Lipopolysaccharide induces human olfactory ensheathing glial apoptosis by promoting mitochondrial dysfunction and activating the JNK-Bnip3-Bax pathway

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Abstract

Olfactory ensheathing glia (OEG) play an important role in regulating the regeneration of an injured nervous system. However, chronic inflammation damage reduces the viability of OEG via poorly understood mechanisms. We aimed to investigate the pathological responses of OEG in response to LPS-mediated inflammation stress *in vitro*. The results indicated that lipopolysaccharide (LPS) treatment significantly reduced the viability of OEG in a dose-dependent fashion. Mechanistically, LPS stimuli induced mitochondrial oxidative damage, mitochondrial fragmentation, mitochondrial metabolism disruption, and mitochondrial apoptosis activation. Furthermore, we verified that LPS modulated mitochondrial apoptosis by promoting Bax upregulation, and this process was regulated by the JNK-Bnip3 pathway. Inhibition of the JNK-Bnip3 pathway prevented LPS-mediated Bax activation, thus attenuating OEG apoptosis. Altogether, our data illustrated that LPS-mediated inflammation injury evoked mitochondrial abnormalities in OEG damage via the JNK-Bnip3-Bax pathway. This finding provides a potential target to protect OEG against chronic inflammation stress.

Keywords Olfactory ensheathing glia · Mitochondrial dysfunction · Bax · JNK-Bnip3 pathway

Introduction

Olfactory ensheathing glia (OEG), a macroglia subtype, are located exclusively in the nervous system. The primary role of OEG is to produce neurotrophic factors to promote neuronal survival (Ramon-Cueto and Avila 1998). OEG are involved in lifelong neuronal regeneration via the transplantation of cultured OEG into the injured nervous system. For example, OEG engraftment attenuates spinal cord injury (Yao et al.

2018), alleviates peripheral nerve damage (Barton et al. 2017), and lessens focal cerebral ischemia (Augestad et al. 2017). This information indicates that OEG are a promising tool to treat nervous system injury. Notably, the viability of OEG is decreased progressively with age due to the chronic inflammation response (O'Neill et al. 2017). However, the search for inflammation-mediated OEG damage has remained futile.

Cellular viability and function are highly regulated by mitochondria, that act in response to stress stimuli, including oxidative stress, the inflammation response, and ischemia in the nervous system (Santuy et al. 2018; Tao-Cheng 2018). There are three general mechanisms by which mitochondria affect cell viability and death: (i) ATP depletion (Li et al. 2018), (ii) mediation of cellular oxidative stress (Zhou et al. 2018e), and (iii) release of proapoptotic factors that initiate caspase-dependent (Zhou et al. 2018d) and/or caspase-independent death pathways (Zhu et al. 2018b). For example, mitochondrial oxidative stress has been associated with endothelial apoptosis in inflammation stress (Cui et al. 2018). Impaired mitophagy is involved in the development and progression of fatty liver disease via promoting hepatocyte death (Li et al. 2018; Zhou et al. 2018a). Moreover, the pathogenesis of diabetic nephropathy has been linked to excessive mitochondrial fission (Sheng et al. 2018).

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Based on these studies, mitochondria damage has been recognized as a major regulator controlling cell viability and organ homeostasis. Although the characterization of mitochondrial stress in response to inflammation has been widely explored, no study is available to explore the alterations of mitochondrial homeostasis in OEG in inflammation injury. Although chronic TNF α exposure increases the proliferation of OEG (Lankford et al. 2017), the TNF α -induced immuno-inflammation response is not similar to pathological damage (Liu et al. 2015). OEG could secrete TNF α to regulate the immuno-inflammation response in olfactory epithelium (Moon et al. 2002) and neuron development (Bauer et al. 2003). Accordingly, it is particularly noteworthy to determine whether the pathological inflammation microenvironment induces OEG damage that occurs, at least, via mitochondrial abnormalities.

At the molecular level, mitochondria-mediated cell damage or death is primarily dependent on the hyperpermeability of the mitochondrial outer membrane (Zhou et al. 2017b). Mitochondrial membrane permeabilization promotes mitochondrial proapoptotic (including cyt-c, Smac, and HtrA2/Omi) release from the mitochondria into the cytoplasm/nucleus where these proapoptotic factors activate the caspase-9-involved mitochondrial death pathway (Li et al. 2017a). Notably, mitochondrial permeability is primarily controlled by Bax and its activator Bnip3. Increased Bax as well as decreased Bcl2 promotes mitochondrial permeabilization, and these processes may further induce the opening of the mitochondrial permeability transition pore (mPTP) that amplifies mitochondria-initiated death signaling (Jing et al. 2018). Accordingly, Bax activation and mitochondrial proapoptotic factor leakage (especially HtrA2/Omi) are regarded as two key upstream molecular events of mitochondria-related cell death. Whether the inflammation mediates OEG damage via Bax-dependent mitochondrial apoptosis is incompletely understood. Previous studies have used LPS to induce the neuroinflammatory (Rigillo et al. 2018) and found that LPS could cause mitochondrial damage in the axons (Errea et al. 2015), microglia (Ho et al. 2018), and spiral ganglion neurons (Zuo et al. 2015). Based on these reports, we posited and analyzed the following three hypotheses: (1) whether LPS-induced inflammation caused apoptosis in OEG, (2) whether mitochondrial dysfunction was required for LPS-mediated OEG death, and (3) whether LPS modulated OEG viability and mitochondrial homeostasis via the JNK-Bnip3-Bax signaling pathways.

Methods and materials

Cell culture and treatment

Immortalized human olfactory ensheathing glia Bmi1/hTERT (OEG; Applied Biological Materials, Inc.; Cat. No. T0282)

and SH-SY5Y cell (ATCC® CRL-2266™) were used in the present study. Different doses of LPS (0–20 μ g/ml) were used for incubation with OEG for 12 h according to a previous study (Cui et al. 2018). OEG treated with 0 μ g/ml LPS (*Escherichia coli* serotype 0127-B8, Sigma Aldrich L3129) were considered the control group. OEG were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco). The culture condition was set at 37 °C in a humidified 5% CO₂ atmosphere, and the medium was replaced with fresh DMEM and 10% FBS every 3 days. In the present study, SP600125 (25 μ M; Selleck Chemicals, Houston, TX, USA) was added into the medium to inhibit the activity of JNK according to a previous study (Jin et al. 2018).

Western blot analysis

Cells were scraped in RIPA lysis buffer (Beyotime, Shenzhen, Guangdong, China). The lysates (50–70 μ g) were separated by 10% SDS-polyacrylamide gel (10–15%) electrophoresis (SDS-PAGE). Proteins were electrotransferred onto the pure nitrocellulose blotting membrane (Life Sciences) (Millipore, Bedford, MA, USA) and then blocked with 5% nonfat milk for 2 h at room temperature. After washing with TBST three times, the membranes were incubated at 4 °C overnight with the following primary antibodies: HrtA2/Omi (1:1000; Abcam; #ab32092), Bnip3 (1:1000, Cell Signaling Technology, #44060), Bax (1:1000; Cell Signaling Technology; #2772), Tom20 (1:1000; Abcam; #ab186735), p-JNK (1:1000; Cell Signaling Technology; #9251), complex III subunit core (CIII-core2; 1:1000; Invitrogen; #459220), complex II (CII-30; 1:1000 Abcam; #ab110410), and complex IV subunit II (CIV-II; 1:1000; Abcam; #ab110268). Subsequently, anti-rabbit- and anti-mouse-conjugated horseradish peroxidase secondary antibodies (1:2000; Cell Signaling Technology; #7074 and #7076) were incubated with membranes for 45 min at room temperature. Next, the membranes were visualized using an enhanced chemiluminescence system (ECL; Pierce Company, USA).

Immunofluorescence staining

Cells were washed twice with PBS, permeabilized in 0.1% Triton X-100 overnight at 4 °C. After the fixation procedure, the sections were cryoprotected in a PBS solution supplemented with 0.9 mol/l of sucrose overnight at 4 °C (Ackermann et al. 2017). After neutralization with NH₄Cl buffer, the sections were permeabilized for 45 min with 0.05% saponin/PBS (pH = 7.4) and incubated overnight with the following primary antibodies: Bax (1:1000; Cell Signaling Technology; #2772), HrtA2/Omi (1:1000; Abcam; #ab32092), Mff (1:1000, Cell Signaling Technology, #86668), cleaved caspase3 (1:1000; Abcam; #ab49822), Tom20 (1:1000; Abcam;

#ab186735), caspase9 (1:1000; Cell Signaling Technology, #9504), and p-JNK (1:1000; Cell Signaling Technology; #9251). The samples were stained with DAPI (10 nM) for 5 min. Confocal immunofluorescence images were taken using the FV10-ASW 1.7 software and the Olympus IX81 microscope.

Transfection of siRNA against Bnip3

In the present study, transfection of two independent siRNAs against Bnip3 was used to repress LPS-mediated Bnip3 activation. The primers for siRNA were as follows: siRNA1-Bnip3, 5'-GAGATTCTAGGATCCTAGATATC-3'; siRNA1-Bnip3, 5'-ACTTACTTGTCTTATCCTCTCAT-3'; and siRNA-control, 5'-GATCTAATCGGCTAAGCGTATC-3'. For transfection, the culture medium was replaced with OptiMEM medium (Invitrogen), and then the siRNA vector was infected with OEG cells using Lipofectamine 2000 (Invitrogen) based on a previous study (Blackburn et al. 2017). Transfection was performed for 48 h, and then the cells were collected. Western blotting was used to analyze the knockdown efficiency.

Flow cytometry assay

ROS production was analyzed via flow cytometry according to a previous study. Cells were washed with cold PBS and cultured with an ROS probe (1 mg/ml, DHE, Molecular Probes, USA) at 37 °C in the dark for 15 min. After the cells were washed with cold PBS three times, the cells were collected using 0.25% pancreatin. After resuspension in cold PBS, the cells were analyzed using a flow cytometer (BD FACSVerse; BD Biosciences, San Jose, CA, USA) (Das et al. 2017). The number of apoptotic cells was analyzed quantitatively using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, USA). After treatment, the cells were harvested, resuspended in 200 µl of binding buffer, and then incubated with 5 µl of Annexin V-FITC/binding buffer mixture (30 min, 37 °C) in the dark. Subsequently, the cells were incubated with 10 µl of propidium iodide for 5 min and immediately analyzed by bivariate flow cytometry using a BD FACSCalibur cytometer (Zhou et al. 2018f)

TUNEL staining and MTT assay

Apoptotic cells were detected with an In Situ Cell Death Detection Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. C1024) according to the manufacturer's protocol. Briefly, cells were fixed with 4% paraformaldehyde at 37 °C for 15 min. Blocking buffer (3% H₂O₂ in CH₃OH) was added to the wells, and then cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. The cells were incubated with TUNEL reaction mixture for

1 h at 37 °C. DAPI (Sigma-Aldrich, St. Louis, MO, USA) was used to counterstain the nuclei, and the numbers of TUNEL-positive cells were recorded (Boone et al. 2017). MTT was used to analyze the cellular viability. Cells (1×10^6 cells/well) were cultured on a 96-well plate at 37 °C with 5% CO₂ (Alghanem et al. 2017). Then, 40 µl of MTT solution (2 mg/ml; Sigma-Aldrich) was added to the medium for 4 h at 37 °C with 5% CO₂. Subsequently, the cell medium was discarded, and 80 µl of DMSO was added to the wells for 1 h at 37 °C with 5% CO₂ in the dark. The OD of each well was observed at A490 nm via a spectrophotometer (Epoch 2; BioTek Instruments, Inc., Winooski, VT, USA) (Chang et al. 2017).

ELISA

Glutathione (GSH, Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. T10095), glutathione peroxidase (GPX) (Beyotime Institute of Biotechnology, China; Catalog No. S0056), and SOD (Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. BMS222TEN) were measured according to the manufacturer's instructions using a microplate reader (Epoch 2; BioTek Instruments, Inc.) (Zhu et al. 2018a). To analyze changes in caspase-9, caspase-9 activity kits (Beyotime Institute of Biotechnology, China; Catalog No. C1158) were used according to the manufacturer's protocol. In brief, to measure caspase-9 activity, 5 µl of LEHD-p-NA substrate (4 mM, 200 µM final concentration) was added to the samples for 1 h at 37 °C. Then, the absorbance at 400 nm was recorded via a microplate reader to reflect the caspase-3 and caspase-9 activities. To analyze caspase-3 activity, 5 µL of DEVD-p-NA substrate (4 mM, 200 µM final concentration) was added to the samples for 2 h at 37 °C.

Quantitative real-time PCR analysis

For mRNA expression analysis, total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to a previous study. Then, cDNA was synthesized using 1 mg RNA and the First-Strand Synthesis Kit (Fermentas, Flamborough, Ontario, Canada) according to a previous study. The cycling conditions were as follows: 92 °C for 7 min, 40 cycles of 95 °C for 20 s, and 70 °C for 45 s. The following primers were used in the study: Drp1 (forward primer 5'-CATGGACGAGCTGGCCTTC-3', reverse primer 5'-ATCC TGTAGTGATGTATCAGG-3'), Fis1 (forward primer 5'-TGTCAGTCCGTAACCTGAC-3', reverse primer 5'-TTCCG ATACCTGACTTAC-3'), Mfn2 (forward primer 5'-CCTC TTGATCCTGATCTTAACGT-3', reverse primer 5'-GGAC TACCTGATTGTCATTC-3'), OPA1 (forward primer 5'-GCTACTTGTGAGGTCGATTC-3', reverse primer 5'-GCCGTATACCGTGGTATGTCTG-3'), Tom20 (forward

prime 5'-TCTACCTAGTCCATGATG-3', reverse prime 5'-CACAGTGGTACCTAGTCTAG-3'), CIII-core2 (forward prime 5'-CTCTAGGAATCCGATAGTCTA-3', reverse prime 5'-GGCAAGTAGATACCAGTA-3'), CII-30 (forward prime 5'-CCTCTAGATACCGATAGCC-3', reverse prime 5'-AACTTACGATAGGCTGATCCG-3'), and CIV-II (forward, 5'-GAGGGTCCTAGATCCGAT-3'; reverse, 5'-GTTGACCAGACCATAGTCCAT-3').

Mitochondrial potential analysis and mPTP opening detection

To observe the mitochondrial potential, JC-1 staining (Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. M34152) was used. Then, 10 mg/ml JC-1 was added to the medium for 10 min at 37 °C in the dark to label the mitochondria. Normal mitochondrial potential showed red fluorescence, and damaged mitochondrial potential showed green fluorescence (Feng et al. 2017). The mPTP opening rate was detected using calcein-AM (Sigma, Cat. No. 17783) as described previously (Li et al. 2018). Briefly, cells were incubated with calcein-AM for 30 min at 37 °C in the dark. Next, PBS was used to wash the cells three times. Finally, the optical density (OD) at an absorbance of 579 nm was recorded using a multifunction microplate reader (Epoch 2; BioTek Instruments, Inc., Winooski, VT, USA). The mPTP opening rate was calculated as a ratio to that of the control group.

Statistical analysis

All results presented in this study were acquired from at least three independent experiments, and the data in the present study were analyzed using SPSS for Windows version 15.0 (SPSS Inc., Chicago, IL, USA). All values are expressed as the means $\pm$ SEM. The statistical significance was determined by one-way analysis of variance followed by Tukey's post hoc multiple comparison tests. $P < 0.05$ was considered statistically significant.

Results

LPS reduces OEG viability in a dose-dependent manner

To induce the inflammation stress, LPS was used in the present study. Next, the cell viability was determined via the MTT assay. As shown in Fig. 1(a), compared with the control group (0 μ g/ml LPS), LPS treatment significantly reduced the cell viability. Interestingly, with the increase in the dose of LPS, the cell viability of OEG was progressively decreased, indicating that LPS dose-dependently reduced the OEG viability. This result was further supported by the LDH cytotoxicity assay.

As illustrated in Fig. 1(b), LPS stress obviously increased the LDH concentration in the medium of OEG. LDH would be released into the medium upon cell membrane breakage. Accordingly, the increased LDH levels in the medium indicated that LPS mediated OEG death in a dose-dependent manner. Subsequently, to reconfirm whether LPS-induced OEG death resulted from cell apoptosis, TUNEL staining and caspase-3 activity detection were performed. Compared with the control group, the number of TUNEL-positive cells was elevated upon LPS exposure (Fig. 1(c, d)). Moreover, caspase-3 activity was also increased after LPS treatment compared with that in the control group (Fig. 1(e)). In addition to caspase-3 activation, the expression of cleaved caspase-3 was also dose-dependently upregulated in answer to LPS treatment (Fig. 1(g–f)). These data highlighted that LPS-mediated OEG death was attributable to excessive apoptosis. Altogether, our results indicated that LPS-related inflammation stress promoted OEG death via apoptosis. Notably, the minimal fatal concentration of LPS was 5 μ g/ml. Accordingly, LPS at 5 μ g/ml was used in the present study.

LPS mediates mitochondrial oxidative stress and energy disorder

Previous studies have identified mitochondria as a potential target of inflammation injury (Yan et al. 2018; Zhu et al. 2018a). The primary consequences of mitochondrial injury include mitochondrial oxidative stress (Zhou et al. 2018c) and ATP depletion (Shi et al. 2018). To investigate whether mitochondrial damage accounted for LPS-induced OEG apoptosis, we observed the alterations in the mitochondrial redox balance and energy metabolism. First, mitochondrial ROS production was measured using a mitochondrial ROS probe. Next, flow cytometry was used to quantify mitochondrial ROS. Compared with the control group, 5 μ g/ml LPS significantly elevated the levels of mitochondrial ROS (Fig. 2(a, b)), suggesting mitochondrial ROS overloading under the LPS-related inflammation microenvironment. Subsequently, cellular antioxidants were measured to further analyze the alterations of redox balance. Compared with the control group, the concentration of antioxidants (SOD, GSH, and GPX) was significantly decreased in response to 5 μ g/ml LPS application (Fig. 2(c–e)). These results further confirmed the existence of redox imbalance in LPS-mediated OEG due to mitochondrial ROS overproduction.

Concerning mitochondrial energy metabolism, ATP production was measured because cellular total ATP was mainly generated by mitochondria (Fuhrmann and Brune 2017). Compared with the control group, 5 μ g/ml LPS significantly reduced the ATP concentration (Fig. 2(f)), indicating ATP depletion under LPS-related inflammation stress. Furthermore, ATP synthesis is closely dependent on the activity of the mitochondrial respiratory complex that consumes

Fig. 1 LPS attenuates OEG viability. (a) OEG viability was measured by the MTT assay. (b) LDH cytotoxicity was used to analyze OEG cell death in response to different concentrations of LPS. (c, d) TUNEL staining for cell death. TUNEL-positive cells were identified as the dead cells. (e) Caspase-3 activity was measured using ELISA. (f, g) Expression of cleaved caspase-3 (Cle. caspase-3) was determined via immunofluorescence. * $p < 0.05$ vs. control group (0 $\mu\text{g/ml}$ LPS treatment was used as the control group)

oxygen and glucose to generate ATP (Gadicherla et al. 2017). Thus, alteration of the mitochondrial respiratory complex was analyzed via western blotting. Compared with the control group, the expression of the mitochondrial respiratory complex was downregulated in LPS-treated cells (Fig. 2(g–j)). This finding was supported via qPCR (Fig. 2(k–m)). We also found that LPS treatment induced cyt-c liberation from mitochondria into the cytoplasm (Supplemental Fig. A–C), and this process was accompanied with an increase in cell apoptosis, as assessed via the flow cytometry (Supplemental Fig. D–E). Collectively, our results demonstrated that LPS-mediated inflammation stress triggers mitochondrial oxidative stress and mitochondrial respiratory complex downregulation.

LPS triggers mitochondrial fragmentation

Mitochondrial dysfunction may be attributed to alterations of the mitochondrial structure. Therefore, we further observed the change in mitochondrial morphology. Mitochondrial immunofluorescence was performed using the mitochondrial specific antibody Tom-20. As shown in Fig. 3(a), during LPS-induced inflammation stress, the mitochondria became smaller, shorter, roundish fragments characteristic of increased mitochondrial fission and/or decreased mitochondrial fusion. Subsequently, the average mitochondrial length was measured. Mitochondria were $\sim 3.5 \mu\text{m}$ in the control cells

(Fig. 3(b)), whereas LPS treatment reduced the mitochondrial length to $\sim 1.7 \mu\text{m}$. To verify the promotive role of LPS in mitochondrial fragmentation, antagonist (Mdivi-1) and agonist (FCCP) were used as the positive control group and negative control group, respectively. In control group, FCCP supplementation obviously induced the formation of mitochondrial debris, and this effect was similar to the action of LPS treatment (Fig. 3(a, b)). However, in LPS-treated cells, Mdivi-1 treatment significantly repressed LPS-mediated mitochondrial fragmentation (Fig. 3(a, b)). These results reconfirmed that LPS mediates mitochondrial fragmentation, a type of mitochondrial structural damage.

According to previous studies (Zhou et al. 2018b, e), the formation of mitochondrial fragmentations results from excessive mitochondrial fission and/or inactive mitochondrial fusion. Mitochondrial fission factor (Mff) has been identified as a primary activator of mitochondrial fission. Accordingly, coimmunofluorescence assay was used to observe the levels of Mff. As shown in Fig. 3(a, c), the level of Mff was significantly increased in response to LPS treatment, and this effect was abolished by Mdivi-1 treatment. Besides, with the help of qPCR, we further demonstrated that the transcription of pro-fission proteins (such as Drp1 and Mff) was significantly augmented by LPS in OEG (Fig. 3(d, e)), an effect that was accompanied by reduced levels of pro-fusion proteins including Mfn2 and Opa1 (Fig. 3(f, g)). These results revealed that

Fig. 2 LPS induces mitochondrial dysfunction as manifested by mitochondrial oxidative stress and mitochondrial respiratory complex downregulation in OEG. (a) Mitochondrial ROS production was stained using a mitochondrial ROS probe. Flow cytometry was used to quantify mitochondrial ROS. (b) Quantification of mitochondrial ROS production. (c) ELISA for cellular antioxidant. Quantification of the GSH concentration in response to LPS treatment. (d) Quantification of the SOD concentration in response to LPS treatment. (e) Alteration of GPX

activity under LPS stress. (f) ELISA for ATP concentration in OEG under LPS treatment. (g) The mitochondrial respiratory complex was evaluated using western blotting. (h) Quantification of complex III subunit core (CIII-core2) expression under LPS treatment. (i) Quantification of complex II (CII-30) expression under LPS treatment. (j) Quantification of complex IV subunit II (CIV-II) expression under LPS treatment. (k–m) qPCR assay for mitochondrial respiratory complex. * $p < 0.05$ vs. control group (0 $\mu\text{g/ml}$ LPS treatment was used as the control group)

LPS stress induces mitochondrial fragmentation via directly upregulating mitochondrial fission and downregulating mitochondrial fusion.

LPS activates mitochondrial apoptosis via Bax upregulation and HtrA2/Omi2 release

Excessive mitochondrial dysfunction has been well recognized as a major apoptosis inducer of inflammation injury. As we mentioned above, mitochondrial Bax activation and HtrA2/Omi release are typical features of mitochondrial apoptosis. Using western blotting, we found that the expression of Bax was significantly upregulated in response to LPS treatment (Fig. 4(a–c)). By contrast, the expression of Bcl-2, an antagonist of Bax, was rapidly downregulated after exposure to LPS (Fig. 4(a–c)). This result indicated that LPS treatment

contributed to Bax upregulation. Notably, LPS-mediated Bax upregulation and Bcl2 downregulation were also noted in SH-SY5Y cells, indicating that LPS-mediated Bax upregulation is specific for the nervous system inflammation injury (Supplemental Fig. F–H). One of the primary consequences of Bax upregulation is mitochondrial membrane hyperpermeability. To detect mitochondrial permeability, mPTP opening was observed. LPS stress promoted the opening of mPTP in OEG (Fig. 4(d)).

Regarding mitochondrial HtrA2/Omi2 release, immunofluorescence assay was used to observe HtrA2/Omi translocation into the nucleus. Compared with the control group, the expression of nuclear HtrA2/Omi was visibly increased in LPS-treated cells (Fig. 4(e)). This result was further validated by western blotting, that showed an increase in the levels of cytoplasmic HtrA2/Omi (cyto-HtrA2/Omi) after LPS

Fig. 3 LPS promotes the formation of mitochondrial fragmentation in OEG. (a) Mitochondria fragmentation formation was determined using immunofluorescence. Mitochondria-specific antibody Tom-20 was used to observe mitochondrial debris. To verify the promotive role of LPS in mitochondrial fragmentation, antagonist and agonist of mitochondrial fragmentation were used as the positive control group and negative control group, respectively. In control group, FCCP, an activator of mitochondrial fragmentation, was applied to incubated OEG. In LPS-treated cells, Mdivi-1, an inhibitor for mitochondrial fragmentation, was added into the medium of OEG. (b) The average length of mitochondria

was evaluated. (c) Expression of Mff (mitochondrial fission factor) was measured via immunofluorescence. The relative immunofluorescence intensity of Mff was determined to quantify Mff expression. (d) Cells were treated with LPS, and then the transcription of mitochondrial fission-related proteins was detected by qPCR. Quantification of Drp1 transcription. (e) Quantification of Fis1 transcription in OEG treated with LPS. (f) Quantification of Mfn2 transcription in response to LPS stress. (g) Quantification of Opa1 transcription in the presence of LPS treatment. * $p < 0.05$ vs. control group (0 $\mu\text{g/ml}$ LPS treatment was used as the control group); # $p < 0.05$ vs. LPS group

treatment (Fig. 4(f–h)). After release into the cytoplasm/nucleus, HtrA2/Omi could activate caspase-9 via suppressing caspase-9 inhibitors (Li et al. 2017a), and the latter, in turn, promotes caspase-3 activation (Hu et al. 2017). In the present study, we found that LPS treatment elevated the expression (Fig. 4(i, j)) and activity (Fig. 4(k)) of caspase-9 compared with that in the control group. In answer to caspase-9 activation, the activity of caspase-3 was also significantly increased in LPS-treated cells (Fig. 4(l)). Altogether, our results

indicated that LPS activates mitochondrial apoptosis by promoting Bax upregulation and HtrA2/Omi release.

LPS modulates Bax activation via the JNK-Bnip3 pathway

According to a recent study, Bax upregulation could be mediated by Bnip3 (Lalier et al. 2007), a proapoptotic factor expressed in the nervous system. Additionally, another study

Fig. 4 LPS activates mitochondrial apoptosis via Bax upregulation and HtrA2/Omi2 leakage in OEG. (a) Cells were treated with LPS, and then the expression levels of Bax and Bcl-2 were determined by western blotting. (b) Quantification of Bax expression in response to LPS treatment. (c) Quantification of Bcl-2 expression in the presence of LPS stress. (d) The mPTP opening rate was determined in OEG treated with LPS. (e) Immunofluorescence assay for HtrA2/Omi. DAPI was used to

indicate the nucleus. Colocation of HtrA2/Omi and the nucleus indicates HtrA2/Omi leakage into the nucleus. (f–h) Mitochondrial proteins were isolated, and the expression of mitochondrial HtrA2/Omi (mito-HtrA2/Omi) was determined. (i, j) Caspase-9 expression was measured via immunofluorescence assay. (k, l) ELISA was used to quantify caspase-9 activity and caspase-3 activity. * $p < 0.05$

further reported that Bnip3 is primarily regulated by the JNK pathway (Jin et al. 2018). Based on this information, we asked whether LPS mediated Bax upregulation via the JNK-Bnip3 pathway. First, western blotting displayed that phosphorylated JNK (p-JNK) and Bnip3 were significantly increased in response to LPS treatment (Fig. 5(a–d)), indicating activation of the JNK-Bnip3 pathway. Next, to verify whether the JNK-Bnip3 pathway was required for LPS-mediated Bax upregulation, SP600125, the selective antagonist of the JNK pathway, was used. As shown in Fig. 5(a–d), SP600125 significantly inhibited LPS-mediated JNK phosphorylation and Bnip3 activation, and this effect was closely associated with a decrease in Bax expression. To explore the role of Bnip3 in Bax activation, two independent siRNAs against Bnip3 were transfected into OEG, and the knockdown efficiency confirmed via western blotting (Fig. 5(e–g)). Transfection of Bnip3 siRNA prevented LPS-mediated Bax activation. Our data indicated that LPS promotes Bax upregulation via the JNK-Bnip3 pathway. This finding was further supported via immunofluorescence. The fluorescence intensity of p-JNK and Bax was significantly augmented in response to

LPS treatment (Fig. 5(h–j)), and these effects could be negated by SP600125. Altogether, our results demonstrated that LPS modulates Bax upregulation via the JNK-Bnip3 pathway.

The JNK-Bnip3 pathway is also involved in LPS-mediated OEG death

Ultimately, we focused on the regulatory effects of the JNK-Bnip3 pathway on LPS-induced OEG death. First, the LDH release assay was conducted to analyze the cell death index with JNK inhibition and Bnip3 siRNA in the presence of LPS-related inflammation stress. Compared with the control group, LPS promoted LDH release, and this effect was inhibited by SP600125 and Bnip3 siRNA (Fig. 6(a)). Subsequently, TUNEL assay was performed to observe the cell apoptotic rate. Compared with the control group, LPS elevated the percentage of TUNEL-positive cells, and this effect was reversed by SP600125 and Bnip3 siRNA (Fig. 6(b, c)). Furthermore, caspase-9 activity and mitochondrial ROS production were determined to confirm the contribution of the JNK-Bnip3

Fig. 5 The JNK-Bnip3 pathway is required for LPS-mediated Bax upregulation in OEG. (a) Proteins were isolated from cells treated with LPS, and then western blotting was performed to analyze the expression of phosphorylated JNK (p-JNK), Bnip3, and Bax. SP600125 was used to inhibit the activity of JNK. (b) Quantification of p-JNK expression. (c) Quantification of Bnip3 expression. (d) Quantification of Bax expression.

(e–g) Proteins were isolated from cells treated with LPS. Then, two independent siRNAs against Bnip3 were transfected into OEG. Then, knockdown efficiency was verified via western blotting. (h–j) Immunofluorescence assay for p-JNK and Bax. OEG were treated with SP600125 or transfected with Bnip3 siRNA. Then, the fluorescence intensities of p-JNK and Bax were measured. * $p < 0.05$

pathway to mitochondrial damage. Compared with the control group, LPS stress elevated caspase-9 activity (Fig. 6(d)) and induced mitochondrial ROS overloading (Fig. 6(e, f)). Interestingly, SP600125 and Bnip3 siRNA could significantly repress caspase-9 activity and neutralize excessive mitochondrial ROS (Fig. 6(d–f)), suggesting that the JNK-Bnip3 pathway was responsible for LPS-mediated mitochondrial damage. Altogether, our results underscored the necessary role played by JNK-Bnip3 in LPS-mediated OEG death.

Discussion

Olfactory ensheathing glia (OEG) have been widely used in the field of nervous system regenerative medicine. At the molecular level, OEG express several extracellular matrix proteins, including but not limited to, laminin, fibronectin, neural/glia antigen 2 (NG2), and galectin-1. These extracellular matrix proteins play pivotal roles in neurogenesis and regeneration (Cao et al. 2010; Tan et al. 2005). Additionally,

Fig. 6 The JNK-Bnip3 pathway regulates LPS-mediated OEG death. (a) The LDH release assay was performed to analyze cell death in response to LPS treatment. OEG were also treated with SP600125 or transfected with Bnip3 siRNA. (b, c) TUNEL staining for apoptotic cells. Green dots in the nucleus indicate apoptotic cells. (d) Caspase-9 activity was measured

using ELISA. OEG were treated with SP600125 or transfected with Bnip3 siRNA in the presence of LPS stress. (e–f) Mitochondrial ROS was measured using flow cytometry. OEG were treated with SP600125 or transfected with Bnip3 siRNA in the presence of LPS stress. * $p < 0.05$

after serious injuries, such as olfactory bulb ablation, OEG proliferate and migrate to the damaged tissues and then produce protective cytokines for organ repair (Chehrehasa et al. 2014; Nan et al. 2001). Moreover, OEG activate the immune response (Lane et al. 2010), alleviate oxidative stress (Jinbo et al. 2013), enhance the clearance of neuron debris (Li et al. 2017b), and promote neuron survival (Wright et al. 2018). This information highlights that OEG-based regeneration

medicine is emerging as a promising approach to treat injuries in the peripheral/central nervous system (Liu et al. 2018). Nevertheless, OEG viability decreases with age due to the chronic inflammation response, that is especially evident in Parkinson's disease and other neurodegenerative disorders (Lei et al. 2018). Therefore, understanding the molecular mechanism by which the inflammation stress induces OEG dysfunction may pave the road to new

treatment modalities that are urgently needed for OEG-based regenerative medicine.

In the present study, our results revealed that LPS treatment dose dependently reduced the viability of OEG due to the increased apoptotic rate. Our conclusion seems to be opposed to the previous observation that chronic TNF α exposure promotes the proliferation of OEG (Lankford et al. 2017). To explain the apparent inconsistent results, several differences should be noted. The previous study used a low TNF α concentration to induce the immuno-inflammation response (Lankford et al. 2017), a finding that is totally different from our model. In the current study, LPS stress was used to mimic the pathological process after neuron injury. More importantly, under physiological conditions, OEG itself could generate and secrete TNF α to regulate immuno-inflammation in olfactory epithelium (Bauer et al. 2003) and neuron development (Moon et al. 2002). Accordingly, several previous studies have argued that OEG should be considered primary immune cells in the healthy and injured nervous system (Lane et al. 2010). Therefore, LPS incubation could be more appropriate to explore the mechanism by which the inflammation stress mediates OEG damage.

Notably, previous studies have used LPS to induce the neuroinflammation model and observe the nerve damage. For examples, LPS is employed to explore whether chronic inflammation plays a role in the progressive dopaminergic neurodegeneration that occurs in Parkinson's disease (Hunter et al. 2017). LPS-mediated neurotoxicity is associated with brain dopaminergic neuronal dysfunction (Kaizaki et al. 2013). LPS administration has been used to induce an in vivo model for neurodegeneration such as the Alzheimer disease (Noh et al. 2014). In the current study, LPS was used to observe the damage mechanism of OEG apoptosis. At the molecular level, we observed the mitochondrial biological response in the presence of LPS stress. Mitochondrial ROS overloading, cellular ATP depletion, mitochondrial respiratory complex downregulation, mPTP opening, and mitochondrial apoptosis activation were noted in the LPS-treated OEG. This information indicated that mitochondria are the potential targets of LPS-related inflammation injury. This information is in accordance with the previous findings. In inflammation-related neurodegenerative disease (Lei et al. 2018), mitochondrial fission is activated and contributes to mitochondrial oxidative stress. Additionally, IL-1 β -mediated neurodegeneration is associated with mitochondrial oxidative stress and mitochondrial depolarization (McFarland et al. 2018). Moreover, inflammation-induced depressive-like behavior is related to mitochondrial abnormalities (Chen et al. 2017). Altogether, these data highlight the key role played by mitochondria in sustaining neuron viability. Accordingly, the preservation of mitochondrial integrity is of utmost importance when designing OEG protective therapies in the clinic.

Furthermore, we explained that LPS repressed mitochondrial dysfunction by activating the JNK-Bnip3 pathway. An augmented JNK-Bnip3 axis elevates Bax expression, an effect that was closely followed by more HtrA2/Omi translocation from the mitochondria into the cytoplasm/nucleus. Notably, Bax-mediated mitochondrial apoptosis and HtrA2/Omi release have been widely investigated in in vivo and in vitro studies (Zhou et al. 2017a, 2018f). Mechanistically, increased Bax accumulates onto the surface of the mitochondrial outer membrane and mediates mitochondrial permeabilization (Westphal et al. 2011); this effect induces mitochondrial potential loss and HtrA2/Omi release (Reyna and Gavathiotis 2016). The primary finding in the present study is that we identified the JNK-Bnip3 pathway as the upstream mediator of Bax upregulation. Bnip3 is a proapoptotic protein whose expression is primarily upregulated by ischemic stress (Jin et al. 2018). Increased Bnip3 could repress Bcl-2 activity and subsequently augment Bax-mediated mitochondrial permeabilization (Zhou et al. 2018e). Additionally, Bnip3 interrupts protective mitophagy, causing accumulation of damaged mitochondria (Jin et al. 2018; Zhu et al. 2018b). In traumatic brain injury, Bnip3 activation has been acknowledged as the pathogenesis of neuron apoptosis (Li et al. 2013). Similarly, in manganese-induced neurotoxicity and ischemic stroke, Bnip3 upregulation exacerbates mitochondrial dysfunction (Prabhakaran et al. 2009) and neuronal death (Cho and Toledo-Pereyra 2008). In the present study, for the first time, we report that LPS triggers mitochondrial injury via the JNK-Bnip3 pathway. The present study highlights the mechanism controlling mitochondrial homeostasis in the presence of LPS-related inflammation stress. Notably, several signaling pathways have been found to be associated with OEG viability, such as TGF β 1 (Li et al. 2017b), Nurr1 (Liu et al. 2018), PI3K/Akt (Tang et al. 2017), and Runx1/3 (Wang and Stifani 2017). Additional studies are required to explore whether these pathways are involved in the inflammation-mediated OEG damage. Our study only explored the caspase-related apoptosis pathway in response to LPS treatment. The caspase-independent mechanisms have also been noted in LPS-mediated cell damage such as myocardial ischemia reperfusion (Zhu et al. 2018b), acute renal injury (Dong et al. 2018), and central nervous system neuroinflammation (Shao et al. 2018). Further investigation is needed to describe the detailed role of caspase-independent pathway in OEG damage in the setting of LPS-mediated inflammation injury.

Altogether, our study found that LPS-induced inflammation stress reduces OEG viability by mediating mitochondrial injury in a manner dependent on the JNK-Bnip3 pathway. These data lay the foundation for detailed study of the molecular mechanisms involving OEG damage and provide new strategies to sustain OEG function during inflammation injury. However, there are several limitations in the present study. Firstly, we did not explain the mechanism by which LPS

induced Bax upregulation. This seems to be associated with Bax mitochondrial relocation and/or LPS-mediated post-transcriptional modification of Bax. Secondly, LPS-mediated Bcl2 downregulation may be a result of Bax upregulation, and this concept needs further investigation. Finally, although we used the inhibitor and knockdown assay to establish the roles of JNK and Bnip3 in LPS-mediated mitochondrial damage, the pathway agonist and overexpression assay are necessary to further support our findings.

Authors' contributions MWH, ZMX, and JMC were involved in the conception and design, performance of experiments, data analysis and interpretation, and manuscript writing. LBX, YTD, FQL, and JMC were involved in data analysis and interpretation.

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Compliance with ethical standards

Ethics approval and consent to participate Not applicable.

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