reactive bands were visualized using ECL plus reagents (Servicebio, Wuhan, China), and the relative intensities of each band were analyzed using the Image J 1.46r software. The bands were incubated with following primary antibodies: GAPDH antibody (1:10000, Proteintech, 60004-1-Ig), anti-HK2 antibody (1:1000, abcam, ab209847), anti-TH antibody (1:5000, abcam, ab137869), anti-LDHA antibody (1:1000, Cell signaling technology, 3582S), anti-CD36 antibody (1:500, Proteintech, 18836-1-AP), anti-Ppar- γ antibody (1:500, Proteintech, 16643-1-AP), anti-Bax antibody (1:1000, abcam, ab32503), anti-Bcl2 antibody (1:1000, abcam, ab32124), anti-AMPK α 1/2 (H-300) antibody (1:500, Santa cruz Biotechnology, sc-25792), anti-Phospho-AMPK α 1/2 (Thr 183/172) antibody (1:500, Santa cruz Biotechnology, sc-101630), anti-Phospho-Akt (Thr308) antibody (1:1000, Cell signaling technology, 4056S), anti-Akt antibody (1:1000, Cell signaling technology, 9272S), anti-Phospho-mTOR (Ser2448) antibody (1:1000, Cell signaling technology, 5336S), anti-mTOR (7C10) antibody (1:1000, Cell signaling technology, 2983S).

Immunofluorescence analysis

The brain was fixed by intra-heart infusion of 4% aqueous buffered formalin. Midsagittal slices from the SNpc were processed for paraffin embedding and sliced into 4- μ m sections. After dewaxing and repairing the antigen, the slides were co-incubated with primary antibodies against anti-HK2 antibody (1:100, abcam, ab209847), anti-TH antibody (1:100, Santa cruz Biotechnology, sc-25269), anti-LDHA antibody (1:200, Cell signaling technology, 3582S), anti-GFAP antibody (1:100, Cell signaling technology, 80788S), anti-Iba1 antibody (1:100, Santa cruz Biotechnology, sc-32725), followed by probing with Alexa 594-labelled or Alexa 488-labelled secondary antibodies (Invitrogen). The tissue slides were assessed by fluorescence microscope (Olympus) in a blinded fashion. Negative controls were prepared by omitting the primary antibody. The percentage of HK2⁺TH/GFAP/IBA1⁺ double positive cells was calculated by the amount of HK2⁺TH/GFAP/IBA1⁺ double positive cells compared to the TH/GFAP/IBA1⁺ single positive cells in each section. The percentage of LDHA⁺TH⁺ double positive cells was calculated by LDHA⁺TH⁺/TH⁺ ratio in each section.

TUNEL assay

One Step TUNEL Apoptosis Assay Kit (Beyotime Biotechnology, Shanghai, China) was used to determine the apoptosis in tissue section according to the manufacturer's instructions. The assay is based on the green fluorescent probe fluorescein (FITC) labeled dUTP (fluorescein-dUTP). After dewaxing and repairing

the antigen, the slides were treated with 20µg/ml proteinase K without DNase for 15min in 37°C. 2.5µl TdT enzyme, 22.5µl fluorescent labeling solution, 25µl TUNEL test solution were added in each tissue sample in the section and incubated for 1h in the dark in 37°C. The tissue slides were assessed by using fluorescence microscope (Olympus) in a blinded fashion. The FITC labeled positive cells were calculated as apoptotic cells.

Lactate and pyruvate production assay:

Lactate and pyruvate fluorometric assay kit (Jiancheng Bioengineering Institute, Nanjing, China) was used to determine lactate and pyruvate levels according to the manufacturer's instructions. The assay is based on the reduction of NAD⁺. Briefly, the culture media of SH-SY5Y was collected. 10 µl medium was collected from each sample and incubated with reaction mix for 30 min at 37°C. The fluorescence was detected at 510nm (Lactate assay) or 530nm (Pyruvate assay) using a plate reader (BioTek). Lactate and pyruvate levels were normalized to protein content in each sample.

Hexokinase activity

Hexokinase activity assay was performed as manufacturer's instructions. The assay is based upon the reduction of nicotinamide adenine dinucleotide (NADH) through a coupled reaction with glucose-6-phosphate dehydrogenase and is determined spectrophotometrically by measuring the increase in absorbance at 340 nm. 150µl reaction mixes was loaded in each well of 96-well microplate. The plate was incubated at room temperature for 15 min to achieve temperature equilibration. 20µl whole-cell lysates sample or positive control were added to start the reaction. Hexokinase solution prepared by dissolving hexokinase (Sigma-Aldrich) in 0.05 M Tris-HCl buffer, pH 8.0, served as positive control. Assay buffer without sample served as negative control. Absorbance (A) at 450 nm was recorded for 30 min and 1 hour. The absorbance from initial linear portion of the curve was used to calculate the enzyme activity. The protein concentration was test by BCA assay (Beyotime Biotechnology, Shanghai, China) to normalize the results.

ATP assay

SH-SY5Y cells are cultured in 12-well plate, treated with MPP⁺ and/or 3-Brpa, incubated for 48h. Medium was discarded after incubation, and placed with 100µl lysate (Beyotime Biotechnology, Shanghai, China) to lyse the cells. Repeatedly pipetting or shaking the culture plate when lysing the cells to make the lysate fully contact and lyse the cells. After lysis, cell lysates were centrifuged at 12000g at 4°C for 5 minutes, and the supernatant was collected for subsequent determination. And 100µl ATP detection working solution was added to the detect in each hole, then leave it at room temperature for 5 minutes, so that all the background ATP is consumed, thereby reducing the background. After 5 minutes, 20µl of sample or standard sample was added to the detect the concentration in each hole, at least 2 seconds later, the RLU value was measured by using an ELX800 microplate reader (Bio-Tek, Winooski, VT, United States).

Statistical analysis

All in vitro experiments were conducted with at least three independent biological replications. When only two experimental groups were compared, the unpaired t-test was used. Multiple comparisons were treated by one-way ANOVA followed by Tukey's test. Statistical analysis of the data was conducted using the GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA), and the results were expressed as mean \pm SEM. In all cases, a *P* value < 0.05 was considered statistically significant.

Results

Hexokinase expression and activity is upregulated in dopaminergic neurons of MPTP-treated mice

We first examined the expression and activity of hexokinase, the first key enzyme in glucose metabolism. We found that the mRNA and protein levels of Hexokinase 2 (HK2), and the activity of HK in brain SNpc were significantly increased in mice treated with MPTP compared to control mice (Fig. 1A-C). This suggests that HK level are upregulated and may be involved in the pathogenesis in the mouse model of PD.

Therefore, we focused on HK2 and investigated whether HK2 will affect the glycolysis in PD. To observe the localization of HK2 in mouse brain SNpc, co-immunofluorescence for HK2 and the markers for dopaminergic neuron, microglia and astrocyte were performed. We found that HK2 was predominantly expressed in dopaminergic neurons and was upregulated in the mouse model than control mice (Fig. 1D and Fig. S1).

Glycolysis and dopaminergic neurons apoptosis are enhanced in MPTP-treated mice

To test our hypothesis that excessive lactate produced by enhanced glycolysis may contribute to the apoptosis of dopaminergic neurons, we first examined the expression of LDHA, a key enzyme in glycolysis, in MPTP-treated mice. We found that LDHA expression was increased in SN of MPTP-treated mice compared to control mice (Fig. 2A). LDH catalyzes the interconversion of pyruvate and lactate. We found that the concentration of pyruvate was decreased and that of lactate was increased in SN lysates from MPTP-treated mice compared to control mice (Fig. 2B). Furthermore, LDHA expression was partially colocalized with tyrosine hydroxylase (TH) expression in SN of MPTP-treated mice, indicating that glycolysis is enhanced in dopamine neurons (Fig. 2C).

Increased apoptosis of dopaminergic neurons is a hallmark feature of PD. We next analyzed the general apoptosis in brain SNpc of MPTP-treated mice using TUNEL assay. We found that the TUNEL-positive cells were markedly increased in MPTP-treated mice compared to control mice (Fig. 2D). Using Western blotting, we found that TH expression was decreased and Bax/Bcl2 ratio was increased in brain SNpc tissue lysates from MPTP-treated mice compared to control mice (Fig. 2E). In support of increased apoptosis, immunofluorescent staining revealed that the number of TH⁺ positive dopaminergic neurons was decreased (Fig. 2F). Our data suggest that enhanced glycolysis and lactate production are associated with the increased apoptosis in PD.

To observe whether fatty acid metabolism could be another source of energy in MPTP-treated mice, we examined the expression of CD36, a fatty acid transporter, and Ppar- γ , a vital transcription factor in lipid metabolism. We found that Ppar- γ and CD36 expression level was decreased in MPTP-treated mice (Fig. S2A). This suggests that fatty acid metabolism was decreased.

Enhanced HK2 expression and lactate production leads to apoptosis in MPP⁺-treated SH-SY5Y cells

We next investigated the role of HK2 upregulation and excessive lactate production in the apoptosis of dopaminergic neurons using MPP⁺ (20mM)-treated SH-SY5Y cells, a dopaminergic neuronal cell line. Consistent with our in vivo findings, HK2 expression and HK activity was increased in MPP⁺ treated SH-SY5Y compared to control group (Fig. 3A and B). The expression of LDHA was also significantly increased in MPP⁺-treated cells (Fig. 3A). The upregulation of LDHA in SH-SY5Y cells were further confirmed by immunofluorescent staining for TH and LDHA (Fig. 3C). Moreover, pyruvate level was decreased and lactate level was increased in culture media of MPP⁺ treated SH-SY5Y compared to control cells (Fig. 3D). These data suggest that glycolysis is upregulated in MPP⁺ treated SH-SY5Y cells.

We found that MPP⁺ treatment significantly increased the apoptosis of SH-SY5Y (Fig. 3E). Moreover, the Bax/Bcl2 ratio was increased after MPP⁺ treatment (Fig. 3F). Since lactate was increased in the culture media of MPP⁺ treated SH-SY5Y, we observed the effect of exogenous lactate, L-lactate, on the apoptosis of SH-SY5Y cells. Interestingly, lactate at the concentration similar to that in culture media of MPP⁺-treated cells induced apoptosis of SH-SY5Y cells dose-dependently (Fig. 3G). This suggests that lactate, the product of glycolysis, may contribute to MPP⁺-induced apoptosis of SH-SY5Y.

It was reported that lactate can induce AMPK activation in PD, and AMPK/Akt/mTOR pathway is involved in dopaminergic neurons apoptosis [14, 15]. Thus, we assumed that lactate accumulation induce the apoptosis of SH-SY5Y cells via AMPK/Akt/mTOR pathway. We found that the phosphorylation of AMPK was increased but the phosphorylation of AKT and mTOR was decreased in L-lactate or MPP⁺ treated SH-SY5Y cells (Fig. 3H) or the SNpc of MPTP-treated mice (Fig. S2B and C). Moreover, an AMPK activator, O-304(20mg/ml), directly induced apoptosis dose-dependently (Fig. S2D). This result further confirmed the role of AMPK pathway in SH-SY5Y apoptosis. These data suggest that HK2 upregulation leads to lactate accumulation and dopaminergic neurons apoptosis by activating AMPK pathway.

To explore the regulation of fatty acid metabolism in the in vitro system, we also examined the CD36, and Ppar- γ . Consistent with our in vivo study, we found that CD36 and Ppar- γ expression were significantly decreased in MPP⁺ treated cells (Fig. S2E), indicating that fatty acid metabolism is downregulated in our in vitro system.

Inhibition of HK2 alleviated the MPP⁺-induced apoptosis in SH-SY5Y cells

To determine the role of HK2 upregulation in apoptosis of dopaminergic neuron, we used small interfering RNA (siRNA) to knockdown *HK2* gene expression (Fig. S3A). We found that *HK2* knockdown substantially

decreased the apoptosis in MPP⁺-treated SH-SY5Y at 48h (Fig. 4A). The decreased cell viability after MPP⁺ treatment was rescued when *HK2* was knockdown (Fig. 4B). In addition, Bax/Bcl2 ratio was decreased after HK2 knockdown (Fig. 4C). Moreover, the lactate concentration was decreased while pyruvate concentration was increased after HK2 knockdown (Fig. 4D). These results suggest that *HK2* knockdown may suppress apoptosis through inhibiting glycolysis and lactate accumulation.

The role of HK2 in apoptosis and lactate accumulation was further tested using 3-Brpa, a specific inhibitor for HK2. Similar to our data from HK2 knockdown experiments, 3-Brpa (5nmol) significantly alleviated the MPP⁺-induced SH-SY5Y apoptosis (Fig. 4E). 3-Brpa treatment also decreased Bax/Bcl2 ratio, and rescued the decreased cell viability after MPP⁺ treatment (Fig. 4F and G). Moreover, inhibition of HK2 with 3-Brpa suppressed the decrease of pyruvate and the increase of lactate after MPP⁺ treatment (Fig. 4H). However, inhibition of HK2 with 3-Brpa at higher concentration (10nmol) resulted in apoptosis (Fig. S3B). The possible explanation is that excessive inhibition of glycolysis leads to a sharp decrease in ATP production in SH-SY5Y.

Further, we examined the role of HK2 upregulation in AMPK signaling pathway. Inhibition of HK2 with 3-Brpa significantly inhibited MPP⁺ induced phosphorylation of AMPK, but increased the phosphorylation of AKT and mTOR (Fig. 4I). This suggested that HK2 upregulation is responsible for MPP⁺ induced activation of AMPK signaling pathway.

Glycolysis is an alternative source of ATP for dopaminergic neurons under the condition of mitochondrial dysfunction. We measured the ATP levels in SH-SY5Y cells and found that MPP⁺ treatment reduced ATP concentration. However, HK2 inhibition with 3-Brpa (5nmol) did not reduce the ATP concentration (Fig. S4A). We next examined whether fatty acid metabolism is a source of ATP when HK2 is inhibited. Using Western blot, we showed that CD36 and Ppar- γ protein expression was decreased when treated with MPP⁺. However, 3-Brpa treatment rescued MPP⁺ induced reduction of CD36 and PPAR- γ expression (Fig. S4B). This indicates that fatty acid metabolism may be an alternative source of ATP when HK2 is inhibited.

Application of 3-Brpa therapeutic effect in PD mouse model

We next observed the effect of inhibition of HK2 with 3-Brpa in MPTP-treated mice. We firstly check the best concentration of 3-Brpa (Fig. S5A) and use pole test and rotaroad test to observe the mice motor behavior (Fig. 5A). The falling time of pole test was increased and latency to fall of rotaroad test was decreased in MPTP-treated mice compared to control mice. This suggests that inhibition of HK2 with 3-Brpa significantly alleviated MPTP-induced motor defects (Fig. 5B and C). MPTP treatment increased the ratio of Bax/Bcl2 protein levels, which was suppressed by 3-Brpa treatment (Fig. 5D). Meanwhile, Immunostaining revealed that the number of TH-positive dopaminergic neurons was decreased after MPTP injection whereas 3-Brpa treatment inhibited the decrease of TH-positive cells (Fig. 5E). The TUNEL⁺ positive cells also decreased after 3-Brpa treatment (Fig. 5F). 3-Brpa treatment also reduce the

lactate concentration in MPTP-induced PD mouse model brain SNpc (Fig. 5G). Our data indicate that inhibition of HK2 alleviates the apoptosis of dopaminergic neurons through inhibiting lactate production.

Discussion

Neuronal metabolism shift from oxidative phosphorylation to glycolysis plays a vital role in neurodegenerative disease like PD and AD [12, 16]. In this study, we demonstrated that inhibition of HK2 ameliorate apoptosis of dopaminergic neurons through AMPK/Akt/mTOR pathway in PD.

The apoptosis of dopaminergic neurons is a major feature of PD. Previous studies have shown that exposure to lactate causes accumulation of α -synuclein in dopaminergic neuroblastoma cells [13]. Moreover, the lactate accumulation was associated with neurons apoptosis in AD [11]. Here, we demonstrated that the increased lactate production from glycolysis induce the apoptosis of dopaminergic neurons. This suggest that lactate plays a vital role in the apoptosis of dopaminergic neurons in PD. Therefore, reducing lactate production might alleviate the progression of PD.

Recently, conflicting results have been reported regarding the role of glucose metabolism in the pathogenesis of PD to be enhanced or impaired [5, 7, 17]. Some studies reported that the general glycolysis was decreased in PD patients brain cortex[18]. However, increased glycolysis of peripheral monocytes and highly expressed HK2 were reported in PD patients' brain [5, 7]. In addition, the elevation of lactate level and alterations of LDH-A/B mRNA-expression-ratio were found to be associated with aging [9]. The metabolic shift from oxidative phosphorylation to glycolysis was also reported affect the development of AD [12]. In considering the findings of mitochondrial dysfunction resulted in the ATP reduction in dopaminergic neurons in PD [19], we test whether glycolysis provide ATP for dopaminergic neurons under this condition. We demonstrate that HK2 and LDHA are enhanced in dopaminergic neurons. In addition, pyruvate is decreased and lactate is increased in in vitro and in vivo systems. These results suggest that glycolysis is increased in dopaminergic neurons in PD. Similar results were also shown in neurons and glia of the AD brain in previous study [20, 21]. Therefore, the benefit of glycolysis is to provide ATP for neurons while the product of glycolysis, lactate, induce the apoptosis of dopaminergic neurons.

The source of lactate was reported from astrocytes by astrocyte-neuron lactate shuttle (ANLS) [22], while it was also reported that the neurons may export rather than import lactate in response to stimulation due to the increased glycolysis [23]. Two-photon imaging in mice has also shown that glucose is processed preferentially in neurons compared with other cell types. Likewise, key glycolytic enzymes such as hexokinase and the insulin-sensitive glucose transporters are also enriched in neurons compared with astrocytes [24]. Based on the theory of the lactate source, we firstly proved the enhanced glycolysis related lactate accumulation also happened in dopaminergic neurons in PD, which then induce apoptosis of dopaminergic neurons.

AMPK can be phosphorylated at Thr172 of AMPK α by an elevated AMP/ATP ratio due to various stress indicating energy stress and starvation. Recently, it was demonstrated that lactic acidosis can also

increase p-AMPK or activate cytosolic Ca^{++} pathway to phosphorylate and activate AMPK [14, 25, 26]. This suggests that lactate can directly activate the phosphorylated AMPK even though the energy is sufficient. Meanwhile, AMPK is a major negative regulator of mTOR pathway, and activated AMPK/AKT/mTOR pathway is related to the apoptosis of dopaminergic neurons [15]. In line with previous studies, we found that lactate (10mM) will activate p-AMPK pathway to induce apoptosis, which further confirms the AMPK pathway plays a role in apoptotic mechanism of dopaminergic neurons.

To identify the role of HK2 in the apoptosis of dopaminergic neurons in PD, HK2 inhibitor, 3-Brpa, or knocking down HK2 was used in vivo and in vitro system. For the first time, we demonstrated that inhibiting HK2 alleviated the apoptosis of dopaminergic neurons and SH-SY5Y cells in in vitro and in vivo systems. In addition, 3-Brpa and si-HK2 reduced the lactate production in in vivo and in vitro systems, respectively. Moreover, AMPK pathway was activated by increased lactate resulted from glycolysis, and this was inhibited by 3-Brpa in SH-SY5Y cells. These results suggest that inhibition of HK2 ameliorate apoptosis of dopaminergic neurons through AMPK/Akt/mTOR pathway. However, it was reported that enhancing glycolysis by terazosin attenuated PD progression [27], which present opposite result. The terazosin enhanced phosphoglycerate kinase 1 (PGK1) activity to generate pyruvate into mitochondrial and restored the TCA cycle, thus leading to the increase production of ATP. However, our study focuses on glycolysis and lactate production under the mitochondrial dysfunction condition. The above conclusions all indicate that ATP and lactate play a vital role in the apoptosis mechanism of dopaminergic neurons.

In considering of the ATP generation, interestingly, we found 3-Brpa inhibited glycolysis, but the expression of CD36, Ppar- γ expression increased, thus present no difference between 3-Brpa and PBS treatment group in SH-SY5Y cells in ATP concentration. This result suggest that fatty acid may be the alternative energy source when glycolysis was inhibited. The mechanism between inhibition of HK2 by 3-Brpa and fatty acid metabolism may provide a better target for the regulation of energy demand in the future.

Our study has several limitations, like those for the HK2 genetic model of PD. We found glycolysis enhanced in dopaminergic neurons in PD, but the mechanism of what cellular mechanisms may allow the transient excess of glycolysis over OXPHOS when neurons are stimulated still remains unclear. In addition, AMPK pathway may affect oxidative stress and autophagy [28, 29], it's still worth to determine whether the lactate activated p-AMPK pathway affect ROS or autophagy function in dopaminergic neurons in the future. Moreover, we found 3-Brpa activated fatty acid metabolism, but the mechanism remains unclear.

In this work, we demonstrated for the first time that inhibition of HK2 can alleviate the dopaminergic neurons death and motor symptoms in PD. These results revealed the metabolic changes play a vital role in PD pathology, not only in ATP generation and mitochondrial dysfunction, but also the metabolism substrate lactate can also be a harmful factor in dopaminergic neurons apoptotic mechanism.

Conclusions

Together, our data suggest that inhibition of HK2 ameliorate the apoptosis of dopaminergic neurons through downregulating the lactate production and AMPK/ Akt/ mTOR pathway activation in PD (Figure 6). Our findings are as follows: (i) the expression and activity of HK2 increases in dopaminergic neurons in in vivo and in vitro systems in PD, (ii) the expression of LDHA and lactate production increase while pyruvate decrease in dopaminergic neurons in PD, (iii) lactate activates AMPK/ Akt/ mTOR pathway and induces apoptosis of dopaminergic neurons in PD, (iv) HK2 inhibitor or HK2 siRNA rescues the apoptosis of SH-SY5Y induced by MPP⁺, (v) HK2 inhibitor or HK2 siRNA alleviates the lactate accumulation and pyruvate deduction in SH-SY5Y induced by MPP⁺, (vi) HK2 inhibitor or HK2 siRNA inhibits the MPP⁺ induced AMPK/ Akt/ mTOR pathway activation in SH-SY5Y, (vii) HK2 inhibitor ameliorate the motor symptoms, apoptosis of dopaminergic neurons and lactate accumulation in MPTP-treated mice. The protective effect of HK2 inhibition on the apoptosis of dopaminergic neurons needs to be further investigated before the application in PD treatment.

Abbreviations

PD: Parkinson's disease; HK2: Hexokinase 2; LDH: Lactate dehydrogenase; SNpc: Substantia nigra pars compacta; AMPK: Adenosine monophosphate activated protein kinase; Akt: Serine/threonine kinase 1; mTOR: Mammalian target of rapamycin; HIF-1 α : Hypoxia-inducible factor 1 α ; AD: Alzheimer disease; TH: Tyrosine hydroxylase; ANLS: Astrocyte-neuron lactate shuttle; PGK-1: Phosphoglycerate kinase 1

Declarations

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Author Contributions

JL were responsible for conducting all of the experiments and data analyses and wrote the paper. LC provided help for flow cytometry and study design. QQ, DW, JZ were involved in the western blot and animal research. HG, XY, JZ and YZ jointly performed some of the experiments. ZM, YX, ZM, MY and CW were involved in study design and review of the paper. ZX contributed to the study design and paper preparation. All of the authors critically reviewed the content and approved the final version for publication.

Consent for publication

Not applicable.

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Availability of data and materials

All raw data used and/or analyzed during the current study are available from corresponding author on reasonable request.

Ethics approval and consent to participate

All the animal work presented has been approved by the Institutional Animal Care and Use Committee of the Tongji Medical College and followed NIH guidelines for the care and use of laboratory animals.

Competing interests: The authors declare that they have no competing interests.

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Figures

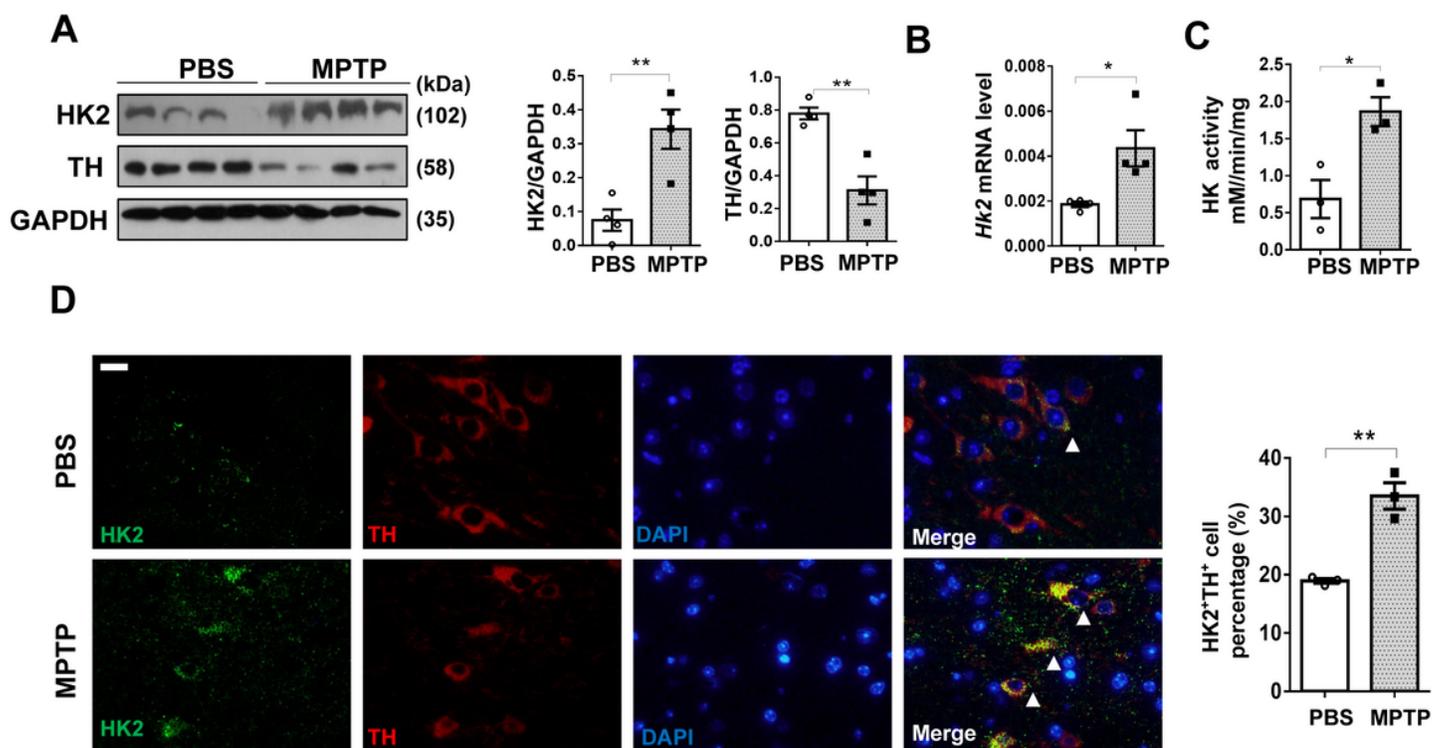


Figure 1

Hexokinase expression and activity is enhanced in SNpc of MPTP-treated mice. (A) HK2 and TH protein expression in brain SNpc lysates of MPTP-treated and control mice were determined using Western blot and quantified using Image J 1.46r software. $n = 4$ per group. (B) HK2 mRNA level in brain SNpc lysates of MPTP-treated and control mice was determined using quantitative PCR. $n = 4$ per group. (C) The activity of HK in brain SNpc lysates of MPTP-treated and control mice were determined using colorimetric tests. $n = 4$ per group. (D) Subsection of substantia nigra in mice. (E) Immunofluorescence of HK2 and TH expression in the section of SNpc from MPTP-treated and control mice. Quantification of cells which were positive for both HK2 and TH were calculated. Location of double positive cells are indicated by arrows. Scale bars: $50\mu\text{m}$. Values are expressed as mean \pm SEM. Unpaired Student's t test was performed to compare between MPTP-treated and control mice. * $p < 0.05$; ** $p < 0.01$.

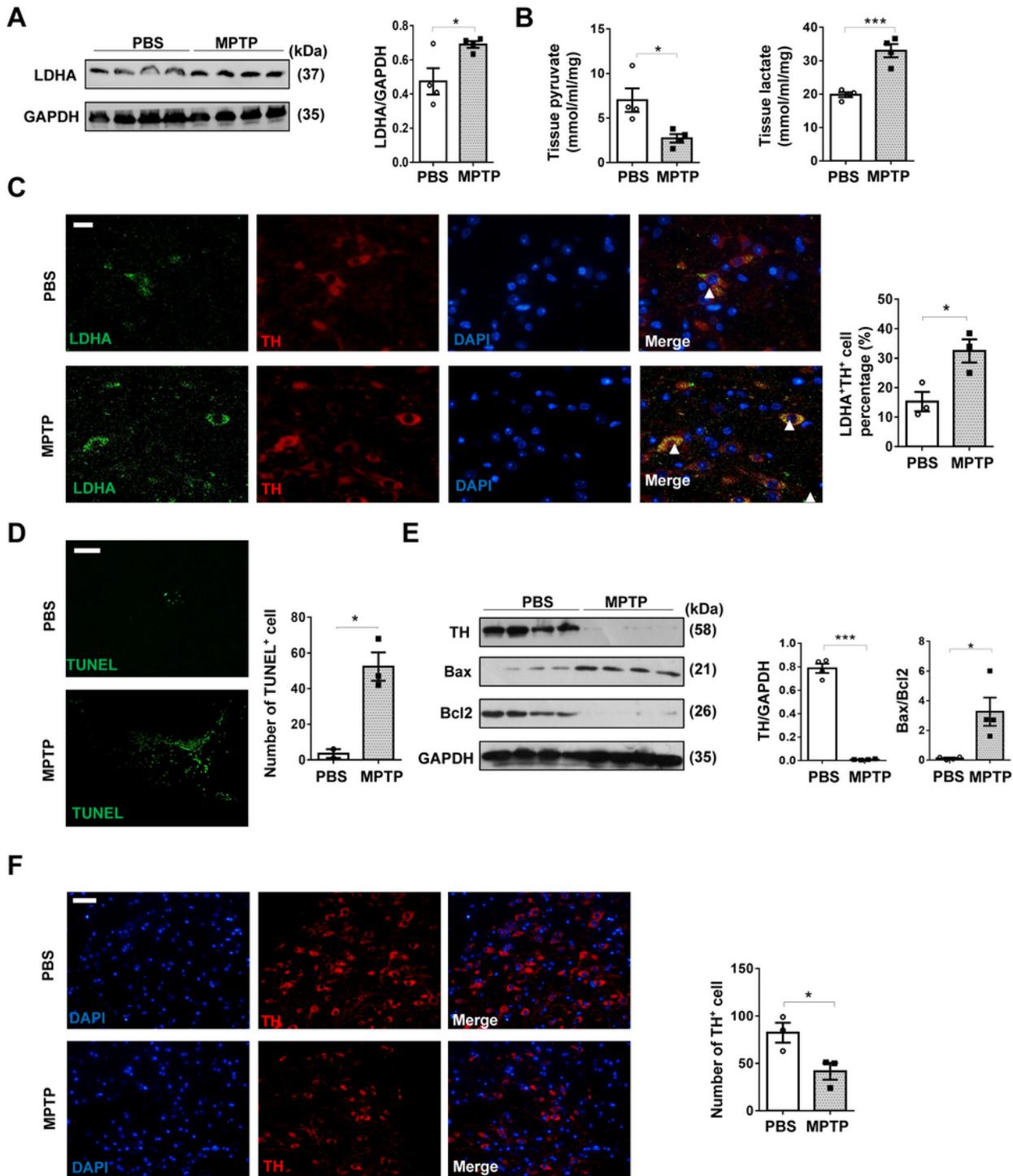


Figure 2

Aerobic glycolysis and apoptosis increased in SNpc of MPTP-treated mice. (A) LDHA expression in brain SNpc lysates of MPTP-treated and control mice were determined using Western blot and quantified using Image J 1.46r software. $n = 4$ per group. (B) Pyruvate and lactate concentration analysis of anaerobic glycolysis in SNpc of MPTP-treated and control mice were measured by colorimetric tests. $n = 4$ per group. (C) Immunofluorescence of LDHA and TH expression in the section of SNpc from MPTP-treated

and control mice. Quantification of cells which were positive for both LDHA and TH were calculated. Location of double positive cells are indicated by arrows. n = 3 per group. (D) Quantification of apoptotic cells which were positive for tunel positive staining were calculated. n = 3 per group. (E) Immunofluorescence showd TH positive cells were calculated as dopaminergic cells. n = 3 per group. (F) TH expression and Bax/Bcl2 ratio was evaluated by western blot to confirm the apoptosis in brain SNpc of MPTP-treated and control mice. Scale bars: 50 μ m (C and F); 100 μ m (D). The comparison for MPTP-treated mice vs control mice was analyzed by unpaired Student's t test; *p<0.05; **p<0.01.

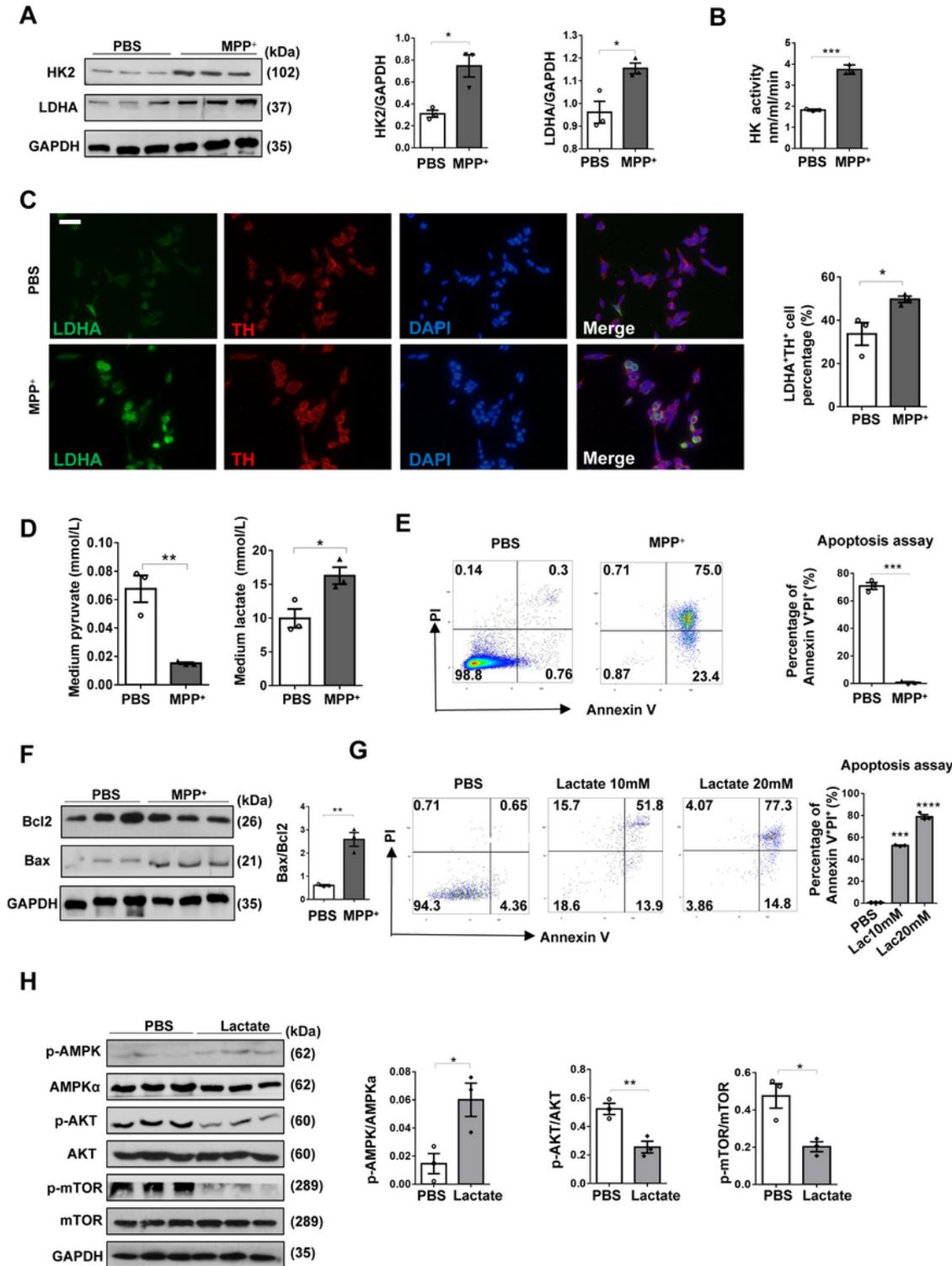


Figure 3

Aerobic glycolysis and apoptosis were increased in MPP⁺-treated SH-SY5Y. (A) HK2 and LDHA expression in cell lysates of MPP⁺ and PBS group were determined by western blot and quantified by Image J 1.46r software. n=3 per group. (B) The activity of HK in cell lysates of MPP⁺ and PBS group were determined using colorimetric tests. n = 3 per group. (C) LDHA expression in MPP⁺ or PBS treated SH-SY5Y was determined by immunofluorescence. Quantification of cells which were positive for LDHA and TH were calculated. n = 3 per group. (D) Pyruvate and lactate concentration in the medium of MPP⁺ or PBS treated SH-SY5Y were analyzed by colorimetric tests. n = 3 per group. (E) The apoptotic SH-SY5Y cells in MPP⁺ and PBS group were analyzed using flow cytometry by FlowJo and quantification of Annexin-V and PI double positive apoptotic percentage in were calculated. n=3 per group. (F) Bax/Bcl2 ratio in MPP⁺ and PBS group was determined by western blot and quantified by Image J 1.46r software. n=3 per group. (G) Flow cytometry analysis of the Annexin-V and PI double positive apoptotic rate after L-lactate treatment were quantified and calculated by FlowJo. (H) Changes in the level of p-AMPK/p-AKT/p-mTOR in SH-SY5Y exposed to lactate (10mM) for 48h were evaluated by WB and quantified by Image J 1.46r software. n=3 per group. Scale bars: 50µm. The comparison for MPP⁺ or PBS group was analyzed by unpaired Student's t test; *p<0.05; **p<0.01.

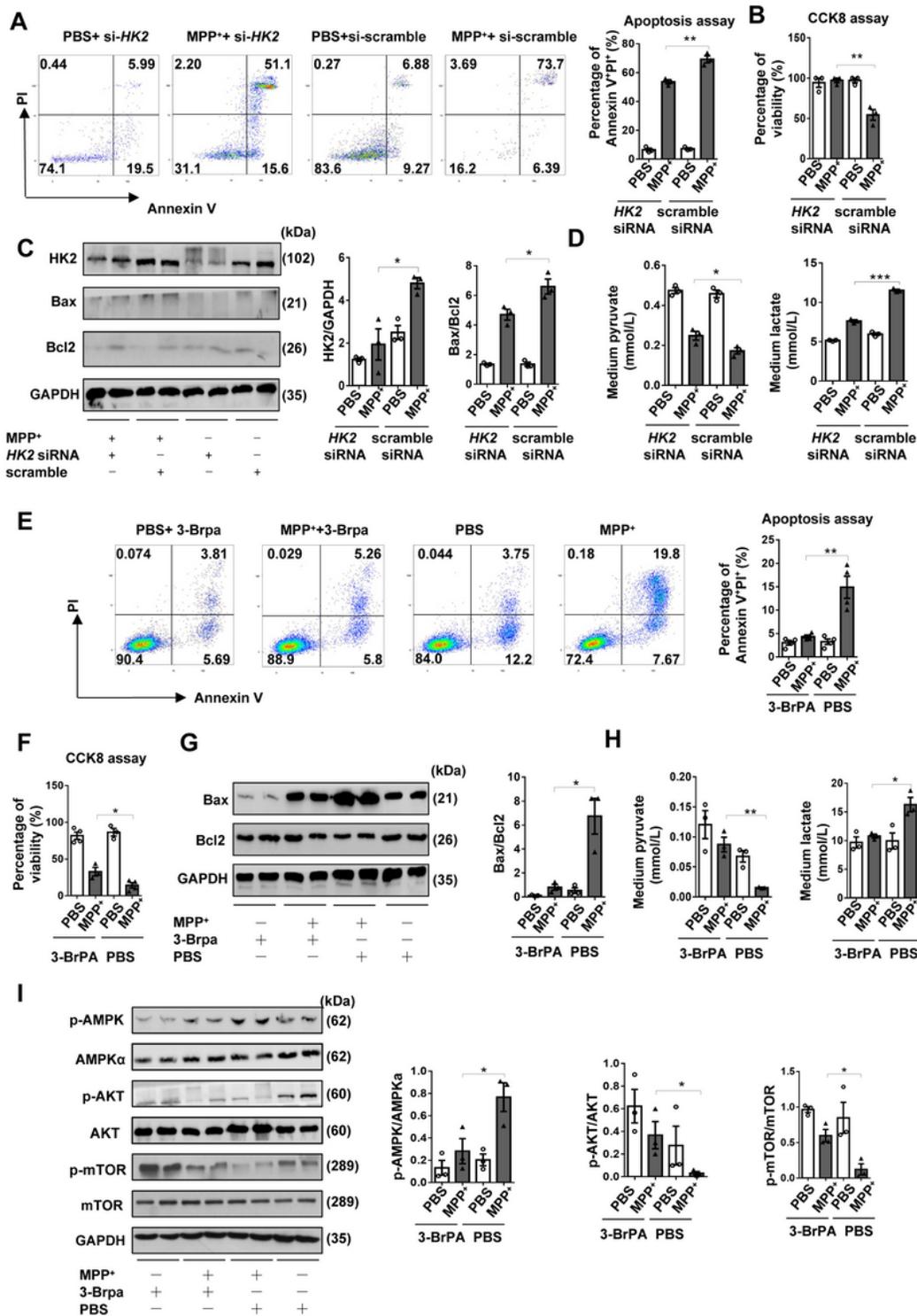


Figure 4

Inhibition of HK2 protect SH-SY5Y from MPP+ induced apoptosis while not affect ATP generation. (A) Apoptosis analysis of SH-SY5Y apoptotic rate after si-HK2 or/and MPP+ treatment was determined by flow cytometry and quantified by FlowJo. n=3 per group. (B) The cell viability of si-HK2 or/and MPP+ treated SH-SY5Y was measured by CCK8 test. n=3 per group. (C) HK2 expression and Bax/Bcl2 ratio in si-HK2 or/and MPP+ group were analyzed using western blot and quantified by Image J 1.46r software. n=3

per group. (D) Medium pyruvate and lactate concentration in si-HK2 or/and MPP+ group were determined by colorimetric tests. n = 3 per group. (E) Flow cytometry analysis of SH-SY5Y apoptotic rate in 3-Brpa or/and MPP+ group was quantified by FlowJo. n=3 per group. (F) CCK8 test analyzed the cell viability in 3-Brpa or/and MPP+ group. n=3 per group. (G) Bax/Bcl2 ratio after 3-Brpa or/and MPP+ treatment was determined using western blot and quantified by Image J 1.46r software. n=3 per group. (H) Medium pyruvate and lactate concentration in 3-Brpa or/and MPP+ group were determined by colorimetric tests. n = 4 per group. (I) The alleviation of p-AMPK/p-AKT/p-mTOR pathway activation by 3-Brpa evaluated by WB and quantified by Image J 1.46r software. n = 3 per group. Values are expressed as mean \pm SEM. Statistical difference was analyzed by unpaired Student's t test; *p<0.05; **p<0.01.

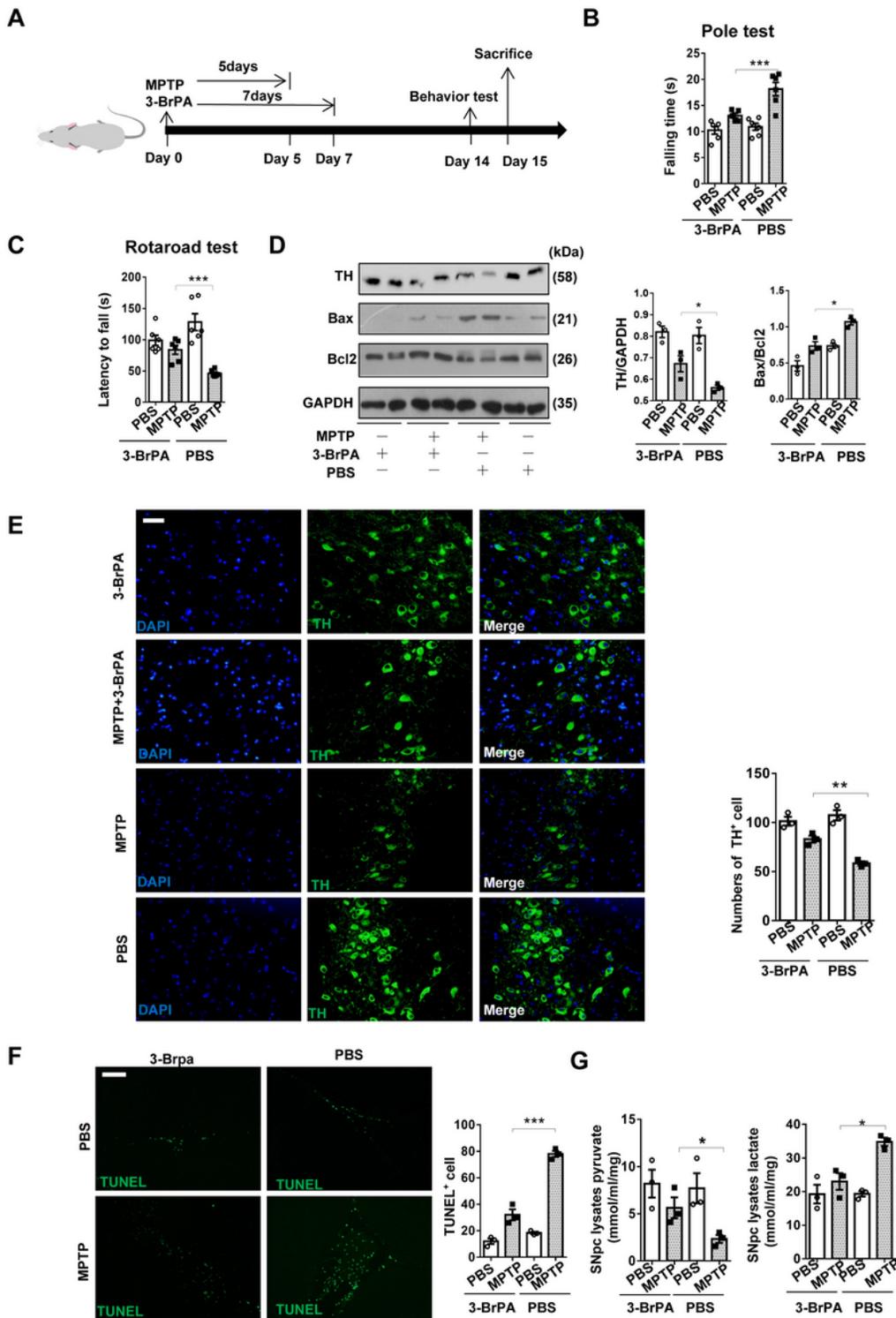
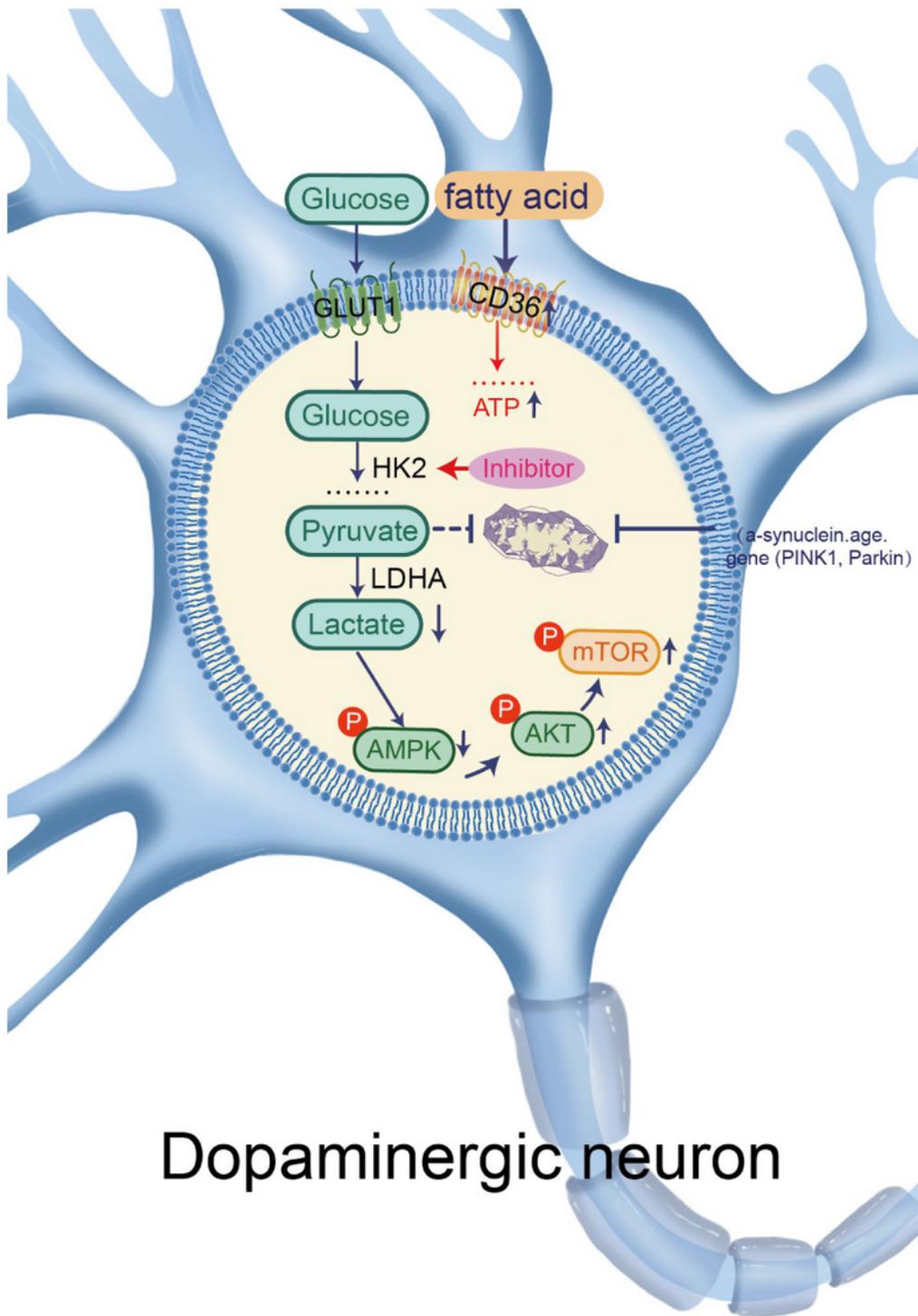


Figure 5

Inhibition of HK2 rescue the motor behavior dysfunction and dopaminergic neurons apoptosis by downregulating aerobic glycolysis. (A) Dosing and treatment plan. (B) Pole test, falling time was tested by the back and forth time of the mouse crawling on the rod to evaluate the behavior. n=6 per group. (C) Rotaroad test, latency to fall was tested by the mice maintaining time in the rotating rod to evaluate the behavior. n=6 per group. (D) TH level, Bax/Bcl2 ratio were evaluated by WB and quantified by Image J

1.46r software. n=3 per group. (E) The amount of dopaminergic neurons was detected using immunofluorescence analysis and TH positive cells were calculated. n=3 per group. (F) The apoptosis of SNpc in each group was evaluated by tunel staining and tunel positive cells were calculated. n=3 per group. (G) Pyruvate and lactate concentration in brain SNpc lysates were tested to by colorimetric tests. n = 3 per group. Scale bars: 50 μ m (E); 100 μ m (F). Values are expressed as mean \pm SEM. Statistical difference was analyzed by unpaired Student's t test; *p<0.05; **p<0.01.



Dopaminergic neuron

Figure 6

Inhibition of HK2 protect the dopmainergic neurons from apoptosis by reducing the aerobic glycolysis product-lactate, and downregulating the lactate activated AMPK/AKT/mTOR pathway.

Supplementary Files

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