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**The variations of Endophilin A2-FoxO3a-autophagy signal
in Ang II -induced dopaminergic neuron injury mouse model
and By Biochanin A**

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Abstract

Biochanin A is a natural plant estrogen, with various biological activities such as anti-apoptosis, anti-oxidation and suppression of inflammatory. In this study, we investigated the protective effects of biochanin A on Ang II-induced dopaminergic neurons damage *in vivo* and molecular mechanisms. Spontaneous activity and motor ability of mice among groups was detected by open-field test and swim-test. The expression of TH, LC3B II/LC3B I, Beclin-1, P62, p-FoxO3a / FoxO3a, FoxO3 and Endophilin A2 were determined by western blot and immunohistochemistry or immunofluorescence staining. Our results showed that Ang II treatment significantly increased the behavioral dysfunction of mice and DA neurons damage. Meanwhile, Ang II treatment increased the expression of LC3B II/LC3B I, Beclin-1, P62 and FoxO3a and decreased the expression of Endophilin A2 and p-FoxO3a / FoxO3a, however, biochanin A treatment alleviate these changes. In summary, these results suggest that biochanin A exerts protective effects on Ang II-induced mouse model may be related to regulating Endophilin A2, FoxO3a and autophagy-related proteins. However, the specific mechanism is not yet clear and needs further study.

Keywords: Biochanin A; Angiotensin II; Endophilin A2; FoxO3a; Autophagy

Introduction

Parkinson's disease (PD), the most common neurodegenerative movement disorder, clinical motor symptoms mainly include tremor, bradykinesia, rigidity and postural instability (Balestrino and Schapira, 2019). Recently, there is cumulative evidence demonstrating that the level of Angiotensin II (Ang II), a chief component of renin-angiotensin system (RAS), is significantly higher in the brain of PD patients (Jiang et al., 2013)(Wright and Harding, 2011). This suggesting that the pathogenesis of PD is closely related to the excessive activation of the intracerebral RAS.

Autophagy is a degradation process to remove redundant or defective cellular components within the cell and plays an important role in various biological processes (Hou et al., 2020)(Liu et al., 2019). It has been reported that activation of autophagy contributes to the Ang II-induced apoptosis of dopaminergic (DA) neurons *in vivo* and *in vitro* (Gao et al., 2016), and there are dysfunctional lysosomes and autophagosomes accumulation in DA neurons of PD patients (Dauer and Przedborski, 2003). A growing number of evidences showed that autophagy is potentially associated with the pathogenesis of PD.

Endophilin A2, which have a highly conserved N-BAR domain and SH3 domain, are conserved endocytic adaptors with membrane curvature-sensing and inducing properties (Gowrisankaran et al., 2020). A role of Endophilin A2 in autophagy dysfunction and neuronal loss has been reported (Liu et al., 2018)(Wang et al., 2019). Endophilin A2 is necessary in the early stage of autophagy and establishes a docking station for autophagy proteins (Soukup and Verstreken, 2017). It was found that mice

of knockout of Endophilin A may contribute to motor impairment, age-dependent ataxia, and neurodegeneration (Murdoch et al., 2016). Meanwhile, RNA sequencing analysis showed that the transcription factor Forkhead box class O3a (FoxO3a) was significantly up-regulated in mice of knockout of Endophilin A (Kuijpers and Haucke, 2016). FoxO3a is a member of Forkhead box class O (FoxO) transcription factors family, which plays an important role in the transcription regulation of autophagy related genes. Activated FoxO3a increases the levels of autophagy core proteins, such as microtubule-associated proteins light chain 3B (LC3B), Beclin-1 and ATG12, thus regulating the occurrence of autophagy and participating in the occurrence and development of many diseases (Li et al., 2017)(O'Neill et al., 2016).

Biochanin A (Bioch A) is classified as a phytoestrogen, which is an O-methylated isoflavone that isolated from chickpea (Tan et al., 2013)(Yu et al., 2019). It has been reported to possess a variety of biological activities, such as anti-apoptosis, anti-oxidation and suppression of inflammatory. Our previous research also showed that Bioch A can protect DA neurons *in vivo* and *in vitro* by inhibiting the activation of microglia (Wang et al., 2016).

In the present study, we aimed to investigate whether Bioch A can exert the neuroprotective effects and attenuate Ang II-induced autophagy impairment, along with possible underlying mechanisms. Our results showed that biochanin A treatment significantly attenuated the behavioral dysfunction, abolished Ang II-induced DA neurons damage, and the mechanisms may be associated with regulating Endophilin A2, FoxO3a and autophagy-related proteins.

89 **Materials and Methods**

90 **Reagents**

91 Bioch A (molecular weight: 284.26, cat. No. D2016) was purchased from
92 Sigma-Aldrich (St. Louis, MO, USA). Primary antibody specific for neuronal
93 Tyrosine hydroxylase (TH, cat. No. GB11181) was obtained from Servicebio (Hubei,
94 China). Primary antibody of FoxO3a (cat. No. 12829s) was purchased from Cell
95 Signaling Technology, Inc (Danvers, MA, USA). The primary antibodies of
96 phospho-FoxO3a (Ser253, cat. No. bs-3140R) was purchased from Bioss (Beijing,
97 China). Primary antibody of Endophilin A2 (cat. No. SC-365704) was obtained from
98 Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary antibody of LC3B
99 (cat. No. ab48394) was purchased from Abcam (Cambridge, UK). The antibodies of
100 P62 (cat. No. 18420-1-AP) and Beclin 1 (cat. No. 11306-1-AP) were obtained from
101 Proteintech Group (Hubei, China). Streptavidin-biotin complex kit (cat. No. SP-9000)
102 and diaminobenzidine (DAB) staining kit (cat. No. ZLI-9019) were purchased from
103 Zhongshan Golden Bridge Biotechnology Co. (Beijing, China). All other chemicals
104 used were of the highest grade commercially available.

105 **Animals**

106 Male C57BL/6 mice, aged 3 to 5 months and weighing 25-30g, were purchased
107 from the Experimental Animal Center of Anhui Medical University. They were kept
108 in a controlled environment with a temperature of 22 ± 2 °C and humidity of 60%
109 under 12 h light/12 h dark cycle. Food and water were available *ad libitum*. All
110 research involving animals was conducted according to Guide for the Care and Use of

Laboratory Animals (8th edition, National Academies Press). All efforts were made to decrease animal numbers and their suffering. All animal operations were approved by Animal Ethics Committee of Anhui Medical University (LLSC20200251).

Neurosurgery and Treatment of Biochanin A

The mice were anesthetized with 1% Pentobarbital Sodium and fixed in the stereotaxic apparatus (RWD Life Science Co. Ltd, Shenzhen, China). Ang II (2.25 μ g in 1.5 μ L of sterile saline; Apexbio) was injected into substantia nigra through a Hamilton syringe (Hamilton Co., 1701RN/SYR 10ul/ ga26s/ 51mm/pst2, Reno, NV) at a rate of 0.3 μ L/min. After each injection, the needle was left in situ for 10 min before complete retraction. The sham-operated mice were injected with saline solution. (Stereotaxic coordinates: 3.0mm posterior to bregma, 1.3mm lateral to the midline, 4.7mm ventral to the surface of the cortex).

The experimental mice were randomly divided into five groups (n=10 each group): sham group, Ang II -treated 1d group, Ang II -treated 3d group, Ang II -treated 5d group and Ang II -treated 7d group. The drug treatment schedule is shown in Fig. 1A. On the 1st, 3rd, 5th and 7th day after the injection of Ang II, behavioral tests were performed.

To explore the role of Bioch A, we designed a new experiment. The mice were randomly divided into six groups (n=10 each group): sham group, Ang II -treated group, Candesartan (1.2mg/kg) +Ang II group, Bioch A (18 mg/kg) +Ang II group, Bioch A (36 mg/kg) +Ang II group, Bioch A (72 mg/kg) +Ang II group. Bioch A was dissolved in distilled water and administered intragastrically (p.o. 0.1 mL/10 g/day) to

different groups of mice at three different concentrations of 18, 36, 72 mg/kg. After Ang II injection, the mice were continuously treated with Bioch A for 7 days according to their body weight. The sham group and Ang II-treated group received equivalent volumes of solvent. The drug treatment schedule is shown in Fig. 4A.

Open-Field Test

Open-field test was performed in a black box (60 cm × 60 cm × 60 cm), which was located in an experimental room with a 50-dB background noise and a white light bulb (15W) located 60 cm high above the center of the open field. Mice were put in the center of the open-field; moving distance, the number of squares crossed and rearing frequency were recorded by ANY-maze Video tracking system (Stoelting Company, USA). After 2 min of pre-adaptation, the motor activity analysis lasted for 3 min.

Swim-Test

Swim-test could be one of the most convenient methods for monitoring the overall motor deficit in rodent models of PD (Chen Y, et al., 2015) (Haobam, et al., 2005). Swim-test was carried out in water tubs (30 cm length × 20 cm width × 20 cm height). The depth of water was kept at 10 cm and the temperature was maintained at 22~25 °C. Swimming scores of mice within 1 min was recorded. Each mouse was measured five times and then the average value was taken. The interval of each measurement was 1 min. Swimming score standard were: 3.0 points: continuous swimming within one min; 2.5 points: occasional floating only and continuous swimming in most of the time; 2.0 points: floating time is more than 30 seconds; 1.5

points: occasional swimming only; 1.0 points: occasional swimming using the hind limbs while floating on one side.

Immunohistochemistry

After behavioral evaluation, the anesthetized mice were given cardiac perfusion with precooled PBS and 4% paraformaldehyde, then brain tissue was quickly removed. Brain samples were postfixed with 4% paraformaldehyde at 4 °C for 48 h, and then transferred to 30% sucrose in PBS before sectioning. The brain tissue was embedded in paraffin and the thickness of continuous sections was 5µm. The coronal sections of substantia nigra compact part were selected according to the mouse brain atlas; from -2.78 to -3.88mm following coordinates from the bregma. The brain coronal sections were dewaxed, hydrated, and incubated with 3% H₂O₂ for 10 min to quench endogenous peroxidase before diaminobenzidine staining. The sections were then sealed with 10% goat serum albumin for 30 min and then incubated with anti-TH antibodies at 4 °C for an additional 24 h. After PBS washing, the sections were incubated for 1 h in secondary antibody: biotinylated-goat anti-mouse IgG or the goat anti-rabbit IgG (Beyotime Biotechnology, China). After been rinsed three times with PBS, a DAB kit was used to detect protein targets according to the manufacturer's instructions. Under the microscope, it can be observed that the positive neurons are stained brown, and the cells were counted with the Image-Pro Plus software (v. 6.1) program (Media Cybernetics, Inc.).

Immunofluorescence Staining

The steps of brain tissue fixation, embedding, sectioning, antigen repair and

sealing are basically consistent with the description of immunohistochemistry. The sections were washed with PBS and incubated with anti-TH, anti-FoxO3a, anti-LC3B, anti-Endophilin A2 antibodies at 4 °C for an additional 24 h. The next day, the sections were placed in corresponding fluorescent secondary antibody and incubated for 1 h at room temperature and under dark conditions. Then, the cell nucleus was stained with DAPI at room temperature and kept away from light for 5 min. The positive neurons were showed green or red fluorescence and were observed and photographed under the microscope. The average fluorescence intensity of positive neurons in each section was analyzed by Image-Pro Plus 6.0 analysis system.

Western blotting

Mice were sacrificed after behavioral testing. The substantia nigra of mice were separated from the brain and homogenized in RIPA lysis buffer prepared with Phenylmethanesulfonyl fluoride. The homogenates were centrifuged at 12000 rpm for 10 minutes at 4°C and the supernatant was collected. Then, Protein concentration was measured by the BCA Protein Assay Kit (Beyotime Biotechnology, China). The protein extract was electrophoresed on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a PVDF membrane (Millipore Corp., Billerica, Massachusetts, USA). The membranes were soaked in blocking buffer (5% skimmed milk in TBST) for 1 h and then probed with primary antibodies of LC3B (1:1000), Endophilin A2 (1:500), FoxO3a (1:1000), phospho-FOXO3a (1:1000), Beclin 1 (1:1000), P62 (1:1000), or β -actin (1:1000) overnight at 4 °C, followed by horseradish peroxidase (HRP) -conjugated secondary

antibody (1: 10,000) for 1 h at room temperature. After washing three times, the protein bands were detected by using Super Signal West Femto chemiluminescence substrate kit (Bridgen, China), and the optical density analysis was performed using Image J. software (National Institutes of Health, USA). The β -actin of the same sample was used as a control to calculate the relative expression of the target protein.

Statistical analysis

Statistical program SPSS 17.0 (Chicago, IL, USA) was used for data analysis. Through the Kolmogorov-Smirnov test, all data distributions conform to the normal distribution. Results were analyzed by one way analysis of variance (ANOVA) accompanied by a post-hoc Dunnett's multiple comparisons test. The data were expressed as mean $\pm$ standard deviation. A value of $P < 0.05$ was considered to be statistically significant.

Results

The survival data taken from this study showed that all the mice used in the experiment survived until the end of the study [100% survival].

Ang II-induced behavioral dysfunction and DA neurons losses in the substantia nigra pars compacta (SNpc) of the mouse

Behavioral tests could be used to evaluate the effect of Ang II treatment on motor dysfunction of animal models. Ang II-treated increased behavioral dysfunction of mice. After Ang II injection, moving distance, the number of crossing the target square and the number of rearing in the open field in a time-dependent manner decreased (Fig. 1B, Fig. 1C, Fig. 1D, $P < 0.05$ or $P < 0.01$). Swim-test also revealed

that Ang II treatment significantly increased immobility time and reduced swimming score (Fig. 1E, $P < 0.05$ or $P < 0.01$). Next, we observed the number of DA neurons in the substantia nigra of mice by TH immunofluorescence staining. Ang II treatment reduced the number of TH positive neurons (Fig. 1F and G, $P < 0.05$ or $P < 0.01$). These results showed that Ang II treatment attenuated the spontaneous activity and motor ability of mice and induced DA neurons damage in the SNpc of mouse model.

The expression of Endophilin A2, FoxO3a and autophagy-related signaling molecules in the SNpc of Ang II -induced DA neuron injury mouse model

Autophagy is a major catabolic process that plays a key role in cell homeostasis, protein quality control, and adaptive response to stress. Recently, a strong association among autophagy and PD has been reported (Moors et al., 2017). We used western blot and immunofluorescence to observe the changes of autophagy-related proteins. These results indicated that Ang II-treatment altered the expression of autophagy-related signaling molecules, including LC3B II/LC3B I (Fig. 2A, $P < 0.05$ or $P < 0.01$), Beclin-1 (Fig. 2B, $P < 0.05$), P62 (Fig. 2C, $P < 0.05$ or $P < 0.01$) and LC3B (Fig. 2H and I, $P < 0.05$ or $P < 0.01$). Interestingly, from the time course, P62 appeared to be two distinct periods of change. The first corresponds to a phase of rapid decrease during the first 3 days Ang II-treatment. The second phase from 3 to 7 days, during which the level of P62 further increased was observed. These results may suggest that early autophagy is compensated for activated, but autophagy flux was blockaged and autophagy substrates and harmful substances could not be normally degraded.

Next, we detected the expression of endophilin A2 in the SNpc of mice by western blot. As shown in Fig. 2D, over the total period of experiment, compared with the sham group, Ang II-treated significantly decreased the expression of endophilin A2 (Fig. 2D, $P < 0.05$ or $P < 0.01$). FoxO3a is a transcription factor that regulates the expression of core autophagy signaling genes. Upon phosphorylation, FoxO3a translocates from the nucleus to the cytoplasm and is subsequently degraded. Thus, we tested the expression of FoxO3a by western blot and immunofluorescence. Elevated FoxO3a and decreased p-FoxO3a levels were detected in the brain of the Ang II-treated mice compared with the sham group (Fig. 2E, $P < 0.05$ or $P < 0.01$). Changes in the level of FoxO3a were further validated via immunofluorescence (Fig. 2F and G, $P < 0.05$ or $P < 0.01$).

The expression of Endophilin A2, FoxO3a and LC3B in DA neurons of Ang II-induced DA neuron injury mouse model

To observe the possible relationship between Endophilin A2 and FoxO3a and DA neurons autophagy, we used immunofluorescence co-localization. As shown Fig. 3, LC3B, Endophilin A2, FoxO3a and DA neurons all had different degrees of co-localization. The LC3B staining showed a diffused pattern with few punctate dots in normal DA neurons while LC3B-positive dots were dramatically increased in the Ang II-treated group. Meanwhile, Ang II treatment induced Endophilin A2 expression in the cytoplasm and enhanced FoxO3a expression in the nucleus. These results indicated that Endophilin A2 and FoxO3a may be associated with autophagy in DA neurons.

Biochanin A attenuated behavioral dysfunction and protected DA neurons in Ang II-induced DA neuron injury mouse model

Herein, after Ang II treatment, moving distance, crossing the target square and rearing of mice during 3 min period in the open field were decreased ($P < 0.01$); however, after Bioch A (36, 72 mg/kg) treatment, moving distance, crossing the target square and rearing of the mice were recovered in varying degrees (Fig. 4B, Fig. 4C, Fig. 4D, $P < 0.05$ or $P < 0.01$). Furthermore, candesartan treatment significantly reduced Ang II effects on moving distance, crossing the target square and rearing of the mice ($P < 0.05$ or $P < 0.01$). Moreover, swim-test revealed that Ang II treatment significantly increased immobility time and reduced swimming score ($P < 0.01$), which was changed by Bioch A (36, 72mg/kg) treatment (Fig. 4E, $P < 0.05$). Candesartan treatment was also increased the swimming score ($P < 0.05$). Candesartan, an Angiotensin II type1 receptor (AT1R) blocker, can directly block the binding of AngII to AT1R, and block the effect of AngII from the source. Although the effect of high dose Bioch A was similar to that of low dose candesartan in this index, as a phytoestrogen, biochanin A may be safer for patients than candesartan. So, we believe it is very meaningful to study the effect of biochanin A on DA neurons. These results demonstrated that Bioch A treatment improves the behavioral dysfunction in Ang II-induced DA neurons damage mouse model.

Next, we observed the number of DA neurons in the substantia nigra of mice by TH immunohistochemical staining. Compared with the sham group, the number of TH positive neurons were significantly reduced in the Ang II-treated group (Fig. 4F

and G, $P < 0.01$). Nevertheless, Bioch A (36, 72mg/kg) treatment attenuated Ang II-mediated DA neurons damage. Candesartan treatment also attenuated DA neurons damage ($P < 0.05$). These results suggested that Bioch A treatment improved the behavioral dysfunction and protected DA neurons in Ang II-induced DA neurons damage mouse model.

Biochanin A alleviated the expression of Endophilin A2 , FoxO3a and autophagy-related signaling molecules in the SNpc of Ang II-induced DA neuron injury mouse model

Similarly, both western blot and immunofluorescence results indicated that Ang II-treatment altered and the expression of autophagy-related signaling molecules, including LC3B II /LC3B I (Fig. 5A, $P < 0.05$), Beclin-1 (Fig. 5B, $P < 0.01$), P62 (Fig. 5C, $P < 0.01$) and LC3B (Fig. 5H and I, $P < 0.05$); Bioch A (36, 72mg/kg) treatment alleviated these changes (Fig. 5A-C, H and I, $P < 0.05$). Candesartan treatment also inhibited the levels of LC3B II /LC3B I , Beclin1, P62 and LC3B ($P < 0.05$). These results indicated that Bioch A may inhibit the expression of autophagy-related protein.

Next, we detected the expression of Endophilin A2 and FoxO3a in the SNpc of mice by western blot. As shown in Fig.5D, the expression of Endophilin A2 was significantly decreased in the Ang II-treated group compared with the sham group (Fig. 5D, $P < 0.01$). However, Bioch A (36, 72mg/kg) treatment can improve the expression of Endophilin A2 (Fig. 5D, $P < 0.05$). In addition, candesartan treatment significantly increased the expression Endophilin A2 ($P < 0.05$). After, we tested the

expression of FoxO3a by western blot and immunofluorescence. Elevated FoxO3a and decreased p-FoxO3a levels were detected in the brain of the Ang II -treated mice compared with the sham group (Fig. 5E, $P < 0.01$), and Bioch A (18, 36, 72mg/kg) treatment changed these effects (Fig. 5E, $P < 0.05$ or $P < 0.01$). Furthermore, the levels of p-FoxO3a/FoxO3a were significantly increased in the candesartan group ($P < 0.05$). Changes in the level of FoxO3a was further validated via immunofluorescence (Fig. 5F and G, $P < 0.05$ or $P < 0.01$). These data implied that Bioch A improved autophagy by restoring Endophilin A2 expression and inhibiting FoxO3a activation.

Discussion

There are several of evidence that the Ang II may be related to the progression of PD, since a significantly higher level of Ang II in the cerebrospinal fluid (CSF) or brain tissues was observed in PD animal models as well as patients with this disease (Konings et al., 1994)(Sonsalla et al., 2013). In this study, Ang II was injected to the left side of substantia nigra of mice, and Ang II-induced motor dysfunction of mice was observed in open-field tests and swim-test. Ang II -treated decreased moving distance, the number of crossing the target square and the number of rearing in open-field test, and decreased swimming score in swim-test. Immunofluorescence and immunohistochemistry was used to evaluate morphological changes in dopaminergic neurons. While Bioch A treatment has protective effect on the behavioral dysfunction and DA neurons of Ang II -induced dopaminergic neuron injury mouse model.

Endophilin A2 is a member of Endophilin A family and plays a vital role in

neurological diseases (Murdoch et al., 2016)(Connor-Robson et al.,2019). FoxO3a is a regulator of core autophagy-related genes. The conversion of FoxO3a between cytoplasm and nucleus, which determines its activity (Sun et al., 2018). In this study, Ang II -treated significantly decreased the expression of endophilin A2, p-FoxO3a and increased the expression of FoxO3a, and Bioch A treatment improved these changes. Simultaneously, some studies found that FoxO3a is also a substrate for basal autophagy degradation (Fitzwalter et al., 2018). This finding adds another interesting feature to the mechanism. When the degradation of autophagy is hindered, FoxO3a will accumulate further, which explains the increased of FoxO3a.

Autophagy is an extremely complex process. Since the autophagy substrates getting into lysosomes involve multiple steps, including synthesis of membrane structures, traffic and fusion of autophagosome with lysosomes and degradation of substrates by lysosomal enzymes, the malfunction in each of them can make autophagy go awry (Nakamura and Yoshimori, 2017). Beclin-1 participates in the early formation stages (nucleation stages) of autophagosomes. LC3B exists as LC3BI under normal conditions, when autophagy is activated, LC3BI converted to LC3BII. Therefore, the amount of LC3B II is closely correlated with the number of autophagosomes, serving as a well indicator of autophagosome formation (Chen et al., 2019). Our results showed that Ang II obviously induced DA neurons damage, accompanied by autophagy excessively activated. The levels of LC3B II and Beclin-1 were significantly increased, but the increased level of P62 seems to contradict with activating autophagy. As a selective substrate for autophagy, P62 is inversely

proportional to the degree of activation of autophagy, so its accumulation is generally considered as an indirect marker of autophagy degradation dysfunction (Lieberman et al., 2012). Therefore, we speculate that Ang II-induced autophagy excessively activated may be accompanied by lysosomal degradation dysfunction.

To further observe between EndophilinA2-FoxO3a-regulated autophagy and DA neurons damage. In this study, EndophilinA2, FoxO3a and LC3B II co-localized with TH, respectively. We observed that FoxO3a probe localized in the nucleus of TH-positive DA neurons, while LC3B II and EndophilinA2 co-localized in the cytoplasm. Combined with the previously detected FoxO3a and autophagy protein levels changes and these results indicate that the dysregulation of autophagy flux of DA neurons may be one of the important pathogenesis factors of PD. Meanwhile, some studies found that Endophilin A2 can co-localized with autophagosomes (Soukup and Verstreken, 2017). Endophilin A is functionally related to proteins such as LRRK2, PARK2/Parkin and SYNJ1 (synaptojanin 1), and these proteins independently participate in all aspects of autophagy and play an irreplaceable role in these process (Guo et al., 2018)(Rui et al., 2015).

Hence, the dysregulation of autophagy flux may be related to the low expression of Endophilin A2. Based on the above evidence, we speculate that Endophilin A2 also plays an indispensable role in autophagy. In summary, the present results suggest that Bioch A protected DA neurons may be related to regulating Endophilin A2, FoxO3a and autophagy-related proteins. However, the specific mechanism is not yet clear and needs further study.

375

376 Abbreviations

377 PD: Parkinson's disease; Ang II: Angiotensin II; Bioch A: Biochanin A; RAS:
378 Renin-angiotensin system; DA: dopaminergic; FoxO3a: Forkhead box class O3a;
379 LC3B: Microtubule-associated proteins light chain 3B; TH: Tyrosine hydroxylase;
380 SNpc: Substantia nigra pars compacta; ANOVA: Analysis of variance; AT1R:
381 Angiotensin II type1 receptor.; CSF: cerebrospinal fluid.

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383 Availability of data and materials

384 The datasets used and/or analysed during the current study are available from the
385 corresponding author on reasonable request.

386

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392 Competing interests

393 The authors declare that they have no competing interests.

394

395 Authors' contributions

396 Y-YY designed the study and analyzed the data and wrote the manuscript. Y-YG,

H-JH and X-HX performed the experiments and wrote the manuscript. Li-WZ and Wu-WN analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval

All experimental procedures were approved by the Ethics Committee for Experimental Animal Use and Care of Anhui Medical University.

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Figure caption

Fig.1 Ang II-induced behavioral dysfunction and DA neurons losses in the substantia nigra pars compacta (SNpc) of the mouse. (A) Drug treatment schedule. (B) Moving distance was recorded in the open-field test. (C) The number of squares crossed in the open-field test. (D) The number of rearing was recorded in the open-field test. (E) The score of swimming. Results are expressed as mean $\pm$ SD (n = 10). (F) TH-Immunofluorescence images. The bar is 200 μ m. (G) Quantitative analysis of TH-positive expression in the SNpc. Results are expressed as mean $\pm$ SD (n = 5). * P <0.05, ** P <0.01 vs the sham group.

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Fig.2 The expression of Endophilin A2, FoxO3a and autophagy-related signaling molecules in the SNpc of Ang II-induced DA neuron injury mouse model. (A), (B) and (C) Representative immunoreactive bands and statistical results showed that Ang II increases the expression of LC3BII and Beclin1 in the SNpc of mice, and the protein level of P62 was inhibited first and then increased in the SNpc of mice. LC3BII /LC3BI(A); Beclin1 (B); P62 (C). The relative density for expression of

Beclin1 and P62 relative to β -actin. (D) Representative immunoreactive bands and statistical indicated that AngII decreases the protein levels of Endophilin A2 in the SNpc of mice. The relative density for expression of Endophilin A2 relative to β -actin. (E) Representative immunoreactive bands and statistical results showed that AngII decreases the relative value of p-FoxO3a/FoxO3a in protein level in the SNpc of mice. (F) Immunofluorescence images results showed that AngII increases the expression of LC3B in the SNpc of mice. The bar is 100 μ m. (G) The bar graphs representing quantitative analysis of LC3B-positive expression in the SNpc of mice. (H) Immunofluorescence images results showed that AngII increases the expression of FoxO3a in the SNpc of mice. The bar is 100 μ m. (I) The bar graphs representing quantitative analysis of FoxO3a-positive expression in the SNpc of mice. Results are expressed as mean $\pm$ SD (n = 5). * P <0.05, ** P <0.01 vs the sham group, # P <0.05, ## P <0.01 vs the AngII-treated 3d group.

Fig.3 The expression of Endophilin A2, FoxO3a and LC3B in DA neurons of Ang II-induced DA neuron injury mouse model. After exposure to AngII, (A) double labeling for TH and LC3B was performed with immunofluorescence. TH colocalized with LC3B. Scale bar = 50 μ m. (B) double labeling for TH and Endophilin A2 was performed with immunofluorescence. TH colocalized with Endophilin A2. Scale bar = 50 μ m. (C) double labeling for TH and FoxO3a was performed with immunofluorescence. TH colocalized with FoxO3a. Scale bar = 50 μ m.

Fig.4 Biochanin A attenuated behavioral dysfunction and protected DA neurons

in Ang II -induced DA neuron injury mouse model. (A) Drug treatment schedule.

(B) Moving distance was recorded in the open-field test. (C) The number of squares

crossed in the open-field test. (D) The number of Rearing was recorded in the

open-field test. (E) the score of swimming. Results are expressed as mean $\pm$ SD (n

=10). (F) Representative images and statistical results showing Bioch A protects

DA neurons (TH-immunoreactive cells) loss in the SNpc of Ang II -induced DA

neurons damage mice (immunohistochemistry). a: sham group; b: Ang II group; c:

candesartan group; d, e, f: Bioch A (18, 36, 72 mg/kg) group. The bar is 200 μ m. (G)

Quantitative analysis of TH-positive expression in the SNpc. Results are expressed as

mean $\pm$ SD (n=5). * P <0.05, ** P <0.01 vs the sham group, $^{\Delta}$ P <0.05, $^{\Delta\Delta}$ P <0.01 vs the

Ang II -treated group.

Fig.5 Biochanin A alleviated the expression of Endophilin A2 , FoxO3a and

autophagy-related signaling molecules in the SNpc of Ang II -induced DA neuron

injury mouse model. (A), (B) and (C) Representative immunoreactive bands and

statistical results showed that Bioch A decreases the expression of LC3BII,

Beclin1 and P62 in the SNpc of mice. LC3BII /LC3BI(A); Beclin1 (B); P62 (C). The

relative density for expression of Beclin1 and P62 relative to β -actin. (D)

Representative immunoreactive bands and statistical results showed that Bioch A

increases the protein level of Endophilin A2 in the SNpc of mice. The relative density

for expression of Endophilin A2 relative to β -actin. (E) Representative

immunoreactive bands and statistical results showed that Bioch A increased the

552 relative value of p-FoxO3a/FoxO3a in protein level in the SN of mice. (F)
553 Immunofluorescence images results showed that Bioch A decreases the expression of
554 LC3B in the SNpc of mice. The bar is 100 μ m. (G) The bar graphs representing
555 quantitative analysis of LC3B-positive expression in the SNpc of mice. (H)
556 Immunofluorescence images results showed that Bioch A decreases the expression of
557 FoxO3a in the SNpc of mice. The bar is 100 μ m. (I) The bar graphs representing
558 quantitative analysis of FoxO3a-positive expression in the SNpc of mice. Results are
559 expressed as mean $\pm$ SD (n = 5). * P <0.05, ** P <0.01 vs the sham group, $^{\Delta}$ P <0.05,
560 $^{\Delta\Delta}$ P <0.01 vs the Ang II -treated group.

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