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ORIGINAL ARTICLE

Trehalose Alleviates PC12 Neuronal Death Mediated by Lipopolysaccharide-Stimulated BV-2 Cells via Inhibiting Nuclear Transcription Factor NF-κB and AP-1 Activation

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Abstract Inflammation is implicated in the pathogenesis of Parkinson's disease (PD). Trehalose is a disaccharide which exhibits a variety of effects like anti-aggregation, autophagy enhancement in PD. It has also been known to suppress inflammation in many experimental models, involving endotoxin shock, murine dry eye and subarachnoid hemorrhage. However, whether trehalose has an anti-inflammation effect on PD is largely unknown. In the present study, we found trehalose inhibited generation of interleukin-1β, interleukin-6, tumor necrosis factor-α, and nitric oxide in the conditioned medium released from lipopolysaccharide (LPS)-stimulated BV-2 cells. LPS-induced nuclear transcription factors of NF-κB and AP-1 activation were also inhibited by trehalose. Then the conditioned medium of BV-2 cells was applied to PC12 neurons. As a result, both MTT and LDH indicated that trehalose decreased PC12 neuronal death. TUNEL assay showed that trehalose suppressed apoptosis of PC12 neurons. These results implied that trehalose exerted a protective effect on PC12 neurons against the neurotoxic effect triggered by BV-2 microglial activation through inhibiting NF-κB and AP-1 activation and inflammatory mediators and cytokines production in BV-2 cells.

Keywords Parkinson's disease · Trehalose · Inflammation · Microglia · Lipopolysaccharides

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disease characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc). However, the cause of PD remains elusive. Recently, emerging evidence has demonstrated that inflammatory responses manifested by glial reactions and increased expression of inflammatory cytokines are recognized as prominent features of PD. Inflammatory mediators such as NO, TNF-α, and interleukin-1β (IL-1β) derived from non-neuronal cells including microglia are believed to modulate the progression of neuronal cell death in PD (Glass et al. 2010).

Microglia are resident macrophages of the central nervous system (CNS) that display high functional similarities to other tissue macrophages acting as the primary responding cells during infection and injury in the CNS (Czeh et al. 2011). LPS, as the major cell wall constituent of gram-negative bacteria, is a potent stimulator of microglia (Kierdorf and Prinz 2013; Mayer 1998). LPS exists almost everywhere in our living environment, such as cigarette, contaminated food and medicine, and non-sterile water. Activated microglia cause neurotoxic effects by accelerating the production of pro-inflammatory mediators such as NO, PGE2, and pro-inflammatory cytokines IL-6, TNF-α, and IL-1. IL-10 is believed to be a protective

cytokine in inflammation response with broad anti-inflammatory properties, which can suppress the production of pro-inflammatory cytokines such as TNF and IL-12 (Graeber and Streit 2010; Stewart et al. 2013; Sanjabi et al. 2009). NF-κB and AP-1 are two important transcriptional factors involved in regulating expression of pro-inflammatory mediators, including cytokines, chemokines, and adhesion molecules, thereby playing a critical role in mediating inflammatory responses (Rothwarf and Karin 1999; Chen et al. 2013). Previous studies have also demonstrated that a decrease in the number of pro-inflammatory mediators in microglia may attenuate the severity of PD disorders (Eikelenboom and van Gool 2004; Liu and Hong 2003). Therefore, elucidating the mechanism for inflammatory responses and identifying specific inhibitor of inflammatory process might potentially prevent or slow down the progression of PD.

Trehalose (α-D-glucopyranoside) is a nontoxic disaccharide which can be administered safely and orally and has been accepted as a safe food ingredient by the European regulation system following approval by the US Food and Drug Administration (Richards et al. 2002). It does not readily cross cell membranes, but can be efficiently loaded into mammalian cells through endocytosis and pinocytosis, exerting a variety of effects such as anti-aggregation and autophagy enhancement (Wolkers et al. 2001; Sarkar et al. 2007). Additionally, it has also been suggested that trehalose has an anti-inflammatory effect and may be useful in future studies on the pathogenesis and prevention of endotoxic shock, subarachnoid hemorrhage and murine dry eye (Minutoli et al. 2008; Echigo et al. 2012; Li et al. 2012). It has been suggested that trehalose has suppressive effects on several pathological events after SAH, including vasospasm, inflammatory responses, and lipid peroxidation (Echigo et al. 2012). Thus, trehalose may be a new therapeutic approach for treatment of inflammation. However, whether trehalose has an anti-inflammatory effect on PD has not yet been clarified.

In this study, we sought to investigate the cross-talk between BV-2 microglial activation and PC12 neuronal death and whether trehalose possesses an anti-inflammatory effect against LPS-stimulated inflammatory responses in BV-2 cells to protect PC12 neurons. Our results indicated that LPS could stimulate BV-2 cells to release pro-inflammatory cytokines and mediators including IL-1, IL-6, TNF-α, and NO through activation of NF-κB and AP-1, and the conditioned medium from the stimulated BV-2 cells could cause PC12 neuronal death. Trehalose inhibited BV-2 inflammatory reactions by suppressing NF-κB and AP-1, and then decreasing the production of IL-1, IL-6, TNF-α, and NO, suggesting that trehalose might be promising for the treatment of neuroinflammatory and neurodegenerative diseases.

Materials and Methods

Materials

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BV-2 microglial cell line and PC12 dopaminergic neurons are commercial sources and are bought from the Cell Bank of the Shanghai Institute of Cell Biology and Biochemistry, Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (Grand Island, NY, USA). All other chemicals and reagents were of the highest grade available from local commercial sources.

Cell Culture and Treatment

The murine BV-2 cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, Grand Island, NY, USA) at 37 °C in a humidified incubator with 5 % CO_2. All experiments were carried out 24 h after the cells were seeded. The cells were treated with the indicated trehalose or sucrose (Sigma, USA) diluted in PBS (pH 7.2) with the final concentrations being 10, 50 mM, respectively, and LPS (1 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) at the same time for 24 h. The supernatants were removed and replaced with fresh DMEM for another 24 h. Then BV-2 cells were harvested for experiments. The conditioned medium of BV-2 cells was removed and added to PC12 neurons, which were cultured in DMEM supplemented with 10 % fetal bovine serum, 5 % horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified 5 % CO_2 and 95 % air.

Enzyme-Linked Immunesorbant Assay (ELISA) for Pro-Inflammatory Cytokines

The conditioned medium collected from BV-2 cells treated with LPS, trehalose and sucrose for 24 h were evaluated for production of pro-inflammatory cytokines using the ELISA. Each well of the microplate was coated with the capture antibodies, IL-1β, IL-6, TNF-α, and IL-10 (BD Biosciences, San Jose, Calif.), incubated overnight at 4 °C, and washed with phosphate-buffered saline containing 0.05 % Tween 20 (PBS-T). After the microplates were blocked with 3 % bovine serum albumin (BSA) in PBS, cultured supernatants and diluted standards were added to the wells and incubated overnight at 4 °C. After any unbound substances were washed away, biotinylated antibodies against IL-1β, IL-6, and TNF-α were added and incubated at room temperature for 45 min. Following

several washes, the plates were incubated with streptavidin–alkaline phosphatase for 30 min. Substrate p-nitrophenyl phosphate (Sigma) solution was then added to each well for color development. The optical density of each well was determined using a microplate reader at 405 nm, and the amount of cytokines was calculated from the standard curve.

Nitric Oxide Detection Assay

Concentrations of NO in the conditioned medium separated from BV-2 cells by centrifugation were determined as nitrite, a major product of NO, using Griess reagent. The conditioned medium were collected and mixed with the same amount of Griess reagent. After 30 min, optical density was measured at 540 nm, and concentrations of NO in the conditioned medium were calculated with reference to a standard curve of sodium nitrite generated by known concentrations.

Western Blot Analysis

Twenty micrograms of protein from the BV-2 cells were subjected to 12 % SDS-PAGE, transferred to PVDF, blocked, and probed overnight at 4 °C with monoclonal rabbit anti-NF-κB (1:1,000, Epitomics, USA) or rabbit anti-AP-1 (1:1,000, Epitomics, USA) and monoclonal rabbit anti-GAPDH (1:8,000, Epitomics, USA). After washing in TBST, the immunoblots were incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 1 h. The immunoblots were developed with an enhanced chemiluminescence (ECL) reagents (Millipore, USA), and measured with Quantity Software (Bio-Rad, CA). To compare protein loading, antibody directed against GAPDH was used.

Immunofluorescence

After BV-2 cells were seeded on the climbing slices for 24 h, the cells were treated with the indicated trehalose or sucrose diluted in PBS with the final concentrations being 10, 50 mM, respectively, and LPS 1 μg/mL at the same time for 24 h. Then after the supernatant was removed, the climbing slices were used for immunostaining analysis. Non-specific binding was blocked with 3 % bovine serum (Serotec, UK), and permeabilized with 0.1 % Triton X-100 in 1 % BSA-PBS for 30 min. The climbing slices were incubated at 4 °C overnight with monoclonal mouse p-NF-κB/p65 (1:1,000; Chemicon, USA), and then incubated with corresponding secondary antibodies at room temperature for 2 h. The nucleus was stained by Hoechst 33,342 (1 μg/ml, Sigma-Aldrich, USA). Control climbing slices were run following identical protocols, but omitting the primary antibodies. For each group, three cell climbing slices were examined in a blinded fashion.

MTT Assay

Cell viability was measured by MTT assay. PC12 neurons were plated at a density of 2×10^3 cells per well in 96-well plates. After being treated by the conditioned medium separated from BV-2 cells for 24 h, MTT solution at the final concentration of 5 mg/mL was added to each well and the plates were incubated for another 4 h. One hundred and fifty microlitre DMSO (dimethyl sulfoxide) was then added to solubilize MTT tetrazolium crystal. Finally, the optical density was determined at 570 nm using a Multiskan MK3 microplate reader (Thermo Electron Corporation) (van Meerloo et al. 2011).

LDH Assay

Cytotoxicity was determined by the release of LDH, a cytoplasmic enzyme released from cells, and a marker of membrane integrity. LDH release into the culture medium was detected using a diagnostic kit (promega, USA) according to the manufacturer's instructions. Briefly, 50 mL supernatant from PC12 neurons from each well was collected to assay LDH release. The samples were incubated with reduced form of nicotinamide adenine dinucleotide (NADH) and pyruvate for 15 min at 37 °C and the reaction was stopped by adding 0.4 mol/L NaOH. The activity of LDH was calculated from the absorbance at 440 nm and background absorbance from culture medium that was not used for any cell cultures was subtracted from all absorbance measurements. The results were normalized to the maximal LDH release, which was determined by treating control wells for 60 min with 1 % TritonX-100 to lyse all cells.

In Situ Apoptosis Detection

PC12 neurons were observed to determine how conditional medium from BV-2 cells cultured wells caused death of PC12 neurons by a tunel method. For in situ cell death detection, PC12 neurons from different treatment groups were stained using an in situ apoptosis detection kit (Roche). Briefly, DNA strand breaks of apoptotic cells were visualized by terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL reaction). PC12 neurons were fixed, permeabilized, and incubated with TUNEL reaction mixture for 60 min at 37 °C in the dark following the kit protocol. All the preparation of solution and buffer was performed following the description for the commercial kit and the TUNEL reaction mixture was prepared immediately before use. After counterstaining with Hoechst 33,342 (1 μg/ml, Sigma-Aldrich,

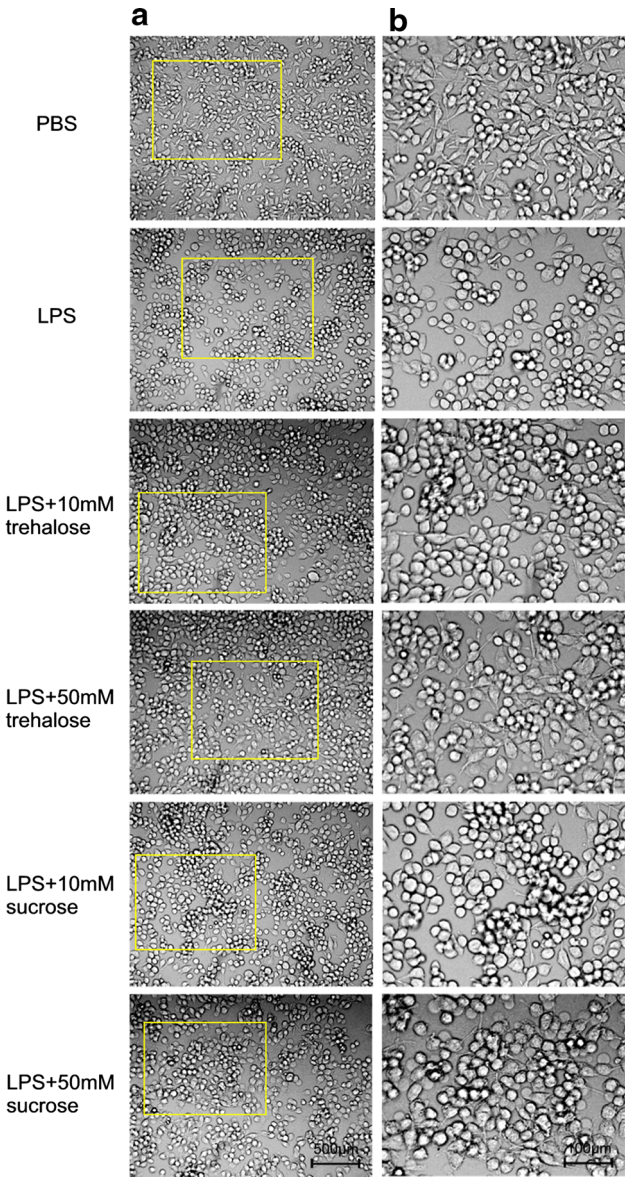

Fig. 1 Morphological changes in LPS-stimulated and trehalose-treated BV-2 cells. **a** *scale bar* is 500 μm. **b** magnification of 5 × within the *yellow rectangles* in (**a**). *Scale bar* is 100 μm (Color figure online)

USA), Fluorescein labels incorporated in the nucleotide polymers were detected and photographed by fluorescence microscopy.

Statistical Analysis

All measurements stated above were repeated at least three times for each experiment, and the data were expressed as mean ± SEM. For all statistical comparisons we first used a one-way ANOVA, with significance set at $P < 0.05$. If ANOVA was significant, all posthoc tests were conducted using Tukey's Multiple Comparison test. Software used to conduct statistical analyses and graph all data was GraphPad Prism 4 (GraphPad Software, Inc.).

Results

Morphological Changes of BV-2 Cells Subjected to LPS Stimulation and Trehalose Treatment

We first observed the morphological change of BV-2 cells stimulated by LPS and trehalose. The normal BV-2 cells exhibited slender, with long projections from the cell bodies. After stimulated by LPS, the cell bodies of activated BV-2 cells became larger and round, and the cellular projections retracted. Ten millimolar trehalose treatment showed some improvement on the retraction of cellular projections. Fifty millimolar trehalose treatment showed obvious improvement on the retraction of cellular projections, as well as recovery of cell bodies from larger and round to slender. 10 and 50 mM sucrose also had some improvement on the retraction of cellular projections mainly because of the high nutrition, but not as obvious as trehalose (Fig. 1a, b).

Trehalose Reduced Inflammatory Cytokines and Mediator Production in LPS-Stimulated BV-2 Cells

To examine potential suppressive effects against neuroinflammation, the effect of trehalose on the expressions of cytokines IL-1β, IL-6, IL-10, and TNF-α and inflammatory mediator NO on LPS-induced BV-2 cells were investigated. BV-2 cells were exposed to LPS (1 μg/mL) for 24 h, co-incubated with 10 mM trelalose, 50 mM trehalose and sucrose (Sucrose was set up as a positive control), and then replaced with fresh medium for another 24 h to get conditioned medium. The cytokines and the amounts of nitrite, which represent the levels of NO, released into the conditioned medium were measured by ELISA and Griess reagent assay, respectively. The results showed that LPS alone markedly induced production of IL-1β, IL-6, TNF-α, and NO from the conditioned medium (Fig. 2a–c, and 3, $P < 0.01$), while had no significant influence on production of anti-inflammatory cytokine IL-10 compared with that of the control (Fig. 2d, $P > 0.05$). However, co-treat with 10 and 50 mM trehalose for 24 h significantly repressed the levels of IL-1β, IL-6, TNF-α, and NO in the conditioned medium from LPS-stimulated BV-2 cells in a concentration-dependent manner compared with that of LPS group (except 10 mM trehalose on IL-6 level) (Fig. 2a–c, and 3, $P < 0.05$). Trehalose seemed to have no effect on IL-10 production (Fig. 2d, $P > 0.05$). Sucrose did not have the

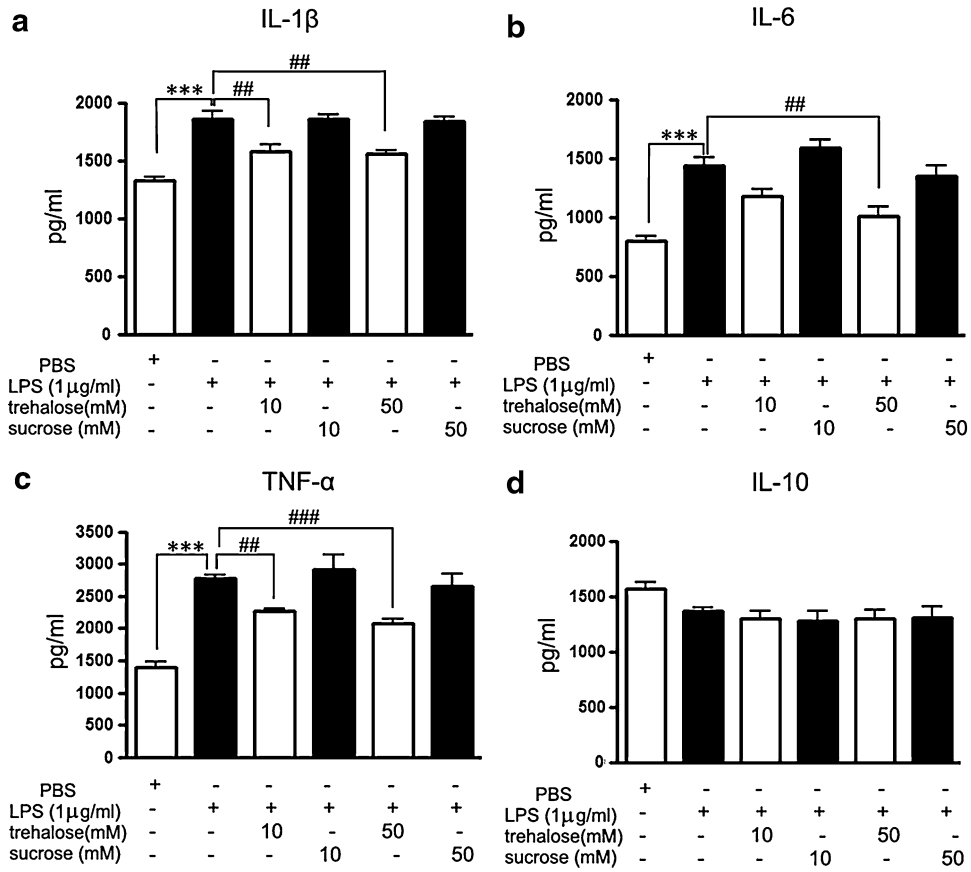

Fig. 2 Effects of trehalose on IL-1β, IL-6, TNF-α, and IL-10 production in LPS-stimulated and trehalose-treated BV-2 cells. After treatment for 24 h, the supernatants were isolated and analyzed by ELISA, for the measurement of IL-1β (**a**), IL-6 (**b**), TNF-α (**c**), and IL-10 (**d**) production, respectively. Results were expressed as mean ± SD ($n = 3$). $^{***}P < 0.001$ versus control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus LPS (1 μg/ml) group

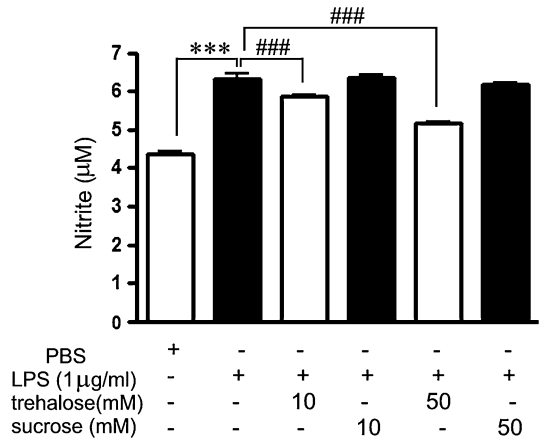

Fig. 3 Effects of trehalose on NO production in LPS-stimulated and trehalose-treated BV-2 cells. After treatment for 24 h, the supernatant were isolated and analyzed by Griess reagent, for the measurement of NO production, respectively. Results were expressed as mean ± SD ($n = 3$). $^{***}P < 0.001$ versus control; $^{\#\#\#}P < 0.001$ versus LPS (1 μg/ml) group

same effect as trehalose (Fig. 2 and 3, $P > 0.05$). These results suggested that trehalose possessed an inhibitory effect against pro-inflammatory cytokine production in activated BV-2 cells.

Trehalose Inhibited NF-κB and AP-1 Activation in LPS-Stimulated BV-2 Cells

Activation of NF-κB and AP-1, which are primary regulators of genes involved in the production of pro-inflammatory cytokines and enzymes involved in the process of inflammation, are crucial for the induction of IL-1β, IL-6, TNF-α, and NO in microglia (Lee et al. 2007; Baima et al. 2010). In this study, Western blot analysis revealed that both the level of NF-κB and AP-1 in BV-2 cells were markedly increased following exposure to LPS for 24 h (Fig. 4a, $P < 0.05$), indicating that LPS could activate NF-κB and AP-1 in BV-2 cells. However, the LPS-induced NF-κB and AP-1 activation could be attenuated by 10 and 50 mM trehalose treatment in a concentration-depend method. Sucrose treatment had no effect on activated NF-κB and AP-1 in LPS-stimulated BV-2 cells (Fig. 4a, $P > 0.05$). Only after NF-κB is phosphorylated, could it can be translocated from the cytoplasm to the nucleus. Thus we stained phosphorylated NF-κB (p-NF-κB) which represented the translocation of NF-κB by immunocytochemical staining. And we found that p-NF-κB located in or around the nucleus. According to our results, LPS stimulated the phosphorylation of NF-κB while trehalose significantly inhibited NF-κB phosphorylation. However,

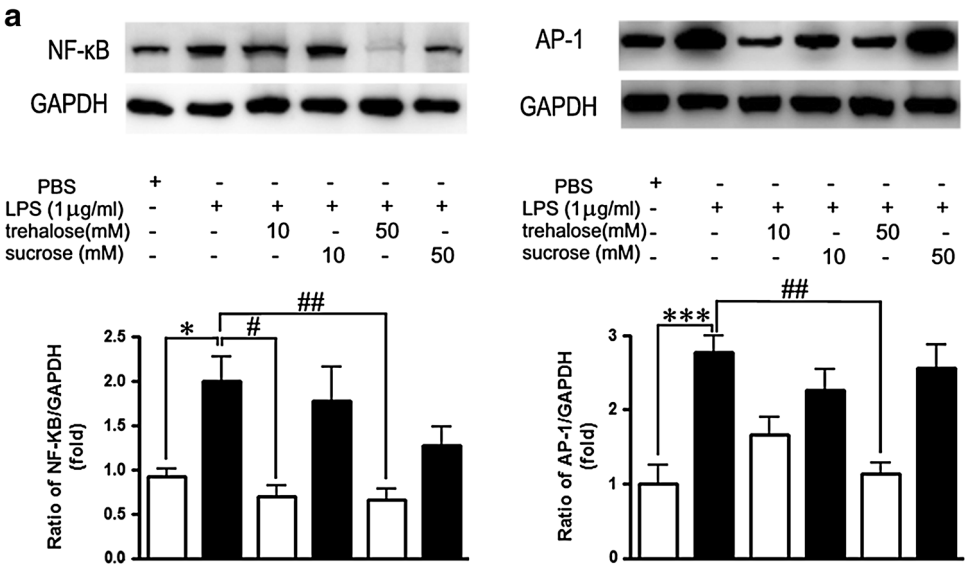

Fig. 4 Effects of trehalose on NF-κB and AP-1 expression in LPS-stimulated and trehalose-treated BV-2 cells. LPS-induced BV-2 microglial cells were treated with different concentrations of trehalose and sucrose for 24 h. **a** the levels of NF-κB and AP-1 were detected by Western Blotting. GAPDH was used as an internal control. Each blot was representative of at least three independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LPS (1 μg/ml) group. **b** immunofluorescence evidence for p-NF-κB (*red*) in LPS-stimulated and trehalose-treated BV-2 cells. *Scale bar* is 200 μm. **c** magnification of 5 × within the *yellow rectangles* in (**b**). *Scale bar* is 40 μm. *Arrows* point to p-NF-κB located in or around the nucleus (Color figure online)

sucrose did not show the same effect (Fig. 4b, c). These findings suggested that trehalose exerted the anti-inflammatory effect by inhibiting the activation of inflammatory transcription factors NF-κB and AP-1 in LPS-stimulated BV-2 cells.

Trehalose Attenuated PC12 Neuronal Apoptosis Exposed to the Conditioned Medium from LPS-Stimulated BV-2 Cells

To further investigate the effect of LPS-stimulated BV-2 cells on PC12 neurons, the conditioned medium from LPS-stimulated BV-2 cells was added to PC12 neurons. Cell viability and LDH release of PC12 neurons were then measured by the MTT assay and LDH assay. The results showed a 16 ± 9.9 % decrease in cell viability and 1.44 ± 0.24 fold increase in LDH release of PC12 neurons exposed to the conditioned medium from LPS-stimulated BV-2 cells compared with PC12 neurons exposed to normal medium (Fig. 5a, $P < 0.001$). A 16 ± 9.7 and 13 ± 2.7 % decrease in cell viability and 1.45 ± 0.24 and 1.41 ± 0.22 fold increase in LDH release were also observed in PC12 neurons exposed to the conditioned medium from LPS-stimulated BV-2 cells treated with 10 and 50 mM sucrose compared with PC12 neurons exposed to normal medium (Fig. 5a, $P < 0.001$). However, decreased cell viability and increased LDH release were reversed in PC12 neurons exposed to the conditioned medium from LPS-stimulated BV-2 cells treated with 10 and 50 mM trehalose (Fig. 5a, $P < 0.01$). According to the results, LPS-stimulated BV-2 cells exerted a cytotoxic effect on PC12 neuronal growth. However, cell viability was increased in trehalose group, indicating that LPS-induced toxicity was reduced by trehalose treatment in a concentration-depend method. Sucrose did not show any effect on PC12 neurons, as indicated in Fig. 5. We further determine how conditioned medium from BV-2 cells caused death of PC12 neurons by a Tunel method. As a result, we found that the conditional medium from LPS-stimulated BV-2 cells could promote the apoptosis of PC12 neurons, while 10 and 50 mM trehalose could attenuate the LPS-induced apoptotic effect (Fig. 5b). Sucrose did not have the same effect as trehalose (Fig. 5b). All the above results indicated that trehalose protected PC12 neurons from apoptosis caused by the cytotoxicity of LPS-stimulated BV-2 cells.

Discussion

Neuroinflammation is thought to be an important contributor to the pathological changes seen in PD (Baima et al. 2010). Inflammation in the brain is induced in part by the activation of microglia, which trigger the neurotoxic effect by increasing the production of inflammatory mediators such as NO, PGE2, and cytokines (IL-1β, IL-6, and TNF-α) (Minghetti and Levi 1998; Le et al. 2001). These activated microglia cluster around dopaminergic neurons in an

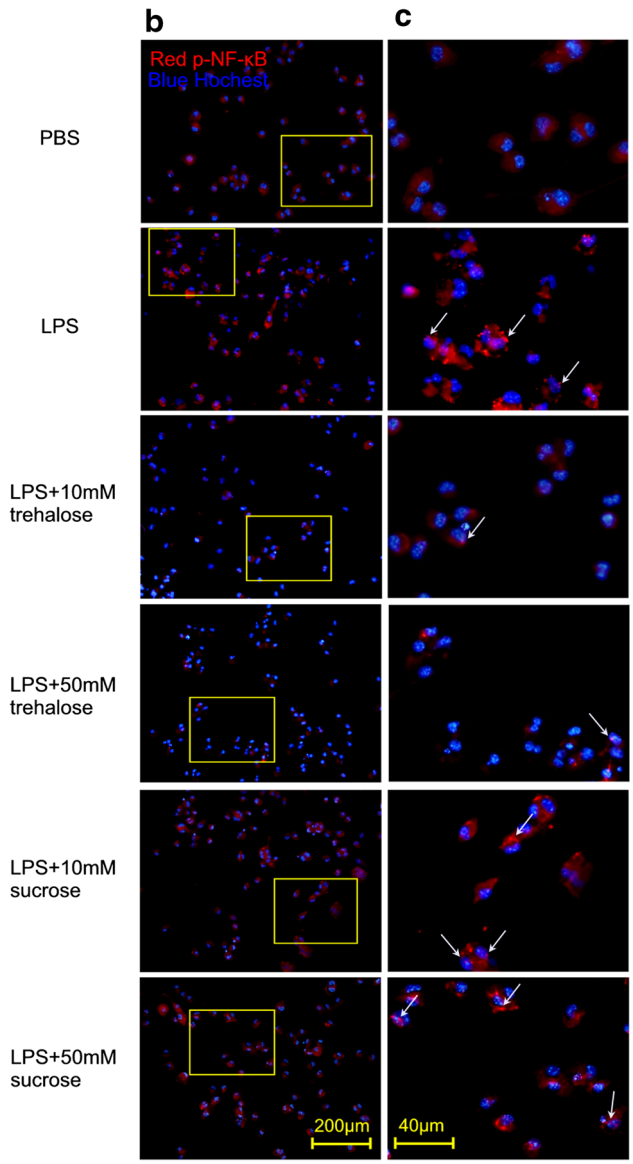

Fig. 4 continued

active mechanism of cell death called phagocytosis, which is considered may be the most critical step in neuronal death. LPS is a major microglial activator and a ligand for TLR4 (Chow et al. 1999). Upon LPS stimulation, microglia is activated by TLR4 through downstream transcription factors, such as NF-κB and AP-1, resulting in the production of a wide range of pro-inflammatory cytokines and chemokines (Ospelt and Gay 2010). Consequently, efficient regulation to microglial cells from over-activation could protect neuronal cells from neurotoxic stimuli and should be a good therapeutic approach to PD.

It is well known that NF-κB and AP-1 play an important role in the pro-inflammatory response. NF-κB can be activated by a series of stimulus. The liberated NF-κB then translocates from the cytoplasm to the nucleus, interacts with κB elements in the promoter region of a variety of inflammatory response genes, and activates their transcription (Rothwarf and Karin 1999; Jeon et al. 2010). Activated AP-1 binds to the promoter region of gene that govern cell inflammation processes, regulating the expression of various cytokines, such as iNOS, TNF-α, and COX-2 (Jochum et al. 2001; Chen et al. 2013). Furthermore, AP-1 and NF-κB have been exploited as molecular targets in drug discovery and development for inflammation-related diseases (Chen et al. 2008; Oh et al. 2013). In our study, we found that LPS could stimulate BV-2 cells to release cytokines and mediators including IL-1β, IL-6, TNF-α, NO, and activates the nucleus transcription factor, NF-κB and AP-1, which are well recognized to be involved in the inflammatory response. The conditioned medium from BV-2 cells challenged with LPS caused PC12 neuronal death. The in vitro study suggested a potential correlation between microglial activation and neuronal damage.

Trehalose is a natural sugar of invertebrates, fungi and many plants. It protects the integrity of the cells against several stress attack (Jain and Roy 2009). Trehalose has been described as an autophagy enhancer through direct protein–trehalose interactions by a large amount of studies (Jain and Roy 2009; Sarkar et al. 2007). Our previous studies have showed that trehalose was effective in disaggregating preexisting A53T-α-synuclein protofibrils and inhibiting the formation of A53T-α-synuclein fibrils in the cell-free system of A53T-α-synuclein incubation (Yu et al. 2012). Lan et al. (2012) also demonstrated that trehalose can enhance degradation of A53T-α-synuclein by promoting macro-autophagy. Recently, trehalose was shown to prevent aggregation of beta-amyloid and polyglutamine in vitro and in vivo models of Alzheimer's disease (AD) and Huntington disease (HD) (Sarkar et al. 2007; Tanaka et al. 2004; Rodriguez-Navarro et al. 2010; Liu et al. 2005). Furthermore, trehalose accelerates the clearance of mutant huntingtin and α-synuclein and inhibited protein misfolding (Sarkar et al. 2007; Singer and Lindquist 1998). Moreover, certain studies demonstrated that trehalose reduced serum TNF-α and prevents mortality in LPS-injected rats (Benaroudj et al. 2001; Arai et al. 2001; Chen and Haddad 2004; Minutoli et al. 2007). A recent study suggested that trehalose significantly suppressed the production of cytokines, including TNF-α, IL-6, IL-1α, and IL-1β, and the level of iNOS through NF-κB pathway in hemolysate-induced experimental subarachnoid hemorrhage model (Echigo et al. 2012). As such, trehalose is considered a potentially powerful therapeutic agent for various diseases. However, it is still unclear whether trehalose has an anti-inflammation and anti-apoptosis effect on PD.

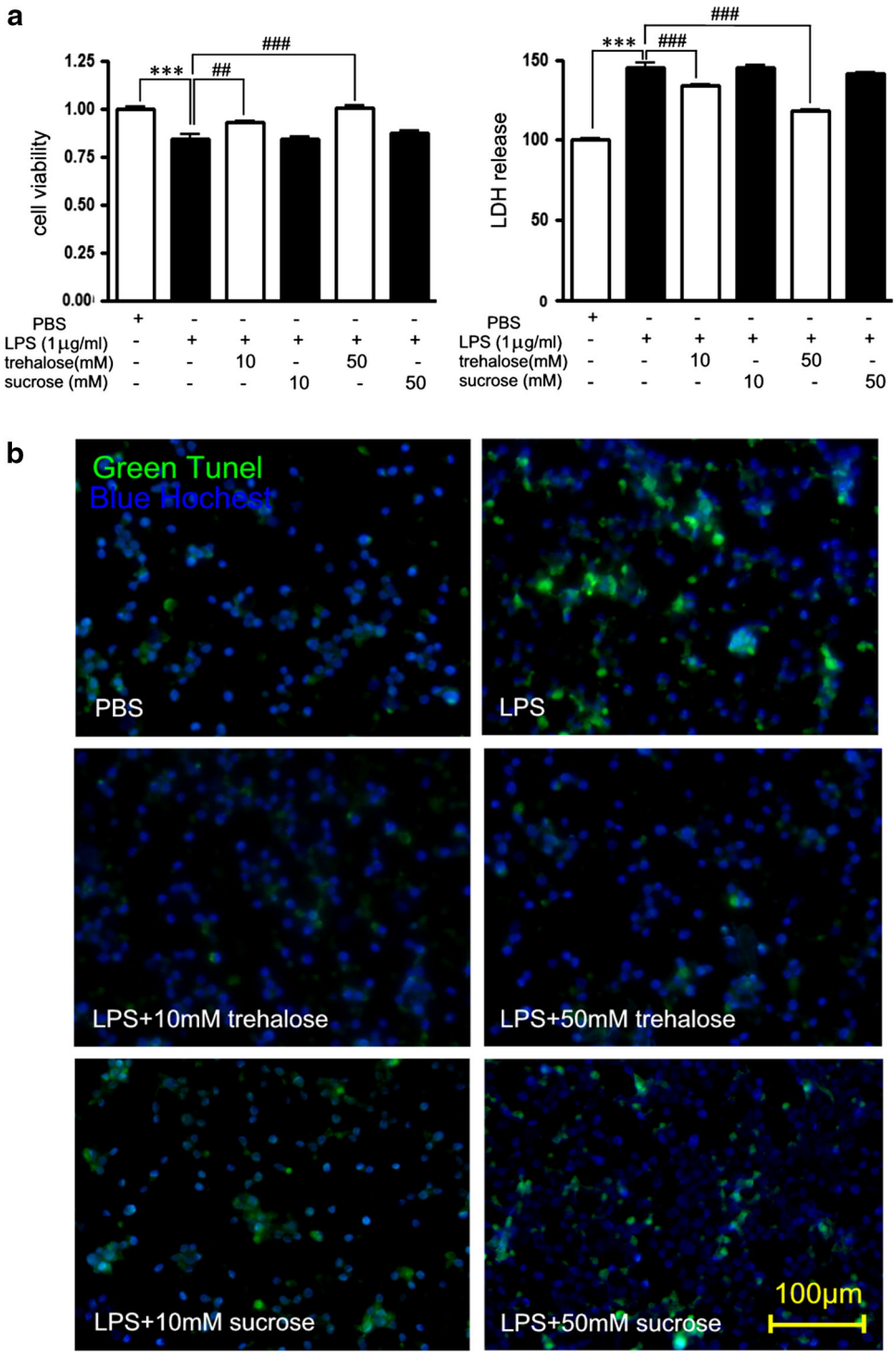

Fig. 5 Effects of the conditioned medium from LPS-stimulated and trehalose-treated BV-2 cells on PC12 neurons. **a** after treatment of the conditioned medium for 24 h, cell viability was measured by the MTT assay and LDH assay. Results were expressed as mean ± SD ($n = 3$). $^{***}P < 0.001$ versus control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus LPS (1 μg/ml) group. **b** PC12 neuronal death induced by the conditional medium from LPS-stimulated and trehalose-treated BV-2 cells was determined by Tunel method. *Scale bar* is 100 μm

In parallel with the previous studies implicating the anti-inflammatory role of trehalose, our experiment also indicated that trehalose significantly attenuated the LPS-induced release of inflammatory mediators and cytokines, including NO, IL-1β, IL-6, and TNF-α and prevented the activation of NF-κB and AP-1 in BV-2 cells. Trehalose was found to protect PC12 neurons from apoptosis by the challenge of the conditioned medium from LPS-stimulated BV-2 cells. Our research further confirmed the anti-inflammatory effect of trehalose.

To evaluate the inhibitive effects of trehalose on the LPS-induced inflammation by activating BV-2 cells, we chose the concentration of 10 and 50 mM, and we found trehalose exerted its protective role in a dose-dependent manner, which was in accordance with the previous studies (Tanaka et al. 2004; Lan et al. 2012). Tanaka et al. found

1,000 μM trehalose were more effective in clearing the abnormal aggregated protein Mb-Gln35 than 50 and 100 μM in in vitro study. Lan et al. also found trehalose cleared A53T α-synuclein in PC12 neurons in a dose-dependent manner. They observed that trehalose at the concentration of 50 and 100 mM other than 10 mM significantly enhanced the clearance of A53T α-synuclein, with 100 mM more powerful. The mechanism underlying the inhibitive role in LPS-induced inflammation of trehalose still needs further investigation. One possibility is that trehalose interacts with the proteins in the NF-κB pathway or some other signaling pathways. A comparative higher concentration might contribute to the binding of trehalose to the proteins in the signaling pathways. We did not apply a higher concentration, since we found higher dosage (more than 100 mM) of trehalose could cause obvious cell death of BV-2 cells due to high osmotic pressure. According to our team's previous work, cell viability of PC12 neurons was measured after trehalose treatment using MTT assay, and the result showed that trehalose reduced MTT tetrazolium salt formation in a concentration-dependent manner. 50 mM trehalose could decrease cell viability to 80 % and 100 mM trehalose to 70 % approximately (Lan et al. 2012). In this study, we found that the concentration of trehalose above 100 mM (including100 mM) could be toxic to BV-2 cells as well and we chose 50 mM as the maximum concentration of trehalose.

In summary, this study identified that BV-2 microglial stimulation could activate inflammatory transcription factor NF-κB and AP-1 to release cytokines and mediators including IL-1β, IL-6, TNF-α, and NO, which in turn damage PC12 neurons through apoptosis effect. While the beneficial effects of trehalose could be linked to its ability to block the above inflammatory response and further to inhibit the apoptosis of PC12 neurons from the cytotoxicity of activated BV2 cells. These experimental findings led us to hypothesize that trehalose is an attractive and safe biophysics approach to modulate inflammatory response and inhibit apoptosis in PD. However, anti-inflammatory and anti-apoptosis mechanisms of trehalose are definitely await exploration and verification in the future.

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