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ORIGINAL ARTICLE

Doxycycline Suppresses Microglial Activation by Inhibiting the p38 MAPK and NF-kB Signaling Pathways

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Abstract In neurodegenerative diseases, the inflammatory response is mediated by activated glial cells, mainly microglia, which are the resident immune cells of the central nervous system. Activated microglial cells release proinflammatory mediators and neurotoxic factors that are suspected to cause or exacerbate these diseases. We recently demonstrated that doxycycline protects substantia nigra dopaminergic neurons in an animal model of Parkinson's disease. This effect was associated with a reduction of microglial cell activation, which suggests that doxycycline may operate primarily as an anti-inflammatory drug. In the present study, we assessed the anti-inflammatory potential of doxycycline using lipopolysaccharide (LPS)-activated primary microglial cells in culture as a model of neuroinflammation. Doxycycline attenuated the expression of key activation markers in LPS-treated microglial cultures in a concentration-dependent manner. More specifically, doxycycline treatment lowered the expression of the microglial activation marker IBA-1 as well as the production of ROS, NO, and proinflammatory cytokines (TNF-α and IL-1β). In primary microglial cells, we also found that doxycycline inhibits LPS-induced p38 MAP kinase phosphorylation and NF-kB nuclear translocation. The present results indicate that the effect of doxycycline on LPS-induced microglial activation probably occurs via the modulation of p38 MAP kinase and NF-kB signaling pathways. These results support the idea that doxycycline may be useful in preventing or slowing the progression of PD and other neurodegenerative diseases that exhibit altered glia function.

Keywords Parkinson's disease · Doxycycline · Microglia · Cytokines

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Introduction

Parkinson's disease (PD) is a neurodegenerative syndrome that is pathologically characterized by the progressive loss of dopaminergic neurons from the substantia nigra (SNc) and the abnormal accumulation in the surviving neurons of cytoplasmic alpha-synuclein (AS) protein inclusions, which are called Lewy bodies (LBs) (Forno 1996). Despite intensive research conducted in the field of PD, the etiology of this neurodegenerative disease remains unknown. Among the proposed underlying pathophysiological mechanisms, oxidative stress, neuroinflammation, protein misfolding, and mitochondrial dysfunction have been credited as major pathways of neurodegeneration (Gandhi and Wood 2005). Oxidative stress is thought to be an

important factor in PD not only due to the well-known toxic effect of free radicals (Zhang et al. 2000; Butterfield and Kanski 2001; Giasson et al. 2002) but also because it has been associated with an enhanced fibrillation of AS (Ostrerova-Golts et al. 2000).

Neuroinflammation is also recognized as a key factor in the initiation and progression of PD pathology (More et al. 2013; Russo et al. 2014) and primarily manifests itself as the excessive activation and proliferation of microglia, as first discovered by pioneering post-mortem studies on PD brains (McGeer et al. 1988). Since then, microglia have been shown to be abundant in the SNc compared with other brain regions (Kim et al. 2000; Lawson et al. 1990). Interestingly, under neuroinflammatory conditions, microglial cells express inducible nitric oxide synthase (iNOS), which is responsible for the production of NO. Superoxide anion, a MAO-B by-product, reacts with NO to generate peroxynitrite, which is ultimately decomposed into the very reactive hydroxyl radical. In parallel, neuron-derived ROS and neuromelanin-iron complexes activate microglia (Langston et al. 1999; Zecca et al. 2008). In that sense, oxidative stress and neuroinflammatory actions are intricately entwined in the pathophysiology of PD.

The neuroinflammatory response during most chronic neurodegenerative diseases is mediated by the activation of glial cells, primarily microglia (Cunningham 2013; Smith et al. 2012; Skaper et al. 2012, 2014). Microglia are the resident immune cells in the central nervous system (CNS) and constitute approximately 10–15 % of the total glial cell population in the adult brain. Under normal conditions, microglia exist in a quiescent stage and are involved in immune surveillance and vigilance (Austin and Moalem-Taylor 2010; Zhang et al. 2013).

Once they are activated, in response to pathogen-associated molecular patterns (PAMPs, e.g., lipopolysaccharide; LPS) or damage-associated molecular patterns (DAMPs), microglia transform from their resting to activated states, and this process is accompanied by marked morphological changes from a ramified to a amoeboid state. Furthermore, stimulated microglia release several proinflammatory and neurotoxic molecules, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), IL-6, NO, eicosanoids, proteinases, and ROS (Zhang et al. 2013; Henry et al. 2009; Dutta et al. 2008).

It has been difficult to determine the causes of PD; thus, there are no treatments aimed at stopping the neurodegenerative processes. Moreover, L-DOPA, the most abundantly used PD treatment, restores the absence of dopamine but does not stop the neurodegenerative process, probably because the neuroinflammatory process that is induced by this drug is exacerbated (Barnum et al. 2008; Bortolanza et al. 2014). Because the factors that are released by activated microglia participate in several neurodegenerative diseases, pharmacological strategies that are aimed at suppressing microglial activity are being explored as new therapies (Zhang et al. 2013; Henry et al. 2009; Dutta et al. 2008; Henry et al. 2008).

Tetracyclines (TC) are bacteriostatic agents that bind to the 30S ribosomal subunit of bacteria, inhibit protein synthesis, and exhibit antibiotic activity against a wide range of microorganisms including gram-positive and gram-negative bacteria (Cunha et al. 1982; Roberts 2003). Semi-synthetic second-generation TC, including minocycline and doxycycline, are currently used as typical antibiotics in humans. In addition to their efficacy in the treatment of multidrug-resistant infections, these antibiotics have good clinical safety and can easily penetrate the blood–brain barrier (Domercq and Matute 2004). However, their therapeutic effects are due to more than just their antimicrobial activity (Ahler et al. 2013). In this respect, an important number of studies have shown that TC have remarkable neuroprotective properties in models of cerebral ischemia, spinal cord injury, PD, Huntington's disease, amyotrophic lateral sclerosis, and multiple sclerosis (Clark et al. 1994; Yrjänheikki et al. 1998; Gordon et al. 2004; Thomas et al. 2004; Metz et al. 2013).

Recently, in an animal model of PD, we showed that doxycycline, when administered at a dose that both induces and represses conditional transgene expression in the tetracycline system, mitigates the loss of dopaminergic neurons in the SNc and nerve terminals in the striatum. Interestingly, this neuroprotection was associated with a reduction in the microglial activation (Lazzarini et al. 2013), which is in agreement with the neuroprotective properties observed in different animal models of neurodegenerative disorders (Wang et al. 2009; Cho et al. 2009). Furthermore, doxycycline has been used for the treatment of central nervous system infections and has protective effects in models of brain injury (Clark et al. 1997; Reasoner et al. 1997; Yrjänheikki et al. 1998; Gordon et al. 2004).

LPS, an endotoxin from the outer membrane of gram-negative bacteria, is known to activate microglia (Hoshino et al. 1999; Lehnardt et al. 2003); thus, it is frequently used as a research agent for this purpose both in vitro and in vivo (Kaneko et al. 2005; Lund et al. 2006, Henry et al. 2008). Through the use of this tool, many research groups have shown that minocycline can inhibit microglia activation and reduce the transcription and release of various cytokines and proinflammatory molecules by microglia (Henry et al. 2008, Kim et al. 2004; Fan et al. 2005; Horvath et al. 2008). Furthermore, doxycycline can attenuate the LPS-induced activation of immortalized BV-2 microglial cells (Cho et al. 2009). However, there is no evidence that doxycycline directly modulates the functions of primary microglial cells. Therefore, in the present study,

we evaluated the in vitro effects of doxycycline on LPS-activated primary murine microglia and the mechanisms involved in these effects. Doxycycline was found to suppress microglial cell activation, and the mechanism of this effect involves the inhibition of p38 MAP kinase and NF-kB-dependent signaling pathways.

Materials and Methods

Drugs

Doxycycline, dexamethasone, and SB 203580 were purchased from Sigma-Aldrich. LPS from *E. coli* serotype 026:B6 (Sigma-Aldrich) was used to induce microglial cell activation. The vehicle of the stock solutions of these drugs was deionized water.

Primary Mouse Microglial Cell Cultures

Animals were treated in accordance with European Directive 86/609/EEC on the protection of animals and the guidelines of the local institutional animal care and use committee. Microglial cultures were prepared from the brains of newborn pups (1 day, P0 microglia) from C57BL/6 J mice (Janvier LABS, Le Genest St Isles, France). Briefly, whole brains were harvested, and meninges were stripped away and then mechanically dissociated and suspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). Cells were seeded at a density of two brains per T75 culture flask (Corning Costar) and maintained at 37 °C in a humidified atmosphere with 5 % CO_2. After 16–18 days of culture, microglial cells were trypsinized, and the cell suspension was centrifuged ($1000\times g$, 5 min, 4 °C) and plated in 48-well plates at a density of 10^5 cells/well in complete medium until stimulation. The purity of obtained microglial cultures was routinely assessed using immunofluorescence with anti-MAC-1, anti-IBA-1, and anti-GFAP antibodies. Using our previously described original procedure, the cultures contained >98 % microglia (submitted).

Microglial Stimulation

Primary microglial cells were pretreated or not with different concentrations of doxycycline (20–250 µM). After 4 h of treatment, cells were stimulated with LPS (1 or 10 ng/mL, final concentration) during 24 h. Dexamethasone (250 µM) was used as a positive control of anti-inflammatory effects in all of the experiments.

Immunocytochemistry

Briefly, cultures were fixed with a fresh formaldehyde solution (4 %, pH 7.4) for 12 min, washed three times with PBS, and processed for immunocytochemistry. Cells were then incubated overnight with a 1:100 dilution of primary rat anti-mouse MAC-1 (AbDSerotec) and 1:400 dilution of rabbit anti-IBA-1 (Wako) to detect microglial cells or with an antibody against GFAP (1:200) to identify astrocytes. On the next day, cultures were incubated with secondary antibodies of goat anti-mouse Alexa 488 (1:500, Invitrogen) or donkey anti-rabbit Alexa 594 (1:400, Invitrogen) for two hours at room temperature. Finally, nuclei were co-stained with 4′,6-diamidino-2-phenylindoledihydrochloride (DAPI) (1:4000; Invitrogen). Cultures were visualized using a Nikon TE 300 inverted microscope (Nikon, Tokyo, Japan) equipped with an ORCA-ER digital camera (Hamamatsu). Phase contrast and fluorescent images were taken using a cooled CCD camera (Hamamatsu Corp., Bridgewater, NJ). Image acquisition and processing were carried out with the HCimage Imaging software (Hamamatsu). The IBA-1 expression was performed using quantitative analysis of the immunofluorescence intensity of individual cells on digitized images using Image J 1.47v software (National Institutes of Health, EUA) as follow: [Integrated Density—(Area of selected cell X Mean fluorescence of background readings)].

Assessment of Cell Viability

To determine cell viability, the colorimetric MTT metabolic activity assay was used (Mosmann 1983). All of the experiments were performed in sextuplicate, and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

Cytokine Release

Conditioned media were collected at the end of treatment periods and frozen at −20 °C until further processing. Cytokine concentration was measured by ELISA assays (mouse IL-6, TNF-α and IL-1β, Invitrogen), according to the manufacturers. The absorbance of each sample was measured at 450 nm with a spectrophotometer SpectraMax M4 (Molecular Devices, Sunnyvale, CA). Standard curves were obtained using a four-parameter logistic curve model (SigmaPlot 12.0 Systat Software, San Jose, CA).

Measurement of Nitric Oxide Production

NO released in the culture medium was quantified using the Griess Reagent System (Promega) that measures nitrite (NO_2^-), which is one of the two primary stable and

nonvolatile breakdown products of NO (Tarpey et al. 2004). One hundred microliters of the medium was mixed with 50 μL of 1 % of sulfanilamide solution in a 96-well plate. After 10 min, the supernatant was allowed to react with 50 μL of 0.1 % NED solution (*N*-1-naphthylethylenediamine). After 15 min, the absorbance was measured spectrophotometrically at 540 nm. Nitrite concentrations were calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay.

Measurement of Intracellular ROS Production

Intracellular ROS were evaluated using fluorescence microscopy with the membrane permeable CellROX Deep Red Reagent (Life Technologies), a fluorogenic probe that produces bright near-infrared fluorescence upon oxidation. Briefly, microglial cultures were exposed to CellROX (10 μM) for 30 min and then washed and fixed with 4 % formaldehyde in PBS before further analysis. For each culture condition, fluorescent images of 10 random fields were acquired using a 20X fluorescence objective. The quantitative analysis representing the corrected cell fluorescence intensity to ROS was calculated using the Image J 1.47v software (National Institutes of Health, EUA) as follows: [Integrated Density—(Area of selected cell X Mean fluorescence of background readings)].

Preparation of the Nuclear Extracts

Microglial cells seeded on 48-well plates that were treated or not with 1 ng/mL of LPS in the presence or absence of doxycycline pretreatment (200 μM). After 30 min of LPS stimulation, cells were removed, and the subcellular fractions were obtained following the protocol adapted from a previous report (Dignam et al. 1983). The cells were lysed with buffer A (10 mM HEPES, 10 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM DDT, 0.2 % NP40, and a protease inhibitor cocktail [Sigma-Aldrich] in ultrapure water). The homogenate was immediately transferred to tubes and vortexed for 1 min. The resulting extract was centrifuged at 20,800×*g* for 5 min, and the supernatant was collected as a cytoplasmic extract. The adhered pellet was washed twice with lysis buffer A, and the supernatants of the centrifugations from these washings were discarded. The resulting pellet was lysed with 50 μL of buffer B (20 mM HEPES, 420 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM DDT, and protease inhibitor cocktail in RIPA buffer [Sigma-Aldrich]) that was maintained in ice and homogenized by vortexing for 30 min. The resulting extract was centrifuged at 20,800×*g* for 5 min, and the resulting supernatant was taken as a nuclear extract.

Western Blotting Analyses

Samples had their protein contents quantified using the Bradford method. Equal amounts of protein were separated by electrophoresis on 10 % polyacrylamide gel (SDS-PAGE), followed by transfer to a nitrocellulose membrane. The membranes were incubated with primary antibody (1:1000) against GAPDH (Sigma-Aldrich), β actin (Sigma-Aldrich), IBA-1 (Wako), p38 MAPK (Cell Signaling), Phospho-p38 MAPK (Cell Signaling), and nucleophosmin (Sigma-Aldrich) in filtered TBS-T buffer containing 5 % milk powder overnight at 2–8 °C with stirring. The membrane containing the nuclear proteins was incubated with p65 (sc-372, Santa Cruz Biotechnology) (1:300) in filtered T-TBS buffer containing 5 % milk powder. After incubation, the membranes were washed with TBS-T buffer and incubated again with the respective secondary antibodies conjugated with peroxidase (Sigma-Aldrich). For measurement, a chemiluminescence system (ECL Western Blotting Systems, GE Healthcare, Little Chalfont, BKM, UK) was used and visualized using the ChemiDoc XRS + System (BioRad, Life Technologies). The bands shown are representative of the groups. The quantification was performed by normalization with a control group (medium).

NF-kB Nuclear Translocation Studies

Microglial cells seeded on 48-well plates were treated or not with 1 ng/mL of LPS in the presence or absence of doxycycline pretreatment (200 μM). After 5, 15, 30, and 60 min of LPS stimulation, cells were fixed as described above, followed by permeabilization with PBS containing 0.05 % Triton X-100 (PBST), and were blocked with 10 % horse serum in PBST. Next, cells were incubated overnight with rabbit anti-NF-kB p65 antibody (Cell Signaling) diluted 1:100 in PBST containing 1 % bovine serum albumin. Samples were then rinsed three times for 5 min with PBS and incubated for 1 h with donkey anti-rabbit Alexa 594 (1:400), washed with PBS and counterstained with DAPI (1 μg/mL). Cultures were visualized using a Nikon TE 300 inverted microscope (Nikon, Tokyo, Japan) equipped with an ORCA-ER digital camera (Hamamatsu). Fluorescent images were taken using a cooled CCD camera (Hamamatsu Corp., Bridgewater, NJ). Regions of interest (ROI) in the acquired images were selected based on DAPI fluorescence in nuclei. The mean fluorescence intensity of the NF-kB p65 signal was then quantified in each ROI. Average values for each condition were calculated and referred to untreated cells. Image acquisition and processing were carried out using the HCimage Imaging software (Hamamatsu) and ImageJ software, respectively.

Statistical Analysis

Data are reported as the mean ± SEM and are representative of two or three different experiments carried out in triplicate. A one-way ANOVA compared the means from different treatments in individual experiments. When significant differences were identified, individual comparisons were subsequently made using Bonferroni's test for unpaired values. The level of significance was set at $p < 0.05$.

Results

Effect of Doxycycline on the Activation and Morphology of Primary Cultures of Microglial Cells

To evaluate the effect of doxycycline on LPS-activated microglia, primary mouse microglial pure cell cultures were pretreated for 4 h with different concentrations of doxycycline. Subsequently, the cells were stimulated with LPS (1 ng/mL) for 24 h. The results showed that most of resting microglia cells in our culture conditions expressed MAC-1 and a very low level of IBA-1. In contrast, LPS-activated microglia showed high IBA-1 fluorescence, and cells treated with doxycycline exhibited no change in MAC-1 but a reduction in IBA-1 staining in a concentration-dependent manner (Fig. 1a, b). The dexamethasone positive control also inhibited the LPS-induced increase in the IBA-1 expression (Fig. 1a, b). Furthermore, the morphology of microglial cells in the control group was almost exclusively amoeboid versions with short thick processes, and these cells most frequently showed an ovoid shape, with a few cells presenting a fusiform shape. Unlike these cells, LPS-stimulated microglia showed a more heterogeneous morphology. Most of the cells had a large, round and flat shape with other cells presenting a ramified morphology. After treatment with dexamethasone and doxycycline in a concentration-dependent manner, microglia cells acquired a morphologic structure typically resembling that seen in the control groups (Fig. 1a). Confirming these results, the Western blotting analysis revealed that doxycycline inhibited the LPS-induced increase in IBA-1 expression by primary microglia (Fig. 1c). Interestingly, these treatments did not alter cell viability (Fig. 1d). Thus, these results clearly show that doxycycline is able to inhibit primary microglial cell activation.

Effect of Doxycycline on the Release of Proinflammatory Cytokines Induced by LPS in Primary Microglial Cells

Next, we investigated the effect of doxycycline on the release of proinflammatory cytokines by microglia in response to LPS. The results showed that this antibiotic reduces the production/release of TNF-α and IL-1β (Fig. 2a, b) in a dose-dependent manner. Dexamethasone was also able to inhibit the production/release of these cytokines. In contrast, the increased levels of IL-6 that were induced by LPS remained unaltered in the presence of doxycycline, whereas a total inhibition of this induction was observed in dexamethasone-treated cultures (Fig. 2c). Notably, doxycycline alone did not increase the production of proinflammatory cytokines (Fig. 2a–c).

Effect of Doxycycline on the Production of Nitrogen and Reactive Oxygen Species that were Induced by LPS in Primary Microglial Cells

In addition to the production of proinflammatory cytokines, activated microglia produce nitrogen and oxygen reactive species that act as mediators in the neuroinflammatory process (Zhang et al. 2013; Sanchez-Guajardo et al. 2013). Thus, we next evaluated the effect of doxycycline on the microglial production of NO and ROS triggered by LPS. Pre-incubation of microglial cells with doxycycline decreased the production of ROS and abolished the NO release induced by LPS (Fig. 3a–c). Doxycycline alone did not increase the production of NO or ROS (Fig. 3a–c). Notably, 1 ng/mL of LPS was not sufficient to generate an increased production of ROS and NO. Therefore, the optimal LPS concentration that was previously standardized for these measurements was 10 ng/mL of LPS.

Effect of Doxycycline on the Activation of the p38 MAPK Signaling Pathway in Primary Microglial Cells Activated by LPS

The mitogen-activated protein kinase (MAPK) signaling pathways, such as p38, control the release of proinflammatory mediators in activated microglial cells (Kang et al. 2014). Therefore, we examined whether doxycycline influences this signaling pathway in LPS-stimulated microglial cultures. We found that LPS treatment significantly increased the p38 MAPK phosphorylation levels compared to unstimulated microglial cells (Fig. 4a). Pre-treatment with doxycycline significantly inhibited the LPS-stimulated upregulation of p38 MAPK phosphorylation (Fig. 4a). To determine whether p38 MAPK is involved in the mechanism by which doxycycline inhibits microglial activation, we investigated the effect of the p38 MAPK inhibitor SB203580. We observed a reduction both in IBA-1 expression and in the production of proinflammatory cytokines TNF-α and IL-1β, but no reduction of IL-6 was observed when microglia cultures were pre-incubated with either doxycycline or SB203580 before LPS stimulation (Fig. 4b–e).

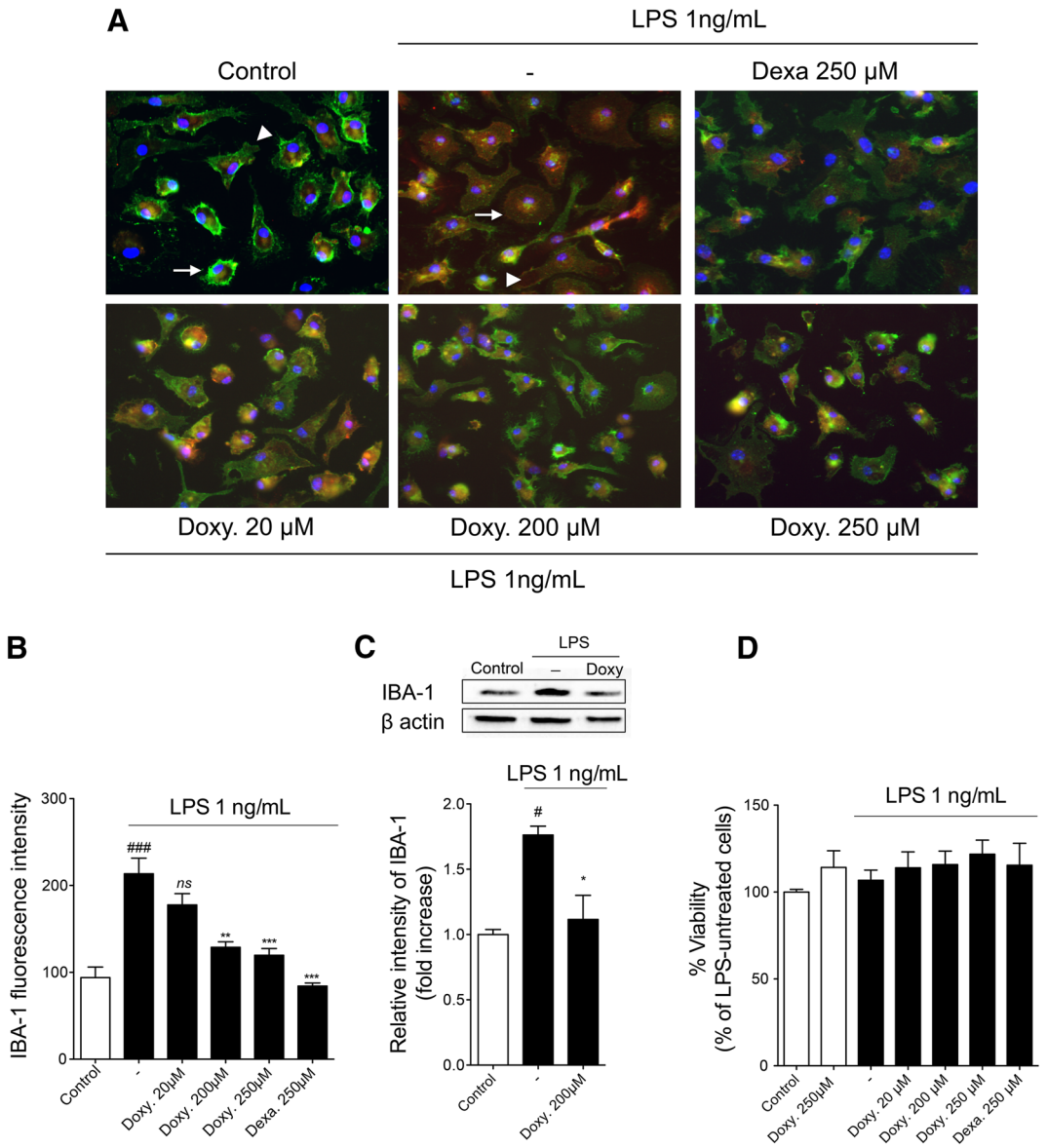

Fig. 1 Effect of doxycycline on LPS-induced microglial cell activation, morphology, and cell viability. **a** Primary microglial cell cultures were pre-incubated for 4 h with doxycycline (20, 200, and 250 μM) followed by stimulation with LPS (1 ng/mL; for 24 h). The total microglial cell population was assessed using immunostaining with MAC-1 (*green*), whereas microglial activation was evaluated using IBA-1 expression (*red*). DAPI (*blue*) was used to visualize all of the cells in culture (100 %). In the control group, microglia were amoeboid with an ovoid shape (*arrow*), and only a few cells presented a fusiform shape (*arrowhead*). LPS-stimulated microglia were large, round and flat shape (*arrow*), with other cells presenting a ramified morphology (*arrowhead*). After treatment with dexamethasone and doxycycline, the microglia cells acquired a morphologic structure that mainly comprised amoeboid versions with short thick processes. **b** Semi-quantitative analysis of IBA-1 expression shows an inhibitory effect of doxycycline (200 and 250 μM). **c** Western blotting analyses of IBA-1 expression in LPS-stimulated microglia and the effect of doxycycline (200 μM). A representative blot is included (*top*), and the relative intensity levels of IBA-1 (%) are shown (*bottom*). **d** Effects of doxycycline on cell viability were measured using an MTT assay and expressed as a percentage relative to the LPS-untreated cells. Data are shown as the mean ± SEM. $^{###}p < 0.001$ versus control and $***p < 0.001$ versus LPS 1 ng/mL (Color figure online)

Effect of Doxycycline on the Activation of the NF-kB Signaling Pathway in Primary Microglial Cells Treated with LPS

To explore other potential anti-inflammatory mechanisms of doxycycline activity, we investigated the effects of doxycycline on the activation of the NF-kB signaling pathway. NF-kB is an inducible transcription factor that plays a crucial role in inflammatory and immune responses (Matsusaka et al. 1993). NF-kB activation is initiated by the signal-induced degradation of IκB proteins, and subsequently, the NF-kB p65 subunit is translocated into the

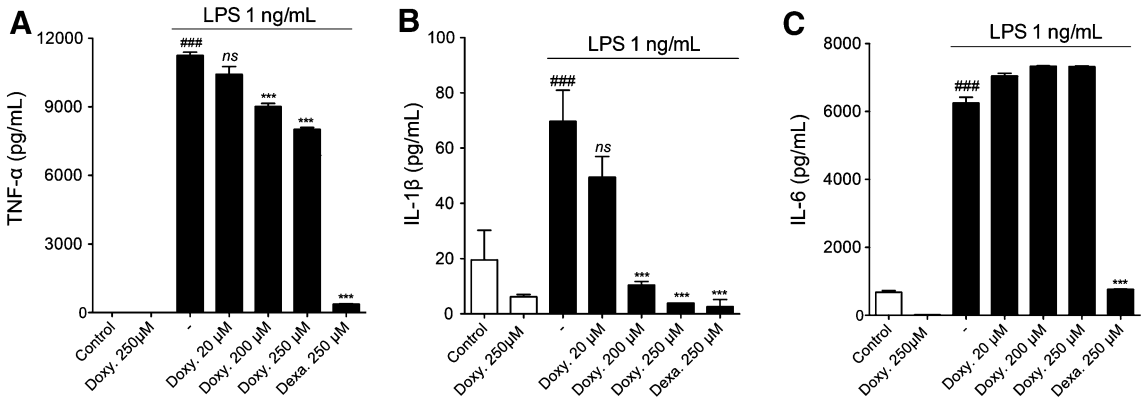

Fig. 2 In vitro effect of doxycycline on the release of proinflammatory cytokines in LPS-stimulated microglial cells. Primary microglial cell cultures were pre-incubated with doxycycline (20, 200, and 250 μM for 4 h) followed by stimulation with LPS (1 ng/mL; for 24 h). Supernatant levels of IL-1β (**a**), TNF-α (**b**), and IL-6 (**c**) were measured using ELISA. Data are shown as the mean ± SEM. $^{###}p < 0.001$ versus control and $^{***}p < 0.001$ versus LPS 1 ng/mL

nucleus, where it can promote the transcription of target genes (Kang et al. 2014). Based on these reports, the time course of NF-kB p65 nuclear translocation in LPS-treated microglial cells was evaluated by immunofluorescence microscopy. A significant increase in p65 nuclear translocation was observed within 5–60 min of LPS stimulation (1 ng/mL) and peaked at 30 min (Fig. 5a). Pretreatment with 200 μM of doxycycline significantly decreased p65 translocation within 30 and 60 min of LPS stimulation compared with the vehicle-treated group (Fig. 5b, c). To confirm these results, Western blotting analyses were performed using the nuclear extract of LPS-stimulated microglial cells. Corroborating the immunofluorescence results, we observed that doxycycline inhibited the LPS-induced translocation of the NF-kB p65 subunit to the nucleus (Fig. 5d).

Discussion

The results of this study show for the first time that the well-tolerated antibiotic, doxycycline, inhibits LPS-induced microglial activation and limits the production of inflammatory mediators by suppressing the p38 MAPK and NF-kB pathways. Neuroinflammation is an important factor in both the pathogenesis and progression of neurodegenerative diseases (Zhang et al. 2013). A vital component of neuroinflammation is the chronic activation of microglia, which are the major immune cells resident in the central nervous system and thus constitute the innate immune system of the brain. Activated microglia can release neurotrophic factors, proinflammatory cytokines, and many cytotoxic molecules, including NO and ROS (Liu and Hong 2003; Wojtera et al. 2005). Acute activation results in tissue repair and protective immune response induction. However, when activation becomes chronic, its outcome can be deleterious to the brain, resulting in neurodegeneration. The mechanisms by which these cells contribute to neuronal damage and degeneration are the subject of intense study directed at finding novel pharmacological strategies (Cunningham 2013).

In addition to the degeneration of dopaminergic neurons, we observed that the neuropathological picture of activated glial cells that are found in the CNS of PD patients (Liu and Hong 2003; Gerhard et al. 2006; Hirsch et al. 1998) is similar to that found in animal models of PD, especially regarding microglial activation (Teismann et al. 2003). We previously demonstrated in a 6-OHDA mouse model of PD that orally ingested or subcutaneously injected doxycycline protects dopaminergic neurons (Lazzarini et al. 2013). Moreover, this neuroprotection was associated with a reduction in microglial cell activation in some brain regions, such as the globus pallidus and the SNc. Based on these findings, we here propose to investigate whether doxycycline could exert a direct effect on microglial cell functions using an in vitro model of neuroinflammation with LPS-activated primary microglial cells.

As a proof of concept, several immunologic stimuli have been used to directly induce microglial activation. The bacterial endotoxin LPS has been the most extensively utilized glial activator for inducing inflammatory dopaminergic neurodegeneration in PD models (Dutta et al. 2008). By using LPS to activate microglia, many research groups have shown that some TC, such as minocycline, can inhibit microglia activation and reduce a wide array of proinflammatory and neurotoxic factors (Henry et al. 2008; Kim et al. 2004; Fan et al. 2005; Horvath et al. 2008). Minocycline may also inhibit the glial activation that is induced by dopaminergic toxins such as MPTP (Wu et al. 2002). However, the administration of minocycline over

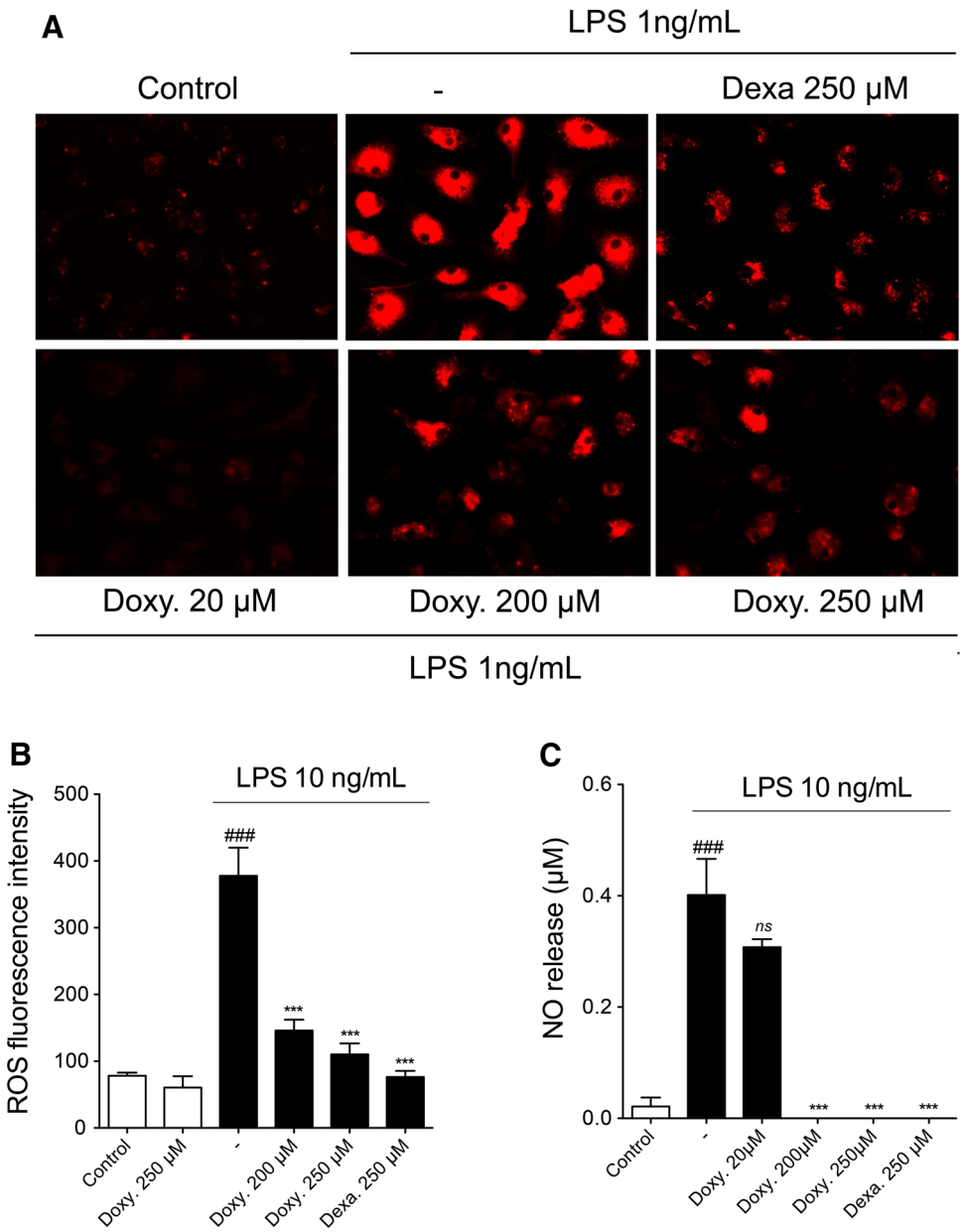

Fig. 3 In vitro effect of doxycycline on the reactive nitrogen and oxygen species in LPS-stimulated microglial cells. Primary microglial cell cultures were pre-incubated or not with doxycycline (20, 200, and 250 µM) for 4 h, followed by stimulation with LPS (10 ng/mL; for 24 h). **a** The probe was added to microglial cells for 30 min. Then, cells were washed with PBS and fixed with 4 % formaldehyde for further analysis using fluorescence microscopy. **b** The quantitative analysis representing the cell fluorescence intensity relative to ROS production showed an inhibitory effect of doxycycline (200 and 250 µM). **c** Inhibitory effect of doxycycline on NO release after stimulation of cells with LPS. Data are shown as the mean ± SEM. $^{###}p < 0.001$ versus control and $***p < 0.001$ versus LPS 10 ng/mL

long periods of time may result in the emergence of undesirable side effects, including disturbances of the commensal microflora (Edan et al. 2013).

To test the effect of doxycycline on the activation of isolated microglia, cells were immunostained with a microglia specific antibody against MAC-1, which is the high-molecular-weight cell surface heterodimeric glycoprotein (CD11b/CD18) (Block et al. 2007). The expression of MAC-1 is restricted to brain microglia, and MAC-1 is considered the microglia surface marker that has the most important functional significance (Roy et al. 2008). Furthermore, the activation status of microglia was investigated using the activation marker IBA-1 (ionized calcium-binding adapter molecule 1), which is highly and specifically expressed in both monocytic and microglial cells (Ito et al. 2001; Singh et al. 2014). The immunostained cells treated with doxycycline showed no change in MAC-1 but very weak IBA-1 staining, whereas cells stimulated with LPS showed high IBA-1 fluorescence. Phenotypic changes in microglia are often accompanied by a morphological transformation, which has been widely used to categorize different activation states (Caldeira et al. 2014). Microglial cells in vitro usually do not have the ramified structure that is typically seen in the normal CNS. They show heterogeneous shapes that range from spindle and rod-shaped or amoeboid versions with short thick processes expanding as

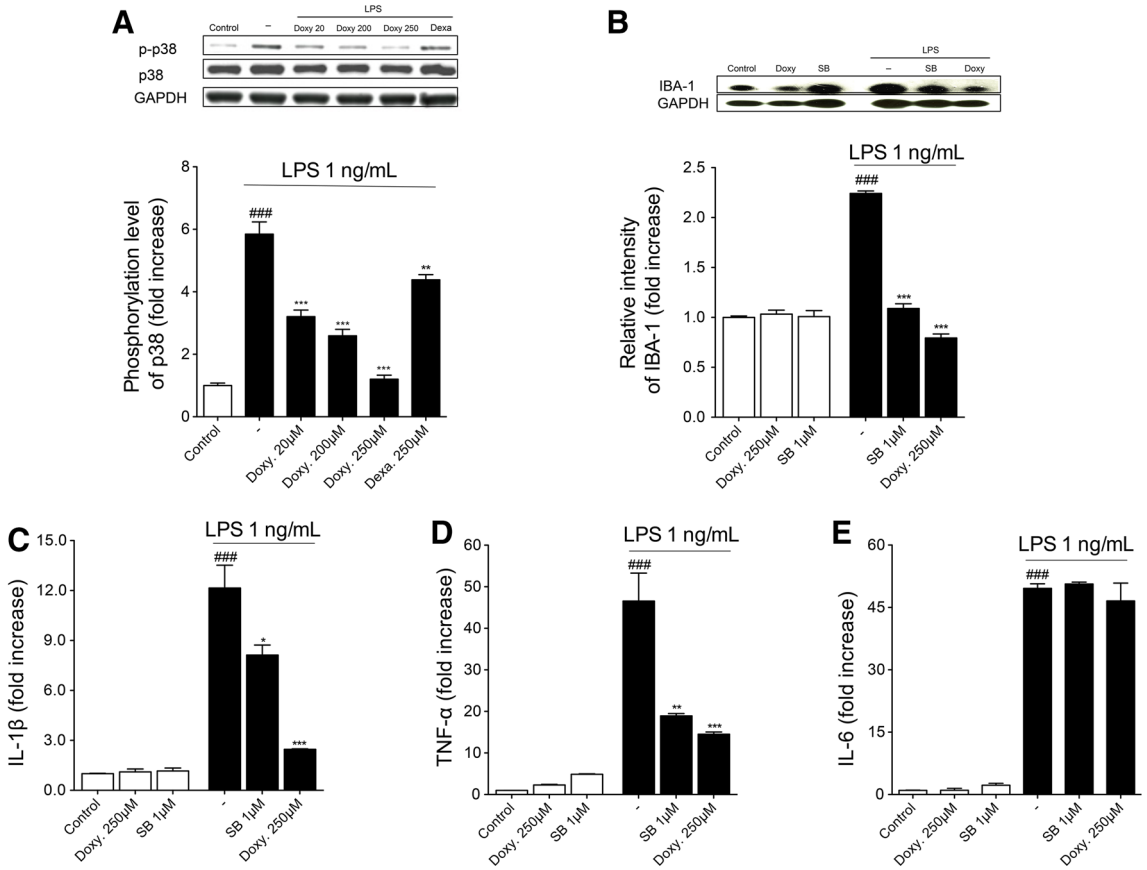

Fig. 4 In vitro effect of doxycycline on the phosphorylation of p38 MAPK in LPS-stimulated microglial cells. Primary microglial cell cultures were pre-incubated or not with doxycycline (20, 200 and 250 μM) or SB203580 (1 μM) for 4 h followed by stimulation with LPS (1 ng/mL; 24 h). Western blotting analysis of p-p38 **(a)** and IBA-1 **(b)** showed an inhibitory effect of doxycycline. A representative blot is included (*top*), and the relative intensity levels of p-p38 and IBA-1 (%) are shown (*bottom*). An inhibitory effect of doxycycline and SB203580 is observed on the release of IL-1β **(c)** and TNF-α **(d),** but not on IL-6 **(e)**, in conditional medium of LPS-activated microglial cells. Values are expressed as fold increase compared to control and are presented as the mean ± SEM. $^{###}p < 0.001$ versus control and $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus LPS 1 ng/mL

lamellipodia to even, round cells (Kettenmann et al. 2011). The microglial cell morphology observed in this work is in agreement with that of Abd-El-basset and Fedoroff (1995). After treatment with doxycycline, the microglia showed a morphology similar to that observed in the dexamethasone group. Therefore, consistent with our in vivo results, doxycycline was able to reduce microglial cell activation in vitro in a concentration-dependent manner.

Furthermore, our findings support the notion that doxycycline limits the production/release of TNF-α and IL-1β. These data corroborate the finding that doxycycline reduced the increase in mRNA of IL-1β and TNF-α in LPS-stimulated BV-2 cells (Cho et al. 2009). Interestingly, doxycycline did not change the production/release of IL-6 by LPS-stimulated microglia in the same manner as that observed in a previous study with mouse thymic epithelial cells (Huang et al. 2011). However, these results are in agreement with other studies using primary human macrophages (Page et al. 2010) or resident peritoneal macrophages (Shi et al. 2015). In fact, Page et al. (2010) showed that although two p38 MAPK inhibitors (SB-731445 and SB-203580) were able to inhibit the production of TNF-α and IL-1β in LPS-stimulated primary human monocytes, they produced a significant increase in IL-6 production. Along the same lines, Shi et al. (2015) showed that the increase in IL-6 production by LPS-stimulated murine peritoneal macrophages was not affected when cells were pretreated with a p38 MAPK inhibitor (SB-203580). Additionally, Kang et al. (2008) showed that macrophages from p38α-deficient mice produce less TNF-α, but not IL-6, when stimulated with LPS. Thus, other signaling pathways, such as the AP-1-dependent pathway, could be involved in the production of IL-6. Indeed, LPS stimulation resulted in the activation of the IL-6 gene in an AP-1-dependent manner (Dendorfer et al. 1994). This finding could also explain why dexamethasone also

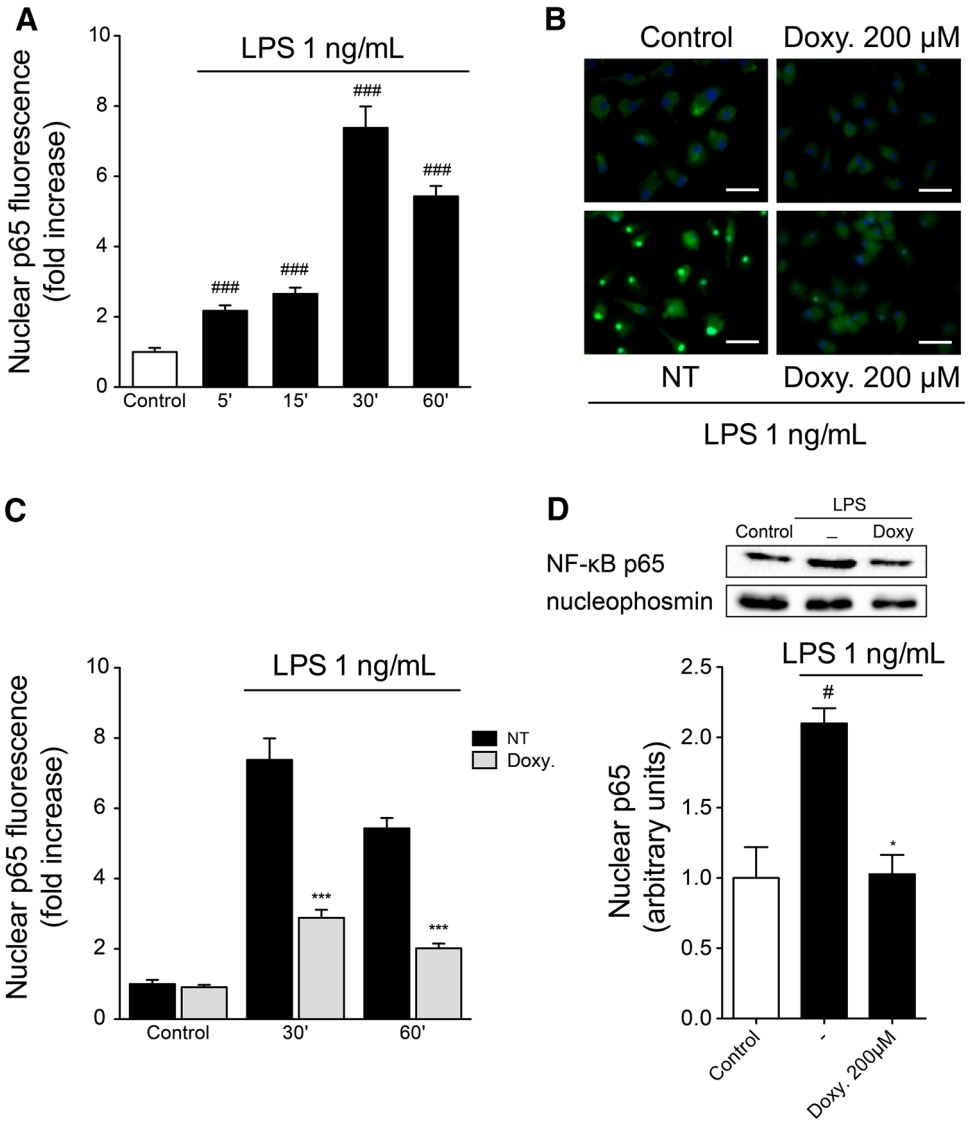

Fig. 5 In vitro effect of doxycycline on NF-kB translocation in LPS-stimulated microglial cells. **a** Time course of NF-kB p65 nuclear translocation in LPS-treated (1 ng/mL) microglial cells measured from immunofluorescence microscopy studies. **b** Nuclear p65 fluorescence is shown in control conditions and at different time points (30 and 60 min) after LPS stimulation (1 ng/mL) in microglial cells pretreated or not (NT) with 200 µM doxycycline. **c** NF-kB subunit p65 (*green*) translocation to the nucleus (DAPI) was followed by immunofluorescence in control conditions and 30 min after LPS stimulation (1 ng/mL) in microglial cells pretreated or not (NT) with 200 µM doxycycline. **d** Levels of NF-kB p65 subunit and nucleophosmin were evaluated in nuclear extracts of LPS-stimulated microglia using Western blot analysis. Data are shown as the mean ± SEM. $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ versus control and $^{*}p < 0.05$; $^{***}p < 0.001$ between LPS (NT) and Doxy. *Bar* size, 50 µm (Color figure online)

affected LPS-induced IL-6 production in primary microglial cells (present results) because it is able to inhibit AP-1 activation (Bosscher et al. 2014).

Oxidative stress, in which the production of highly reactive oxygen species (ROS) and reactive nitrogen species (RNS) overwhelms antioxidant defenses, can directly oxidize and damage macromolecules such as DNA, proteins, and lipids, culminating in neurodegeneration in the CNS (Emerit et al. 2004). Because ROS/RNS are vital proinflammatory mediators and play an important role in neuroinflammatory diseases, we tested the ability of doxycycline to inhibit the ROS and NO microglial production triggered by LPS. The results showed that doxycycline reduced the production of ROS and completely abolished the production of NO induced by LPS. Our results matched a similar finding, in which doxycycline decreased the release of NO by BV-2 microglial cells in response to LPS (Cho et al. 2009).

We further evaluated the effect of doxycycline on upstream p38 MAPK and NF-kB signaling pathways to investigate the underlying molecular mechanisms. MAPKs play critical roles in the integration and processing of cellular responses to a number of diverse extracellular signals that lead to inflammatory responses (Kang et al. 2014). A key signal transduction pathway involved in the production of proinflammatory cytokines is p38 MAPK, which is also one of the kinase pathways that regulates the production of IL-1β and TNF-α (Bachstetter and Eldik 2010). NF-kB is also a key transcription factor that has been implicated in the regulation of proinflammatory cytokines (Blackwell and Christman 1997). NF-kB activation involves IκB-α phosphorylation and the subsequent

translocation of the NF-kB p65 subunit into the nucleus to promote the transcription of target genes (Kang et al. 2014). Our results suggest that the inhibition of p38 MAPK and NF-kB signaling might be one of the possible molecular mechanisms contributing to the effect of doxycycline in LPS-stimulated microglial cells. These data are consistent with related findings suggesting that minocycline's mechanism of action on neuroinflammation seems to be dependent on the modulation of MAPK and NF-κB signaling pathways in primary microglia cell cultures (Nikodemova et al. 2006). Although these findings suggest that TC can down-modulate the MAPK and NF-kB signaling pathways, the exact molecular target of these compounds remains to be elucidated.

It is noteworthy that similar to doxycycline, p38 inhibition did not change the production of IL-6 by LPS-stimulated microglia, in agreement with other studies using primary human macrophages (Page et al. 2010) or resident peritoneal macrophages (Shi et al. 2015). For example, Page et al. (2010) showed that although two p38 MAPK inhibitors (SB-731445 and SB-203580) were able to inhibit the production of TNF-α and IL-1β in LPS-stimulated primary human monocytes, they produced a significant increase in IL-6 production. Further, Shi et al. (2015) showed that the increase in IL-6 production by LPS-stimulated murine peritoneal macrophages was not affected when cells were pretreated with a p38 MAPK inhibitor (SB-203580). Additionally, Kang et al. (2008) showed that macrophages from p38α-deficient mice produce less TNF-α, but not IL-6, when stimulated with LPS. Thus, other signaling pathways such as AP-1-dependent signaling could be involved in the production of IL-6. Indeed, LPS stimulation resulted in the activation of the IL-6 gene in an AP-1-dependent manner (Dendorfer et al. 1994). This finding could explain why dexamethasone also affects LPS-induced IL-6 production in primary microglial cells (present results), as it can inhibit AP-1 activation (Bosscher et al. 2014).

From a therapeutic point of view, both minocycline and doxycycline have neuroprotective activity that is likely mediated through the inhibition of microglial activation. However, doxycycline has an advantage because it is absorbed rapidly, penetrates very well into the brain (Yim et al. 1985) and has less toxic side effects than minocycline does (Smith and Leyden 2005).

Conclusions

The present results indicate that doxycycline has a direct effect on microglial cell activation through the reduction of the production of proinflammatory cytokines and reactive nitrogen and oxygen species. These effects might be mediated by the inhibition of MAP kinase p38 and NF-kB signaling pathways. The development of agents that reduce microglial activation and their proinflammatory responses is considered an important therapeutic strategy for neuroinflammatory disorders such as cerebral ischemia, Alzheimer's disease, and PD (Block and Hong 2005; Stolp and Dziegielewska 2009). Therefore, our results reinforce that doxycycline should be explored as a useful therapeutic target to regulate microglial activation and suggest that doxycycline, which is a drug that protects dopaminergic neurons, could be proposed for the treatment of PD.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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