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ORIGINAL ARTICLE

Damage and Phenotype Change in PC12 Cells Induced by Lipopolysaccharide Can Be Inhibited by Antioxidants Through Reduced Cytoskeleton Protein Synthesis

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Abstract— The present study investigated changes in cellular phenotype and oxidative stress during the inflammatory response in PC12 cells stimulated by lipopolysaccharide (LPS) and assessed the effects of minocycline, astragalus (AST), and baicalin on inflammation. PC12 cells were exposed to LPS with or without minocycline, AST, or baicalin. Cell viability was measured by a thiazolyl blue tetrazolium bromide (MTT) assay. Contrast and laser confocal microscopy were used to analyze changes in cellular phenotype and cytoskeleton synthesis. Western blotting tested the expression of α7nAChR and vimentin. Inhibitory ratio of superoxide dismutase (SOD) activity and leakage of lactate dehydrogenase (LDH) were detected to evaluate cellular oxidative stress. Results showed that LPS could attenuate PC12 cell viability in a time- and dose-dependent manner, which could be rescued by minocycline. In addition, minocycline could reverse PC12 cell phenotypic change and the synthesis of the mesenchymal cytoskeleton protein vimentin, both induced by LPS. During LPS-initiated inflammation, α7nAChR and vimentin expression were obviously inhibited by minocycline, AST, or baicalin. The inhibitory rate of SOD activity and LDH leakage in PC12

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Abbreviations: *LPS*, Lipopolysaccharide; *AST*, Astragalus; *EMT*, Epithelial-to-mesenchymal transition; *SOD*, Superoxide dismutase; *LDH*, Lactate dehydrogenase; *ROS*, Reactive oxygen species; *MTT*, Thiazolyl blue tetrazolium bromide; *Mino*, Minocycline; *α7nAChR*, α7 nicotinic acetylcholine receptor.

cells were increased by LPS and attenuated significantly when exposed to minocycline, AST, or baicalin. These findings suggest phenotype change, altered cytoskeleton protein synthesis, and oxidative stress are all involved in the inflammatory response in PC12 cells during which α7 nicotinic acetylcholine receptor (α7nAChR) is induced by LPS stimulation. Minocycline, AST, and baicalin have a protective effect against PC12 cell injury, acting as antioxidants and inhibitors of mesenchymal proteins.

KEY WORDS: lipopolysaccharide (LPS); inflammation; phenotype change; vimentin; superoxide dismutase (SOD).

INTRODUCTION

Inflammation leads to tissue damage, which is part of the basic pathology of various diseases, including neurodegenerative and allergic diseases such as diabetic neuropathy or asthma [1, 2]. Lipopolysaccharide (LPS) is a major membrane component and toxin in Gram-negative bacteria, able to trigger inflammatory responses and increase cellular oxidative stress and superoxide [3–5]. LPS in the microenvironment can cause cellular inflammation; thus, it can be used to generate a useful model to investigate this process. LPS-induced injury of PC12 cells is a model widely used to study inflammation-related diseases [6, 7].

Cells in tumor tissues under persistent inflammation may undergo a phenotype switch, described as the epithelial-to-mesenchymal transition (EMT), and then acquire enhanced invasion and migration ability, leading to further deterioration. Inflammation and EMT can not only stimulate each other but also synergistically promote metastasis in malignant tissues. However, whether PC12 cells under inflammatory stimulation can undergo EMT remains unknown. In the present study, the possible occurrence of EMT in PC12 cells stimulated by LPS, and the potential as a therapeutic target intended to rescue cells from damage during this process, was investigated.

Increased oxidative stress is a major feature of the inflammatory response; it results in the excessive production of reactive oxygen species (ROS), causing damage to cellular structure and function. The possible association between oxidative stress, inflammation, and EMT was also explored in this study.

Numerous natural compounds have various biological activities, including antioxidant and cytoprotection properties. In the present study, a wide spectrum antibiotic, minocycline, and two traditional Chinese medicines, baicalin and astragalus, were used to treat PC12 cells injured by LPS in order to detect the inhibitory effect on EMT and assess the antioxidant properties. Additionally, we investigated these pathological processes to determine whether there are any possible targets for these agents to aim at.

MATERIALS AND METHODS

Cell Culture and Treatment

PC12 cells were purchased from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China). PC12 cells were cultured in 10% fetal bovine serum (FBS)-containing RPMI-1640 (Invitrogen, Carlsbad, CA, USA) in 37 °C with 5% CO_2. LPS (Sigma, USA) was added to induce an inflammatory reaction in PC12 cells. Minocycline, baicalin, or astragalus (all purchased from Sigma, USA) was added to the cell medium 30 min before LPS for the treatment groups.

Cell Viability Assay

A 100-μl PC12 cell suspension was seeded onto 96-well plates (Costar, USA) at a density of 3000 cells/well. After treatment, cell viability was detected by thiazolyl blue tetrazolium bromide staining (MTT; Sigma, USA). Briefly, 20 μl of 0.5 mg/ml MTT was added to each well. Then the plate was cultured at 37 °C with 5% CO_2 in the atmosphere for 4 h, after which the medium containing MTT was discarded and 100 μl DMSO (Sigma, USA) was added to the wells. After 10 min, the absorbance at OD_{560} was detected.

Immunofluorescence, Confocal Microscopy, and Contrast Microscopy Analysis

A detached cell suspension was seeded onto multiple glass-bottom tissue culture plates (10 mm, Shengyou Biotechnology, China) and cultured for 24 h with complete medium containing 10% FBS. Then, the cells were exposed to different kinds of treatments for 48 h. Subconfluent cells were rinsed with PBS rapidly at room temperature (RT). Four percent of paraformaldehyde was then used to fix cells, followed by washing with cold PBS and then blocking in 1% BSA for 30 min, after which cells were washed again and then stained overnight at 4 °C with a primary antibody (anti-vimentin 1:1000). Next, cells

were washed and stained with FITC-conjugated secondary antibodies (1:100) at 37 °C for 1 h, then washed with PBS. The samples were mounted in 1:2000 DAPI and analyzed using a Zeiss LSM 710 confocal laser-scanning microscope (Zeiss, Thornwood, NY, USA).

Adherent cells were seeded into a 24-well plate (Costar, USA) after treatment, observed with a Leica DMI 3000B microscope 2005 (Leica, Wetzlar, Germany), and then scanned at a magnification of × 20. Representative images were generated using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Western-Blot Analysis

Cultured cells were first rinsed with ice-cold PBS, then lysed in 150 μl RIPA buffer containing 1 mM PMSF (Beyotime, China) on ice. Then cells were scratched and the lysates were collected after centrifugation at 14,000 rpm for 20 min at 4 °C. Supernatants were collected, and protein concentrations were determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). The lysates were solubilized with 5X SDS-PAGE sample loading buffer (Beyotime, China) and boiled to denature the protein. Equal amounts of lysates were separated *via* 10% SDS-PAGE and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA) by electroblotting. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline and 0.1% Tween 20 (TBST) at room temperature, washed with 1X TBST buffer, and incubated overnight at 4 °C with primary antibodies (anti-vimentin, 1:2000; anti-GAPDH, 1:2000; anti-α7AChR, 1:500). Membranes were then washed with TBST several times, incubated with secondary antibodies (1:1000) for 1 h at room temperature, and finally washed again with TBST to develop with an ECL reagent (Pierce, Rockford, IL, USA). GAPDH was used as the internal loading control. Immunoblots were visualized and scanned using an Odyssey FC Imaging System (LI-COR Biosciences, NE, USA).

Superoxide Dismutase Activity and Lactate Dehydrogenase Assay

Following treatments, PC12 cells were harvested from a 6-well plate and homogenized in PBS on ice. After centrifugation at 10,000×*g* for 15 min, the supernatant was removed and the total protein concentration was measured. The inhibitory ratio of superoxide dismutase (SOD) activity was detected using an SOD assay kit (Dojindo laboratory, Japan). One unit of SOD activity was defined as the amount causing 50% inhibition of the initial rate of reduction of WST-1 (2-(4-iodophenyI)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) dissolved in water. The absorbance at 450 nm of samples and blanks were detected. Inhibitory ratio of SOD activity was calculated according to the instruction of SOD kit, which was 100% - (absorbance of sample - absorbance of blank2)/(absorbance of blank1-absorbance of blank3) × 100%. (Blank1: blank control without inhibitor; blank2: sample blank; blank3: reagent blank). A 100-μL cell suspension was seeded onto a 96-well plate (5×10^4 cells/ml) followed by cell adherence and different treatments. Lactate dehydrogenase (LDH) was measured using an LDH assay kit according to the instructions (Dojindo, Japan).

Statistical Analysis

All experiments were repeated a minimum of three times. Data are presented as the means ± SEM. The images presented are representative of the experiments. Statistical analysis was conducted using GraphPad Prism 6.0 software (La Jolla, CA, USA). The Student's *t* test was used to examine the differences between two groups. Differences were considered significant if $P < 0.05$.

RESULTS

LPS-Induced PC12 Cell Damage in a Dose- and Time-Dependent Manner, which Could Be Reversed by Minocycline

Following stimulation with LPS for different times, PC12 cell viability was decreased at 24 and 48 h with a dose of 125 ng/ml and 1 μg/ml, compared with the control (Fig. 1a). PC12 cell viability was tested after 48 h of LPS stimulation with a series of increasing concentrations ranging from 62.5 ng/ml to 1 μg/ml. With an increased dose of LPS, PC12 cell viability decreased and showed obvious attenuation at 125 ng/ml. At the dose of 1 μg/ml, we observed the most damage we saw in PC12 cells according to the dose range (Fig. 1b). Minocycline, a broad-spectrum antibiotic, was then used to test the effect on PC12 cell viability together with 125 ng/ml LPS for 48 h. As can be seen in Fig. 1c, with the increased dose of minocycline, cell viability attenuated by LPS was reversed, recovering to the normal level when the dose of minocycline increased to 50 μM, in comparison with the control group, suggesting a protective effect of minocycline against LPS damage to cells.

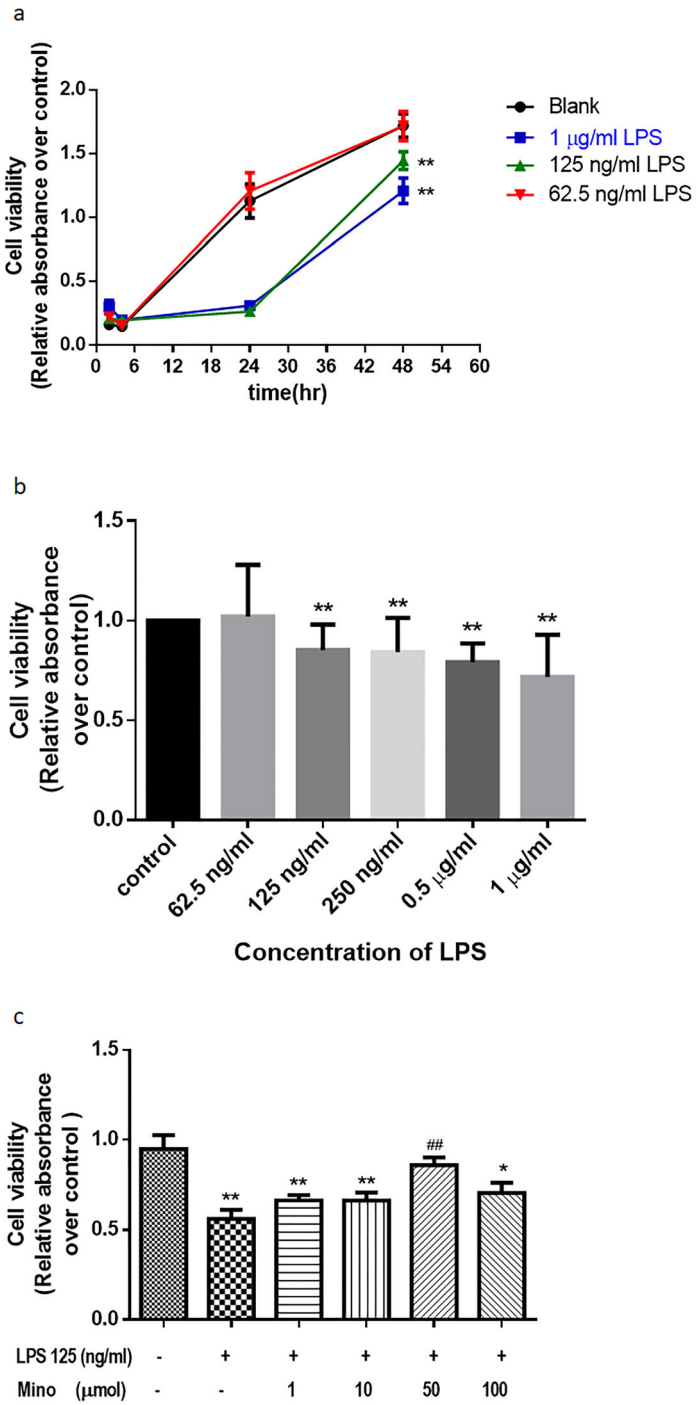

Fig. 1. Protective effect of minocycline on PC12 cells stimulated by LPS. **a** LPS-induced cell damage in a time-dependent manner. PC12 cell viability decreased obviously at 24 and 48 h at a LPS dose of 125 ng/ml and 1 μg/ml, compared with that of the control. **b** Viability of PC12 cells cultured with LPS for 48 h decreased in a dose-dependent manner at the dose of 125 ng/ml. **c** Minocycline exerted a protective effect on PC12 cell viability in a dose-dependent manner following stimulation with LPS (125 ng/ml); 50 μM gave the most obvious protective effect. (*, compared with the control group, $P < 0.05$, $^{**}P < 0.01$; $^{\#}$, compared with the group treated with LPS alone, $P < 0.05$, $^{\#\#}P < 0.01$).

Minocycline Abolishes LPS-Induced Phenotypic Switch and Cytoskeleton Changes in PC12 Cells

In addition to cell viability, we investigated changes to PC12 cell phenotype and cytoplasmic structure. PC12 cell morphology under LPS stimulation alone or together with minocycline was observed using a phase-contrast microscope, as shown in Fig. 2a. The control group exhibited completed cell outlines with strong refractivity, and the space among cells was clear. However, cells exposed to LPS alone showed darker cell bodies with black granules in the cytoplasm and there was almost no refractivity, suggesting weak viability and that the space among cells was not as clear as that of the control, thus revealing damage among the cells. When treated together with minocycline at a concentration of 50 μM, cell morphology was healthy, as was that of the control group. This cytoprotective dose of minocycline is the same as that used to test cell viability, suggesting that LPS-induced inflammation in PC12 cells could be abolished by minocycline.

It has recently been reported that inflammation and EMT shows a synergistic relationship. EMT plays a key role in the control of inflammatory responses, steering the inflammation towards deterioration and resulting in a cell phenotype switch [8, 9]. Therefore, the synthesis of vimentin, which is a cytoskeleton protein known as a marker of EMT development, was detected by using confocal microscopy. As shown in Fig. 2b, LPS-induced vimentin expression in PC12 cells, which was inhibited by 50 μM minocycline. However, when minocycline was used alone, the new vimentin synthesis was undetectable. These results suggest the damage induced by LPS is partly due to an inflammatory response during which PC12 cells underwent a phenotype switch, including morphological changes and increase vimentin expression that indicates the degree of EMT. This could all be reversed by minocycline used together with LPS.

Increased Vimentin Synthesis in LPS-Stimulated PC12 Cells Is Alleviated by Minocycline, Astragalus, and Baicalin, Partially Involving α7nAChR

Increased vimentin expression is a known marker of phenotype switch, indicating EMT. In abnormal tissues or cells, EMT and inflammation co-exist and are mutually inducible [8]. Western blotting was performed to confirm the expression of vimentin induced by LPS. In addition to minocycline, many herbal formulations of traditional Chinese medicine have anti-inflammatory effects; astragalus (AST) and baicalin are commonly used [10, 11]. In the present investigation, AST, baicalin, and minocycline were used, with or without LPS, in PC12 cells to investigate their anti-inflammatory effects. Figure 3a shows LPS-induced vimentin expression, which was abolished by combining LPS with minocycline, AST, or baicalin. Figure 3b showed the quantitated expression of vimentin increased significantly induced by LPS when compared with that in control. When combined with minocycline or astragalus or baicalin in PC12 cells stimulated with LPS, the level of vimentin showed an obvious tendency of attenuation especially in the group of astragalus suggesting that EMT in LPS-stimulated PC12 cells is sensitive to these agents.

As previously identified, increased vimentin expression could be mediated by α7nAChR in some tumor cells [12, 13]. We aimed to discern whether vimentin expression induced by LPS in PC12 cells was associated with α7nAChR expression. Results showed that α7nAChR expression could be induced by LPS, but attenuated by a combination with minocycline, baicalin, or AST (Fig. 3c). AST decreased the expression of α7nAChR after LPS stimulation, though not as obviously as the other two agents. In accordance with the change tendency of vimentin, the quantitated expression of α7nAChR was enhanced obviously by LPS stimulation in PC12 cells when compared with control. There were obvious abatements in the expression of this receptor when cells damaged by LPS were treated together with minocycline or astragalus or baicalin as Fig. 3d showed.

Antioxidative and Protective Effects of Minocycline, AST, or Baicalin Against LPS Injury in PC12 Cells

As a well-known inflammatory stimulus, LPS can induce acute neuroinflammation and reactive oxygen species (ROS) generation, resulting in an imbalance in cell oxidative stress [14, 15]. In different kinds of pathophysiological processes, superoxide is widely known to cause many types of cell damage. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals into molecular oxygen or hydrogen peroxide in order to defend tissues and cells against oxidant damage. Thus, SOD is viewed as an antioxidant, and the level of SOD is usually measured to evaluate the antioxidant ability of cells or tissues [16]. In our experiments, the inhibitory rate of SOD activity was examined. High or low doses of AST or baicalin were applied. As shown in Fig. 4a, following LPS stimulation, the inhibitory rate of SOD in PC12 cells was clearly increased compared with the control group. However, cells treated with minocycline or AST or baicalin alone, no matter the dose, showed no difference in the level

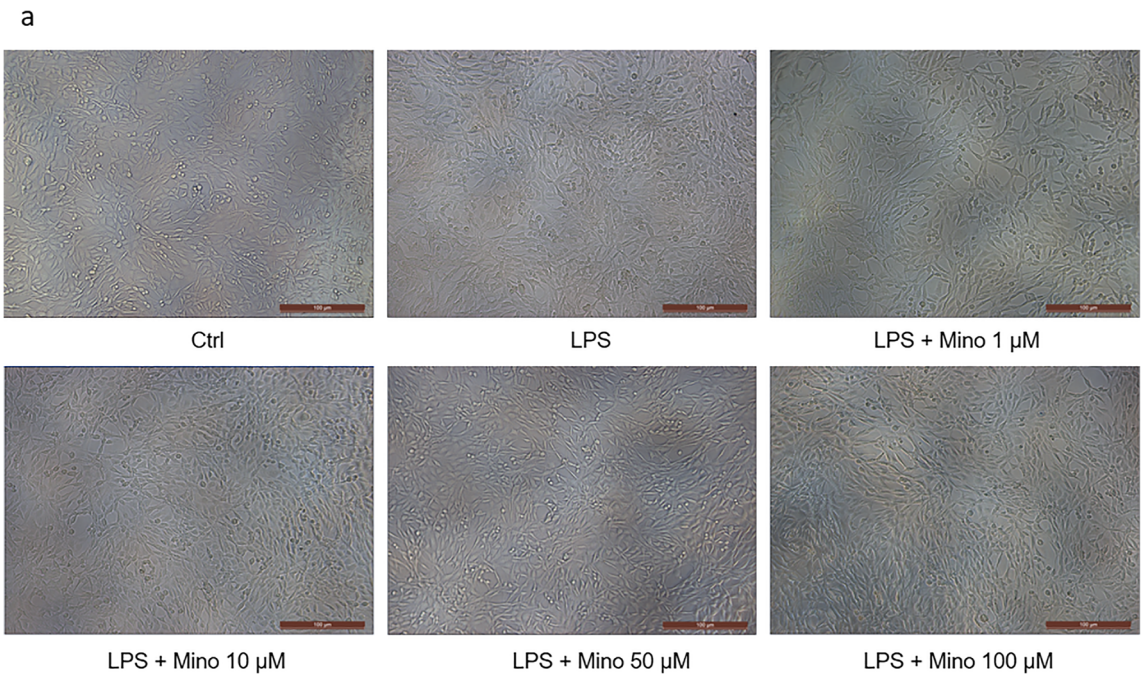

Fig. 2. PC12 cells undergo a phenotypic switch when stimulated by LPS, which is abolished by minocycline. **a** PC12 cell morphology when damaged by LPS at a concentration of 125 ng/ml for 48 h. PC12 cells in the control group showed a complete outline with strong refractivity and the space among the cells was clear. Cells exposed to LPS alone showed darker cell bodies with black granules in the cytoplasm. When treated with LPS combined with 50 μM minocycline, the injury caused by LPS could be reversed. Cells were scanned and representative areas were photographed at ×10 magnification. **b** No vimentin expressed in the control group. Vimentin (red) was induced by 48 h of LPS in PC12 cells, which then underwent EMT. Increased vimentin stimulated by LPS in the PC12 cell cytoplasm was abolished through the combined usage of minocycline at a concentration of 50 μM. Cells were scanned at a magnification of × 20.

of SOD when compared with the control group, indicating that LPS induces a significant increase in oxidative stress and contributes cellular damage of cells; however, AST, baicalin, or minocycline do not have any notable negative effects on cells when used alone.

Furthermore, treatment of PC12 cells with LPS together with AST or baicalin for 24 h (Fig. 4b) and 48 h (Fig. 4c) could effectively attenuate inhibitory rate of SOD compared with that in LPS alone. These results suggest the protective and the antioxidative effects of AST, baicalin, or minocycline against LPS damage in PC12 cells. However, Fig. 4c shows that after 48 h of treatment with LPS together with AST or baicalin, the inhibitory rate of activity of SOD was even lower than that of the control. These phenomena should be contributed to the protective effects of AST or baicalin along with the longer culture time when considering the result of Fig. 4a together.

Lactate dehydrogenase (LDH) is a valuable enzyme and a sensitive indicator of cellular metabolic status [17]. LDH level in the culture medium shows not only the state of a damaged cellular membrane but also the direction of oxygen usage involved in aerobic or anaerobic metabolism and even immune surveillance [18]. Therefore, LDH was quantified when PC12 cells were treated with LPS alone or together with minocycline or AST or baicalin (Fig. 4d). After 24 h, the large amount of LDH released from cells induced by LPS was obviously inhibited when cells were treated together with AST, minocycline, or baicalin (Fig. 4e). When these same treatments were performed for 48 h, similar results were observed. Combination of AST or baicalin with LPS could obviously attenuate LDH release compared with LPS alone (Fig. 4f).

DISCUSSION

Neuron damage is common in various neurodegenerative diseases [19] including diabetic neuropathy [7]. LPS is a known component of the Gram-negative bacterial cell wall, this can be recognized by the immune system, inducing neuroinflammation [3]. The PC12 cell line is derived from a rat pheochromocytoma of the adrenal medulla, which possessed neuron-like characteristics. It provides a cellular model for use in studying a series of neurological

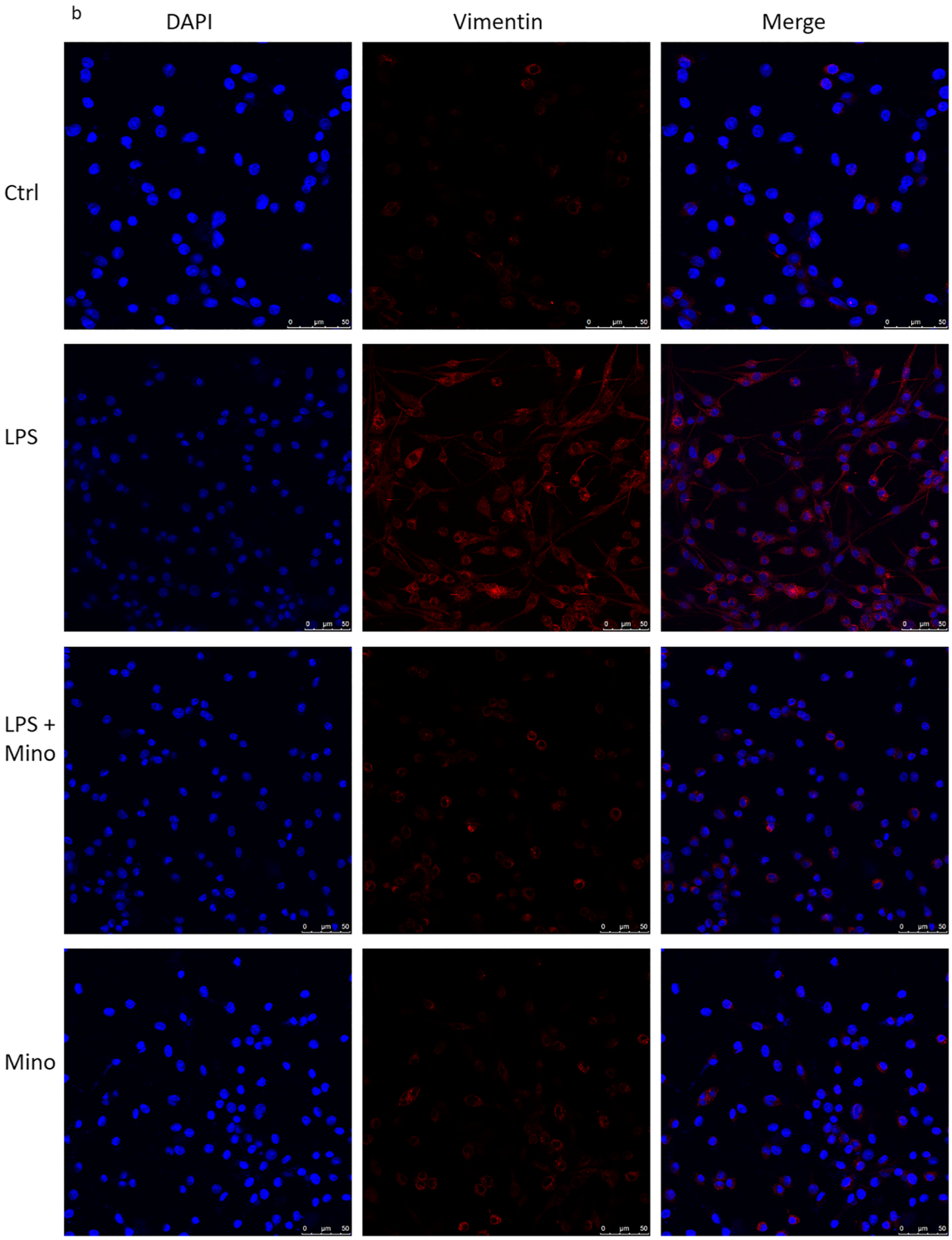

Fig. 2. (continued)

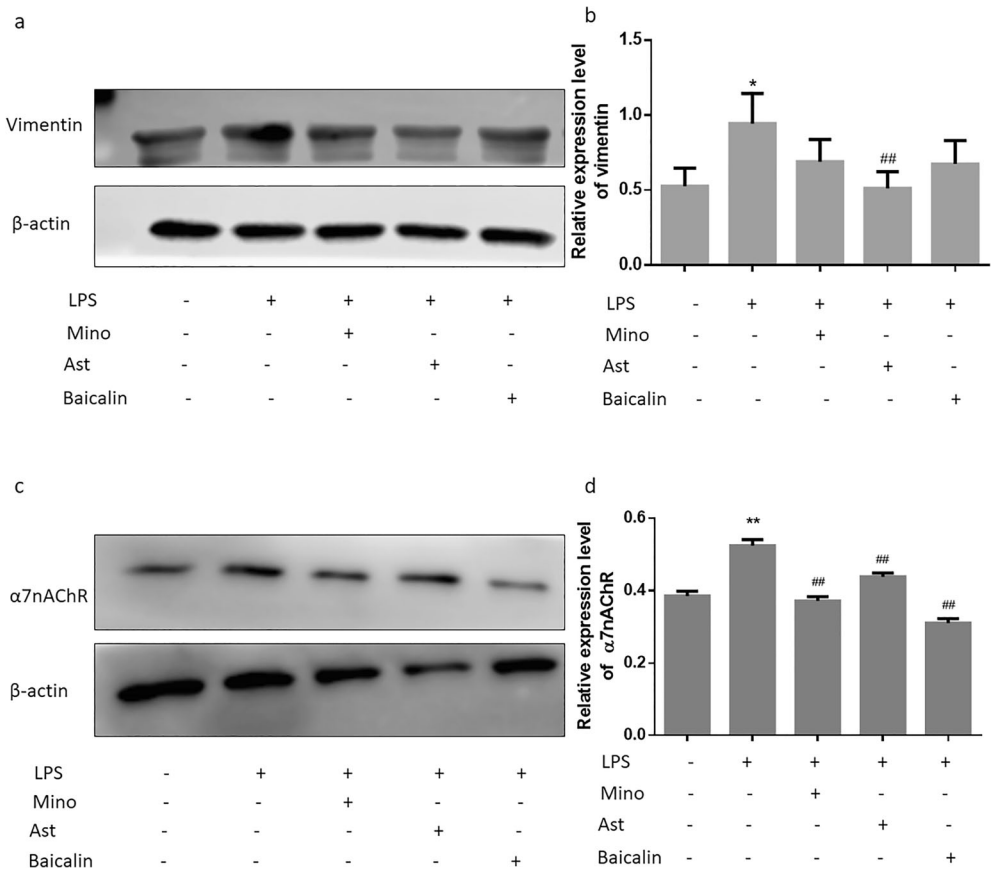

Fig. 3. Effect of minocycline, astragalus or baicalin on vimentin and α7nAChR synthesis in LPS-treated PC12 cells. **a** Increased vimentin expression in PC12 cells induced by LPS treatment for 48 h was inhibited by co-administration with minocycline, astragalus, or baicalin. **b** The quantitated expression of vimentin in PC12 cells of figure3a. **c** Minocycline, baicalin, or astragalus decreased the expression of α7nAChR following LPS induction for 48 h. **d** The quantitated expression of α7nAChR in PC12 cells of Fig. 3c.

diseases, including neurodegeneration, apoptosis, and cytotoxicity. In the present study, LPS-induced inflammation in PC12 cells in a dose- and time-dependent manner. Damage caused during the inflammatory response occurs *via* ≥ 3 routes, including altered cell viability, morphological transition, and cytoskeletal synthesis, leading to a phenotypic switch and increased oxidative stress.

The protective effect of several agents on PC12 cells during this process was observed and the underlying possible mechanisms were investigated. Minocycline is a wide spectrum antibiotic. Minocycline, when used to treat PC12 cells stimulated with LPS, showed a protective effect on cell viability at a dose range from 1 to 50 μM. At the concentration of 50 μM, minocycline shows an obvious protective effect on PC12 cell viability and the capacity to reverse cellular morphology changes brought about during inflammation, as determined by phase-contrast microscopy. Cellular morphology changes are usually accompanied by alterations in cytoskeletal synthesis, especially under certain detrimental micro-circumstances that lead to EMT [8]. When PC12 cells were exposed to LPS together with minocycline at the same dosage, the increased expression of vimentin stimulated by LPS was attenuated, as detected by confocal microscopy. These results suggest minocycline has the ability to abolish EMT promoted in PC12 cells by inflammation though inhibiting vimentin synthesis. Simultaneously, both AST and baicalin showed an ability to decrease vimentin expression, suggesting a regulative effect on the cytoskeleton. Our previous study verified the ability of α7nAChR to mediate vimentin expression, a marker of EMT [12, 13]. Our present results show that α7nAChR expression could be induced in PC12 cells by LPS and be decreased by minocycline, baicalin, or AST, suggesting α7nAChR is at least partly involved in LPS-induced EMT mediation in PC12 cells and its sensitivity to minocycline, baicalin, or AST.

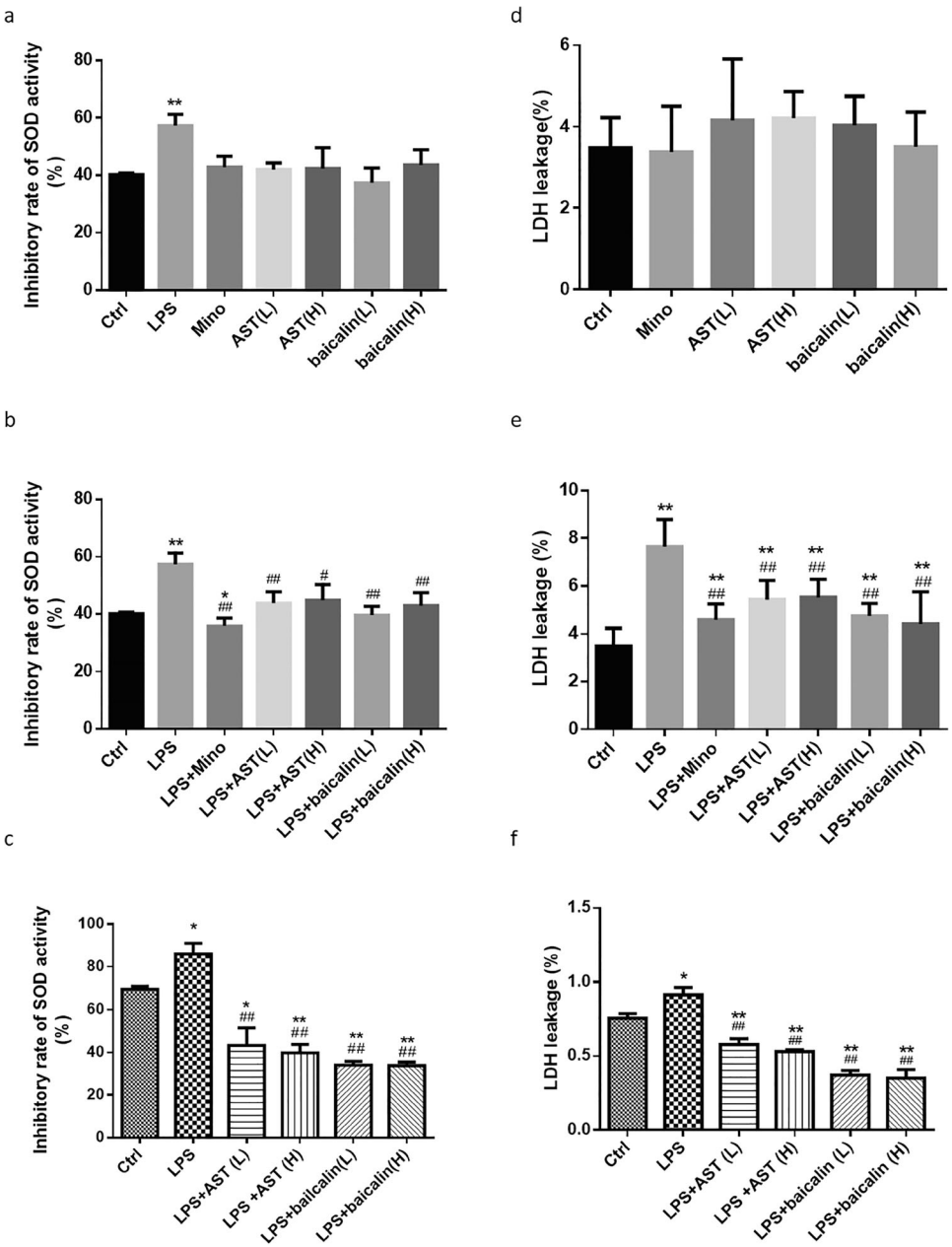

Fig. 4. Antioxidative and protective effects of minocycline, astragalus or baicalin on LPS injury of PC12 cells. **a** Inhibitory ratio of SOD in PC12 cells when exposed to LPS, minocycline, astragalus, or baicalin alone for 24 h. Under stimulation with LPS, the inhibitory ratio of SOD in PC12 cells was increased compared with the control group. However, cells treated with minocycline or AST or baicalin alone, no matter the dose, showed no significant difference in the SOD level relative to the control group. **b** Inhibitory ratio of SOD in PC12 cells was attenuated when exposed to LPS combined with minocycline, astragalus, or baicalin for 24 h. **c** Inhibitory ratio of SOD in PC12 cells decreased significantly when treatment with LPS combined with baicalin or astragalus for 48 h. **d** Leakage of LDH from PC12 cells when exposed to LPS, minocycline, astragalus, or baicalin alone for 24 h. The leakage of LDH was increased by stimulation with LPS, whereas cells treated with minocycline, astragalus, or baicalin alone showed no difference compared with the control. **e** The leakage of LDH in PC12 cells exposed to LPS together with minocycline, astragalus, or baicalin for 24 h was decreased compared with cells treated with LPS alone. **f** The leakage of LDH in PC12 cells stimulated with LPS combined with baicalin or astragalus for 48 h was attenuated significantly when compared with the control or cells treated with LPS alone. Minocycline: 50 μM; astragalus (L), astragalus low dose: 50 μM; astragalus (H), astragalus high dose: 100 μM; baicalin (L), baicalin low dose: 0.2 mM;baicalin (H), baicalin high dose: 1.0 mM. (*, compared with the control group, $P < 0.05$, * $P < 0.01$; $^{\#}$, compared with the group treated with LPS alone, $P < 0.05$, $^{\#\#}P < 0.01$).

AST has been reported to show various pharmacological effects, including anti-inflammatory, immune-stimulant, antioxidative, and anti-diabetic activities [20]. Baicalin is also a widely used component in traditional Chinese Medicine. It has been established to have anti-inflammatory and neuroprotective properties [11, 21, 22]. However, the effects of both AST and baicalin on the damage to PC12 cells have not been clearly demonstrated. Therefore, the antioxidative effects of both were investigated. Results revealed that AST and baicalin significantly reduced the inhibitory rate of SOD, which was obviously increased by LPS, showing an ability to balance oxidative stress during inflammation. Even at high concentrations, no obvious cellular toxicity occurred with AST and baicalin treatment, indicating a good safety profile. The basic structure of AST includes glucoside indican, the oxygen-containing groups of which could replace the aglycon of cycloartane-type saponins to balance increased oxidative stress [20, 23]. This antioxidant effect contributes to offsetting the development of LPS-induced inflammation and may inhibit the synthesis of vimentin. However, the underlying mechanism needs to be further investigated.

We investigated the damage caused by LPS, which occurs due to altered cell viability, phenotypic switch, and oxidative stress. Minocycline, AST, and baicalin exert protective effects on PC12 cells, mediated by inhibited cell EMT and increased activity of SOD and attenuated LDH leakage from cytoplasm following inflammatory stimulation. The three agents not only have antioxidant characteristics[24–26] but also have regulatory effects on the expression of α7nAChR, which is involved in decreased vimentin synthesis. However, in the present investigation, the specific antagonist of α7nAChR was not applied directly to PC12 cells following LPS stimulation to identify the expression of vimentin due to present unavailability of α-bungarotoxin in China (according to a revised policy). α7nAChR only can be considered as a possible mechanism for regulating vimentin in PC12 cells; further investigations should be performed.

In summary, the results showed that minocycline, AST, and baicalin could decrease LPS-induced vimentin expression, showing the ability to inhibit and reverse the development of EMT in which α7nAChR is involved. This receptor and the process of EMT during the inflammatory response may be looked upon as potential treatment targets. Furthermore, we verified that oxidative stress contributes to cell damage in inflammation. Certain traditional Chinese medicines possess both antioxidant characteristics and the ability to inhibit changes to cytoskeletal proteins under inflammatory conditions, hinting at a relationship between oxidative stress and cell phenotype switch. The process of switching, which includes α7nAChR, may be considered as potential target for an adjuvant regimen, although further research is required.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

REFERENCES

1. Glass, C.K., K. Saijo, B. Winner, M.C. Marchetto, and F.H. Gage. 2010. Mechanisms underlying inflammation in neurodegeneration. *CELL* 140 (6): 918–934.
2. Mishra, V., J. Banga, and P. Silveyra. 2018. Oxidative stress and cellular pathways of asthma and inflammation: therapeutic strategies and pharmacological targets. *Pharmacology & Therapeutics* 181: 169–182.
3. Lopes, P.C. 2016. LPS and neuroinflammation: a matter of timing. *Inflammopharmacology* 24 (5): 291–293.
4. Thangavel, J., S. Samanta, S. Rajasingh, B. Barani, Y.T. Xuan, B. Dawn, et al. 2015. Epigenetic modifiers reduce inflammation and modulate macrophage phenotype during endotoxemia-induced acute lung injury. *Journal of Cell Science* 128 (16): 3094–3105.
5. Park, J., J.S. Min, B. Kim, U.B. Chae, J.W. Yun, M.S. Choi, I.K. Kong, K.T. Chang, and D.S. Lee. 2015. Mitochondrial ROS govern the LPS-induced pro-inflammatory response in microglia cells by regulating MAPK and NF-kappaB pathways. *Neuroscience Letters* 584: 191–196.
6. Tian, R., and R. Shi. 2017. Dimercaprol is an acrolein scavenger that mitigates acrolein-mediated PC-12 cells toxicity and reduces acrolein in rat following spinal cord injury. *Journal of Neurochemistry* 141 (5): 708–720.
7. Xu, H., C. Xiong, L. He, B. Wu, L. Peng, Y. Cheng, et al. 2015. Trans-resveratrol attenuates high fatty acid-induced P2X7 receptor expression and IL-6 release in PC12 cells: possible role of P38 MAPK pathway. *Inflammation* 38 (1): 327–337.
8. Suarez-Carmona, M., J. Lesage, D. Cataldo, and C. Gilles. 2017. EMTand inflammation: inseparable actors of cancer progression. *Molecular Oncology* 11 (7): 805–823.
9. Lopez-Novoa, J.M., and M.A. Nieto. 2009. Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. *EMBO Molecular Medicine* 1 (6–7): 303–314.
10. Auyeung, K.K., Q.B. Han, and J.K. Ko. 2016. Astragalus membranaceus: a review of its protection against inflammation

and gastrointestinal cancers. *The American Journal of Chinese Medicine* 44 (1): 1–22.
11. Jin, X., M.Y. Liu, D.F. Zhang, X. Zhong, K. Du, P. Qian, et al. 2019. Baicalin mitigates cognitive impairment and protects neurons from microglia-mediated neuroinflammation via suppressing NLRP3 inflammasomes and TLR4/NF-kappaB signaling pathway. *CNS Neuroscience & Therapeutics* 25 (5): 575–590.
12. Zhang, C., P. Yu, L. Zhu, Q. Zhao, X. Lu, and S. Bo. 2017. Blockade of alpha7 nicotinic acetylcholine receptors inhibit nicotine-induced tumor growth and vimentin expression in non-small cell lung cancer through MEK/ERK signaling way. *Oncology Reports* 38 (6): 3309–3318.
13. Zhang, C., X.P. Ding, Q.N. Zhao, X.J. Yang, S.M. An, H. Wang, et al. 2016. Role of alpha7-nicotinic acetylcholine receptor in nicotine-induced invasion and epithelial-to-mesenchymal transition in human non-small cell lung cancer cells. *Oncotarget* 7 (37): 59199–59208.
14. Shah, S.A., M. Khan, M.H. Jo, M.G. Jo, F.U. Amin, and M.O. Kim. 2017. Melatonin stimulates the SIRT1/Nrf2 signaling pathway counteracting lipopolysaccharide (LPS)-induced oxidative stress to rescue postnatal rat brain. *CNS Neuroscience & Therapeutics* 23 (1): 33–44.
15. Hussain, T., B. Tan, Y. Yin, F. Blachier, M.C. Tossou, and N. Rahu. 2016. Oxidative stress and inflammation: what polyphenols can do for us? *Oxidative Medicine and Cellular Longevity* 2016: 7432797.
16. Hayyan, M., M.A. Hashim, and I.M. AlNashef. 2016. Superoxide Ion: Generation and chemical implications. *Chemical Reviews* 116 (5): 3029–3085.
17. Jurisic, V., S. Radenkovic, and G. Konjevic. 2015. The actual role of LDH as tumor marker, biochemical and clinical aspects. *Advances in Experimental Medicine and Biology* 867: 115–124.
18. Ding, J., J.E. Karp, and A. Emadi. 2017. Elevated lactate dehydrogenase (LDH) can be a marker of immune suppression in cancer: interplay between hematologic and solid neoplastic clones and their microenvironments. *Cancer Biomarkers* 19 (4): 353–363.
19. Irie, T., T. Kawakami, K. Sato, and M. Usami. 2017. Sub-toxic concentrations of nano-ZnO and nano-TiO2 suppress neurite outgrowth in differentiated PC12 cells. *The Journal of Toxicological Sciences* 42 (6): 723–729.
20. Li, X., L. Qu, Y. Dong, L. Han, E. Liu, S. Fang, Y. Zhang, and T. Wang. 2014. A review of recent research progress on the astragalus genus. *Molecules* 19 (11): 18850–18880.
21. Wan, D., and H. Ouyang. 2018. Baicalin induces apoptosis in human osteosarcoma cell through ROS-mediated mitochondrial pathway. *Natural Product Research* 32 (16): 1996–2000.
22. Luo, J., B. Dong, K. Wang, S. Cai, T. Liu, X. Cheng, et al. 2017. Baicalin inhibits biofilm formation, attenuates the quorum sensing-controlled virulence and enhances Pseudomonas aeruginosa clearance in a mouse peritoneal implant infection model. *PLoS One* 12 (4): e176883.
23. Hu, T., Q.M. Liu, X.W. He, F. Huang, M.W. Zhang, and J.G. Jiang. 2017. Identification of bioactives from *Astragalus chinensis* L.f. and their antioxidant, anti-inflammatory and anti-proliferative effects. *Journal of Food Science and Technology* 54 (13): 4315–4323.
24. Cheong, C.U., C.S. Yeh, Y.W. Hsieh, Y.R. Lee, M.Y. Lin, C.Y. Chen, and C.H. Lee. 2016. Protective effects of costunolide against hydrogen peroxide-induced injury in PC12 cells. *Molecules* 21 (7): E898.
25. Kumar, P., T. Raman, M.M. Swain, R. Mishra, and A. Pal. 2017. Hyperglycemia-induced oxidative-nitrosative stress induces inflammation and neurodegeneration via augmented tuberous sclerosis complex-2 (TSC-2) activation in neuronal cells. *Molecular Neurobiology* 54 (1): 238–254.
26. Xu, W., F. Li, Z. Xu, B. Sun, J. Cao, and Y. Liu. 2018. Role of peroxiredoxin 2 in the protection against ferrous sulfate-induced oxidative and inflammatory injury in PC12 cells. *Cellular and Molecular Neurobiology* 38 (3): 735–745.