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Minocycline inhibits apoptotic cell death via attenuation of TNF-α expression following iNOS/NO induction by lipopolysaccharide in neuron/glia co-cultures

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Abstract

We attempted to ascertain the neuroprotective effects and mechanisms of minocycline in inflammatory-mediated neurotoxicity using primary neuron/glia co-cultures treated with lipopolysaccharide (LPS). Neuronal cell death was induced by treatment with LPS for 48 h, and the cell damage was assessed using lactate dehydrogenase (LDH) assays and by counting microtubule-associated protein-2 (MAP-2) positive cells. Through terminal transferase deoxyuridine triphosphate-biotin nick end labeling (TUNEL)-staining and by measuring caspase-3 activity, we found that LPS-induced neuronal cell death was mediated by apoptosis. We determined that pre-treatment with minocycline significantly inhibited LPS-induced neuronal cell death. In addition, LPS induced inducible nitric oxide synthase (iNOS) expression significantly, resulting in nitric oxide (NO) production within glial cells, but not in neurons. Both nitric oxide synthase (NOS) inhibitors (N^G-monomethyl-L-arginine monoacetate (L-NMMA) and S-methylisothiourea sulfate (SMT)) and minocycline inhibited iNOS expression and NO release, and increased neuronal survival in neuron/glia co-cultures. Pre-treatment with minocycline significantly inhibited the rapid and extensive production of tumor necrosis factor-alpha (TNF-α) mediated by LPS in glial cells. We also determined that the signaling cascade of LPS-mediated iNOS induction and NO production was mediated by TNF-α by using neutralizing antibodies to TNF-α. Consequently, our results show that the neuroprotective effect of minocycline is associated with inhibition of iNOS induction and NO production in glial cells, which is mediated by the LPS-induced production of TNF-α.

Keywords: inducible nitric oxide synthase, lipopolysaccharide, minocycline, neuronal cell death, nitric oxide, TNF-α.
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Inflammation has an important role in the pathogenesis of such neurodegenerative diseases as Alzheimer's disease (O'Banion and Finch 1996), Parkinson's disease (McGeer *et al*. 1988), and multiple sclerosis (Raine 1994) as well as in post-traumatic brain and spinal injuries (Giulian and Vaca 1993; Dirnagl *et al*. 1999; Lee *et al*. 1999). During the progress of these diseases, the frequently observed activation of glia produces a variety of proinflammatory factors. These factors include nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and reactive oxygen species (ROS). Therefore, inflammation is believed to contribute to neurodegeneration (Kreutzberg 1996; Aloisi 1999).

Minocycline, a semisynthetic tetracycline derivative, has an anti-inflammatory property, in addition to its antibacterial effect. Some reports have shown that minocycline provides neuroprotection against global ischemia in gerbils and focal brain ischemia in rats (Yrjänheikki *et al*. 1998, 1999). We

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Abbreviations used: GFAP, glial fibrillary acidic protein; IFN-γ, interferon-γ; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; L-NMMA, N^G-monomethyl-L-arginine monoacetate; LPS, lipopolysaccharide; MAP-2, microtubule-associated protein-2; NO, nitric oxide; SMT, S-methylisothiourea sulfate; TNF-α, tumor necrosis factor-alpha; TUNEL, terminal transferase deoxyuridine triphosphate-biotin nick end labelling.

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have previously shown that minocycline inhibits neuronal apoptotic cell death, and improves hindlimb motor function after spinal cord injury in rats (Lee *et al*. 2003). It has also been reported that minocycline delays mortality in a transgenic mouse model of Huntington's disease (Chen *et al*. 2000), and reduces the loss of hippocampal pyramidal neurons in a gerbil model of ischemia (Yrjänheikki *et al*. 1998). Furthermore, the report by Yrjänheikki *et al*. (1998) showed that minocycline treatment inhibited inducible nitric oxide synthase (iNOS) expression in astrocytes and microglia and reduced ischemic damage. These observations suggest that minocycline provides neuroprotection from neural injuries via anti-inflammatory mechanisms.

Lipopolysaccharide (LPS) has been used in both *in vivo* and *in vitro* studies of inflammatory disorders associated with neurodegenerative diseases. Some reports indicate that LPS-induced neuronal cell death is closely associated with iNOS/NO-dependent toxicity (Jeohn *et al*. 2000c; Kim *et al*. 2002), and that glial cells are the main source of LPS-induced iNOS/NO in both neuron/glia cell cultures, and in *in vivo* systems (Jeohn *et al*. 2000a; Kim *et al*. 2002; Liu *et al*. 2002). While the relative contributions of iNOS activation in microglia and astrocytes to brain inflammation is not clear, it has been suggested that activated microglia and astrocytes produce NO, and mediate neuronal cell death in neurodegenerative disorders (Boje and Arora 1992; Liu *et al*. 2002).

NO is involved in a number of physiological and pathological processes within the CNS (Bredt and Snyder 1990; Nathan 1992; Bruhwyler *et al*. 1993; Liu *et al*. 2002). Three isoforms of nitric oxide synthase (NOS) exist in the CNS. Among them, iNOS is most rapidly transcribed and expressed in macrophages, microglia, and astrocytes after stimulation with LPS and proinflammatory cytokines such as TNF-α, interleukin-1 (IL-1), and interferon-γ (IFN-γ) (Stuehr *et al*. 1989; Galea *et al*. 1992; Simmons and Murphy 1992; Coyle 1996; Bhat *et al*. 1999). It has been reported that NO produced by iNOS in mixed neuron/glia cultures leads to NO-mediated neuronal cell death (Dawson *et al*. 1994; Skaper *et al*. 1995; Jeohn *et al*. 2000c). NO-induced apoptosis has been reported in macrophages (Sarih *et al*. 1993), astrocytes (Hu and Van Eldik 1996), and numerous other mammalian cells.

The purpose of this study was to explore the potential neuroprotective effects of minocycline utilizing neuron/glia co-cultures. Also, we wanted to explore a possible mechanism underlying LPS-induced neuronal cell death. For this purpose, we investigated the following: (1) whether minocycline reduces the iNOS/NO production which contributes to LPS neurotoxicity in neuron/glia co-cultures; (2) whether the activation of glia which can be inhibited by minocycline in neuron/glia co-cultures contributes to LPS-induced neuronal cell death; and (3) whether an early induction of TNF-α by LPS up-regulates iNOS expression and NO production in activated glial cells. Our data suggest that minocycline has great potency in the protection of LPS-induced neurotoxicity. The protection is achieved by attenuating TNF-α production, which occurs in part through iNOS induction and NO production in glial cells.

Materials and methods

Materials

Cell culture ingredients, TRIZOL reagent and MMLV reverse transcriptase were purchased from GibcoBRL (Gaithersburg, MD, USA). The apoptag peroxidase kit for terminal transferase deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) was purchased from Oncor (Gaithersburg, MD, USA). Z-DEVD-AFC, a caspase-3 enzyme substrate, was purchased from Enzyme Systems Products (Livermore, CA, USA). NOS inhibitors, N^{G}-monomethyl-$_{L}$-arginine monoacetate (L-NMMA) and S-methylisothiourea sulfate (SMT) were purchased form Calbiochem (San Diego, CA, USA). The neutralizing antibody to TNF-α was purchased from R & D Systems (Minneapolis, MN, USA). Minocycline, lipopolysaccharide (LPS; *Escherichia coli* 0111:B4), and other compounds were purchased from Sigma (St Louis, MO, USA).

Primary cell cultures

Cultures substantially enriched in neuronal cells were prepared from the cerebral cortices of embryonic day 16 (E16) rats [Sprague-Dawley rats; Sam:TacN (SD) BR, Korea] according to the previously reported method, but with a small modification (Jeohn *et al*. 2000b,c). Briefly, meninges were removed and dissociated by trituration in HBSS media. After trypsinization, cells were harvested and seeded at a density of 5×10^5 cells in 24-well plates (Nalge Nunc International, Rochester, NY, USA) coated with 20 μg/mL of poly-D-lysine (Sigma) in DMEM media, supplemented with 10% fetal bovine serum (FBS), and were grown in humidified 5% CO_2 incubator at 37°C. After 24 h, half the media was removed and 10 μM cytosine arabinoside (Ara-C) was added.

Primary mixed glia cultures were prepared from SD rat brains on postnatal day 1–2 according to the protocol for neuronal cell preparation described above. Cells (5×10^5 cells) were plated onto 24-well plates and the DMEM medium was changed every 3 days thereafter until the glial cells formed a completely confluent layer in each culture well. It usually took ~11 days to obtain a confluent layer of glia in a given well. As measured by immunocytochemistry, approximately 34% of glial culture cells stained positively for CD-11b, and about 60% stained positively with glial fibrillary acidic protein (GFAP).

For neuron/glia co-cultures, neuronal cells were also prepared according to the protocol described above. Cells (5×10^5 cells) were seeded on the confluent glial cell layers in 24-well tissue culture plates. The DMEM media was changed after 1 day, and co-cultures were continued for 5 days. The neuron/glia co-cultures were stained with MAP-2 antibody (1 : 100; Upstate Biotechnology, Lake Placid, NY, USA), and approximately 110 ± 8 neurons were counted per mm^2.

Pure microglia cultures were prepared from 7 to 10-day-old-mixed glial cultures as described above. The mixed glial cells were seeded at a density of 10^7 cells per 75 cm^2 flask. After 7–10 days in cultures, the flasks were shaken at 200 r.p.m. for 3 h. Floating cells were harvested and seeded onto poly-D-lysine-coated cover glasses at a density of 5×10^4 cells. By immunocytochemical analysis,

more than 98% of the cells stained positively for anti-OX-42 antibody (1 : 100; Chemicon, Temecula, CA, USA).

To obtain pure astrocytes, the cells remaining in the flasks after removal of microglia were harvested by trypsinization (0.25% trypsin/0.02% EDTA). Cells were plated on poly-D-lysine-coated cover glasses at a density of 2.5×10^4 cells. Greater than 98% of cells were GFAP-positive by immunocytochemistry using anti-GFAP antibody (1 : 100; Dako, Carpintera, CA, USA).

Assessment of neuronal cell death

Neurotoxicity was measured by counting the number of the MAP-2 positive cells per field of each well. Cells were fixed with 4% paraformaldehyde and stained with MAP-2 antibody. After incubating with HRP-conjugated secondary antibody, diaminobenzibine (DAB) substrate was used for visualization. The MAP-2 positive cells were counted using an inverted microscope at 200 × magnification. Three fields of each well were counted with three repetitive experiments to achieve an average count per field. Neurotoxicity was also assessed using an LDH assay kit (Sigma) according to the manufacturer's instructions. Absorbance was read at 490 nm with a microplate reader (Spectra Max 340; Molecular Devices, Sunnyvale, CA, USA). Values were expressed relative to measurement from maximal LDH.

Terminal transferase dUTP nick end labeling (TUNEL) staining

For TUNEL staining, we used an *in situ* apoptag peroxidase kit, according to the manufacturer's instructions. Cells were rinsed with PBS and fixed in 4% paraformaldehyde for 30 min followed by quenching with 3% H_2O_2. Thereafter, cells were incubated in a mixture of terminal transferase, digoxygenin-dUTP in TdT buffer at 37°C for 2 h. After the reaction was terminated with stopping buffer, cells were incubated in peroxidase-antidigoxygenin antibody at 37°C for 30 min, and then stained with DAB reagents. The TUNEL-positive cells were visualized with an inverted microscope at 200 × magnification, and analyzed by counting the number of TUNEL-positive cells per field of each well. Four fields of each well were counted and averaged.

Caspase-3 enzyme activity

Caspase-3 enzyme activity was analyzed according to the previously described method with slight modifications (Lee *et al*. 2003). Cells were incubated as indicated above, then lysed in lysis buffer (100 mM HEPES, 10% sucrose, 0.1% 3-[3-cholamidopropylammonio]-1-propanesulfonate (CHAPS), pH 7.5, 10 mM DTT, and 10 mg/mL leupeptin). Following sonication (10 s, duty cycle 100%, output control 10%), lysates were centrifuged 10 000 *g* at 4°C for 5 min Protein extract (200 μg) was added to 1 mL of caspase homogenization buffer, containing caspase-3 substrate, Z-DEVD-AFC (15 μM). After incubation at RT for 5 min, relative fluorescence (excitation at 400 nm and emission at 505 nm) was measured using a K2 multifrequency phase fluorometer (ISS Inc., Champaign, IL, USA) for 1 h. The specific activity of the samples was calculated relative to recombinant caspase-3 standard curves (Upstate Biotechnology). The experiment was repeated three times.

Immunocytochemistry

Mixed neuron/glia co-cultures were grown in chamber slides (Nalge Nunc International) for immunocytochemistry, and cultured for 5 days as described above. After LPS treatment, cells were fixed with 4% paraformaldehyde in PBS and kept on ice for 15 min. After washing with PBS, cells were blocked with 1% normal goat serum, and incubated with MAP-2 antibody (1 : 200). Incubation was performed at 4°C overnight. After several washes with PBS, cells were incubated with anti-mouse secondary antibodies at RT for 1 h. After washing, staining was visualized using DAB. For double immunostaining, antibodies for iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 100 dilution) and TNF-α (Santa Cruz Biotechnology; 1 : 100 dilution) were used. Secondary antibodies (FITC for TNF-α, TRITC for iNOS) were used for double immunostaining.

Nitrite assay

NO production was measured as nitrite accumulated in the culture medium by using a colorimetric reaction with Griess reagent (Sigma). One hundred μL supernatants were collected after 48 h of LPS treatment. Equal volumes of Griess reagent were used. Absorbance was measured at 540 nm with a microplate reader (Spectra Max 340; Molecular Devices). Activity was expressed in terms of the nitrite concentration as determined from a standard sodium nitrite curve.

RNA isolation and RT-PCR

RNA was purified using TRIZOL Reagent according to the manufacturer's instructions. To ascertain that all RNA samples were DNA-free, samples were treated with RNase-free DNase I (Sigma). After spectrophotometric quantification, purified RNA was separated on a formaldehyde-agarose gel to check the extent of degradation. One microgram of total RNA was reverse-transcribed to first-strand cDNA in each 20 μL reaction mixture using MMLV (Invitrogen, Groningen, the Netherlands) according to the manufacturer's instructions. A 20-μL PCR reaction contained 2 μL of first-strand cDNA, 0.6 U Amplitaq polymerase (Perkin-Elmer, Branchburg, NJ, USA), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM dNTP, and 10 pmol of each specific primer. Primers for iNOS (Yune *et al*. 2003), tumor necrosis factor-α (TNF-α) (Estler *et al*. 1992) and β-actin (Nudel *et al*. 1983) were subjected to PCR reactions on a thermocycler (Perkin-Elmer). Primers with the target gene sequences were synthesized by the Genetech Corp. (Daejeon, Korea). The primer sequence for iNOS was 5′-CTCCATGACTCT-CAGCACAGAG-3′ (sense) and 5′-GCACCGAAGATATCCTCAT-GAT-3′ (antisense). Primers for tumor necrosis factor-α (TNF-α) were 5′-CCCAGACCCTCACACTCAGAT-3′ (sense) and 5′-TTGTCCCTTGAAGAGAACCTG-3′ (antisense). Primers for β-actin were 5′-CTTCTGCATCCTGTCAGCGATGC-3′ (sense) and 5′-AGAAGAGCTATGAGCTGCCTGACG-3′ (antisense). After amplification, PCR products were subjected to 2% agarose gel electrophoresis, and visualized by ethidium bromide staining. The relative density of bands was analyzed by the ChemiImager™ 4400 (Alpha Innotech Corporation, San Leandro, CA, USA). Quantification of PCR bands was performed as previously described (Murphy *et al*. 1993; McCombe *et al*. 1998; Lee *et al*. 2003). Experiments were repeated three times and the values obtained for relative intensity were subjected to statistical analysis.

Western blot analysis

For western blot analysis, cells were lysed with 500 μL of buffer containing 10 mM HEPES, 250 mM sucrose, 0.1% CHAPS, 1 mM EDTA, 5 mM DTT, 1.5 mM $MgCl_2$, 10 mM KCl, and protease inhibitor cocktail (Sigma). The lysates were centrifuged at 12 000 *g*

at 4°C for 10 min, and the supernatants were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Membranes were blocked in 5% skim milk in TBS/0.05% Tween for 1 h, and incubated overnight with anti-iNOS antibody (BD Transduction Laboratory, Palo Alto, CA, USA) at a 1 : 1000 dilution. The blots were visualized by chemiluminescence using Supersignal (Pierce, Rockford, IL, USA).

Statistical Analysis

Data are presented as mean ± SD values. Data were evaluated for statistical significance using ANOVA with a *post hoc* Tukey test. In all analyses, a p-value of < 0.05 was considered statistically significant.

Results

Minocycline protects neurons from LPS-induced neurotoxicity

Kim *et al*. (2002) previously reported that LPS (10 μg/mL) induced neuronal cell death in primary neuron/glia co-cultures. After 48 h of LPS treatment, LDH assays were performed. Results showed that LPS treatment induced LDH release levels to about five times those of the control. Minocycline treatment 1 h prior to LPS treatment at concentrations ranging from 1 to 20 nM resulted in significant reductions in LDH release (Fig. 1a). Maximal neuroprotective effect without toxicity was detected at 1 nM minocycline. Although these data are not shown, there was no significant neuroprotective effect at low concentrations (for example, 0.1 or 0.5 nM) minocycline. Therefore, 1 nM of minocycline was used for the experiments. For the detection of neuronal cell death, MAP-2 positive neurons were also counted after immunocytochemistry. The number of MAP-2 positive cells in neuron/glia co-cultures increased after minocycline pre-treatment (71 ± 15 per field) from untreated levels (39 ± 10) (Fig. 1e).

Minocycline prevents LPS-induced apoptotic neuronal cell death

To examine apoptotic cell death, TUNEL staining and caspase-3 enzyme activity assays were performed on neuron/glia co-cultures after 48 h of LPS treatment. LPS treatment resulted in an increase in TUNEL-positive cells (18 ± 3 per field), whereas pre-treatment with minocycline resulted in a reduction in TUNEL-positive cells (3.6 ± 0.5) (Figs 2a–d). LPS treatment also increased specific caspase-3 activity at 24 h (1.662 ± 0.49 pmole/min) and 48 h (4 ± 0.5 pmole/min). Pre-treatment with minocycline significantly reduced LPS-induced caspase-3 specific activity at 48 h (2.32 ± 0.27 pmole/min) (Fig. 2e).

Minocycline inhibits iNOS expression and NO production

Since previous reports indicated that iNOS expression/NO production were critical in LPS-induced neurotoxicity (Jeohn *et al.* 2000 a,b,c; Kim *et al*. 2002; Liu *et al*. 2002), we first

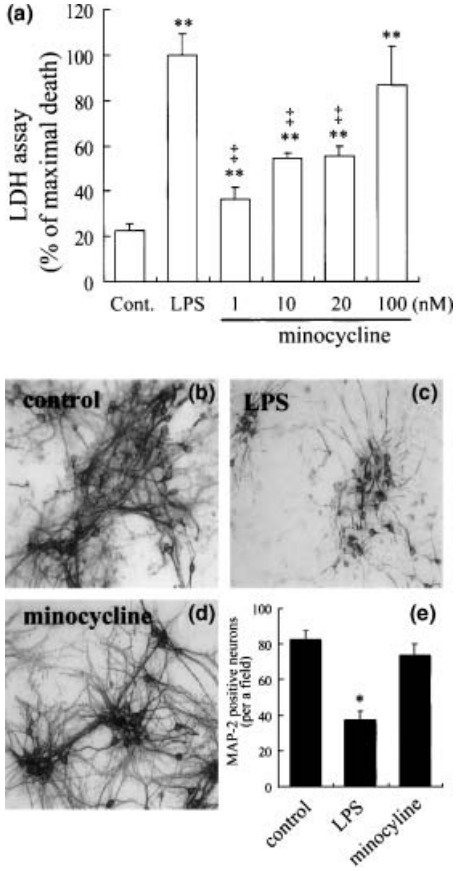

Fig. 1 Cell cytotoxicity and viability in mixed neuron/glia co-cultures after LPS treatment. Cells were incubated with minocycline (1, 10, 20, and 100 nM) before LPS (10 μg/mL) treatment. After 48 h, culture media was assayed for LDH release (a). Neuroprotective effects of minocycline were determined by immunostaining with MAP-2 antibody (b-d). Cells were incubated with minocycline (1 nM) for 48 h, the cells were fixed in 4% paraformaldehyde and then immunostained with MAP-2 antibody. The numbers of MAP-2 positive neurons were counted in a 200 × magnification field (e). MAP-2 positive neurons were reduced by LPS toxicity (c), but in the presence of minocycline, neurons were conserved (d). Values are expressed as mean ± SD. *$p < 0.05$, **$p < 0.005$ vs. control; ‡$p < 0.005$ vs. LPS-treated.

examined the expression of iNOS mRNA and protein in neuron/glia co-cultures following NO production mediated by LPS-treatment. As shown in Figs 3(a) and (b), LPS treatment increased iNOS mRNA and protein expression in neuron/glia co-cultures in a time-dependent manner, and pre-treatment with minocycline inhibited such expression (Figs 3a,b). LPS also induced NO production in a time-dependent manner, and this was also inhibited by pre-treatment with minocycline (Fig. 3c). LPS treatment markedly increased NO production to levels of about 30 μM, but minocycline significantly decreased LPS-induced NO production to final levels of 12 μM at 48 h (Fig. 3c). Based on

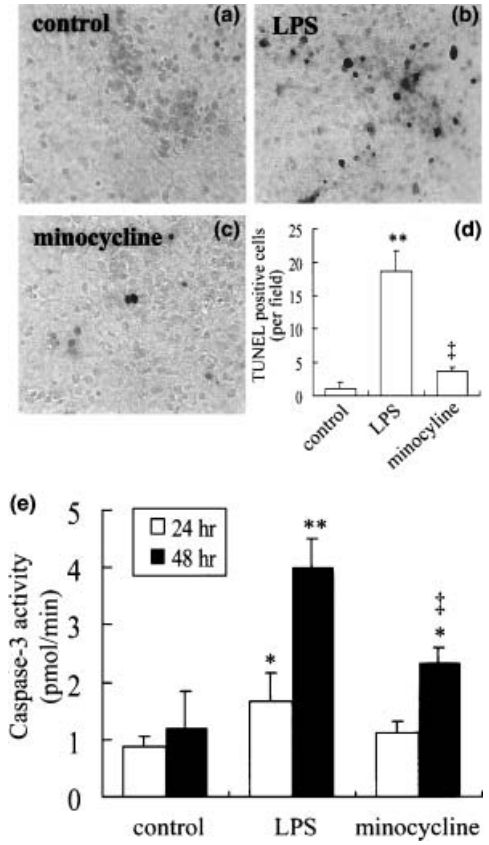

Fig. 2 TUNEL-staining in mixed neuron/glia co-cultures after LPS treatment. After LPS treatment (10 μg/mL) for 48 h either with or without minocycline (1 nM) pre-treatment, cells were fixed and TUNEL stained, according to manufacturer's protocols (a-d). The number of TUNEL-positive cells was counted in four fields, and the results were expressed as number in each field (x 200 magnification) (d). Figures presented indicate that TUNEL-positive cells were detected after LPS (b). Note that treatment with minocycline significantly reduced the number of TUNEL-positive cells compared to those seen in the LPS-treated cells (c, d). Caspase-3 activity in mixed neuron/glia co-cultures after LPS treatment. After cells were stimulated by LPS for 24 and 48 h, cells were harvested, following homogenization. Caspase-3 activity was measured by cleavage of Ac-DEVD in a fluorometric assay. Note that caspase-3 specific activity was lower in minocycline treated samples than in LPS-treated cells (e). Values are expressed as mean ± SD. $*p < 0.05$, $**p < 0.005$ vs. control; ‡$p < 0.005$ vs. LPS treated.

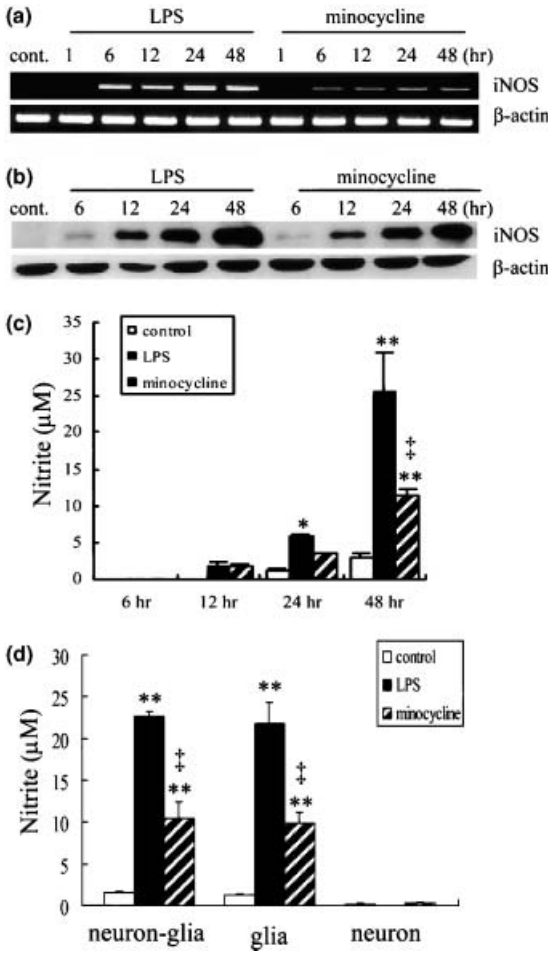

Fig. 3 Inducible nitric oxide synthase (iNOS) expression and NO production in mixed neuron/glia co-cultures, glia cultures and neuronal cell culture after LPS treatment. Cells were stimulated with LPS (10 μg/mL) and pre-treated with minocycline (1 nM). After incubation, total RNA was prepared and analyzed by RT-PCR for levels of iNOS mRNA. β-actin mRNA levels were used to normalize RT-PCR. The gel shown is representative of results from three separate experiments (a). iNOS protein expression in cell lysate was determined by western blotting (b). NO production from culture media was determined by Griess reagent (c, d). Data indicated that LPS induced high levels of iNOS mRNA and protein expression (a, b). In addition, NO production was occurred in the glia culture, not in neuron culture after LPS treatment for 48 h (d). However, minocycline reduced iNOS expression and NO production (a-d). Values are expressed as mean ± SD. $*p < 0.05$, $**p < 0.005$ vs. control; ‡$p < 0.005$ vs. LPS treated.

previous reports indicating that LPS-induced NO production occurs in glial cells (Jeohn *et al*. 2000b,c; Kim *et al*. 2002), we examined whether NO was produced by glial cells or neuronal cells. As shown in Fig. 3(d), NO was produced significantly in neuron/glia co-cultures and in glial cultures after LPS treatment, but not in neuronal cultures. These results suggest that there is a correlation between iNOS expression/NO production and the extent of neuronal cell death, as previous reports had also concluded (Jeohn *et al*. 2000b,c; Kim *et al*. 2002).

NO-mediated neurotoxicity is ameliorated by pre-treatment with minocycline

To determine the role of NO in LPS-induced neurotoxicity, we pre-treated neuron/glia co-cultures with the NOS inhibitor

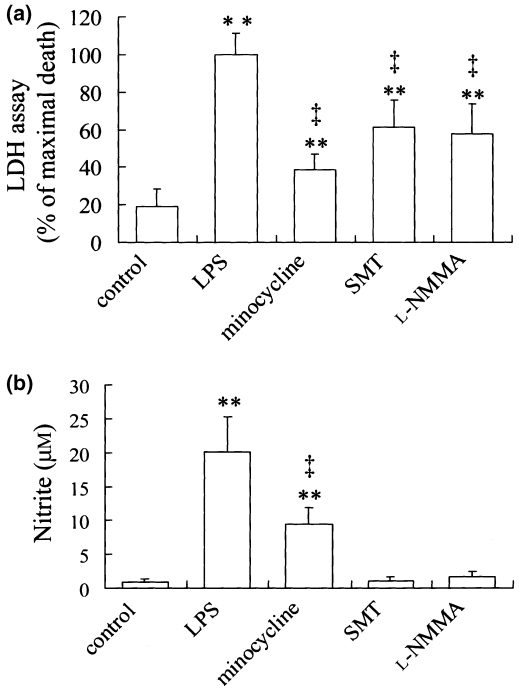

Fig. 4 The effect of minocycline on NO-mediated neurotoxicity after LPS treatment in neuron/glia co-cultures. Cells were incubated with minocycline (1 nM), SMT (500 μM, iNOS inhibitor), and L-NMMA (1 mM, NOS inhibitor) before LPS stimulation (10 μg/mL). At 48 h, cytotoxicity was determined by LDH release (a). NO release was determined by Griess reagent (b). Note that minocycline, SMT, and L-NMMA significantly inhibited NO production and reduced LDH release after LPS treatment (a, b). Values are expressed as mean ± SD. $^{**}p < 0.005$ vs. control; $‡p < 0.005$ vs. LPS-treated.

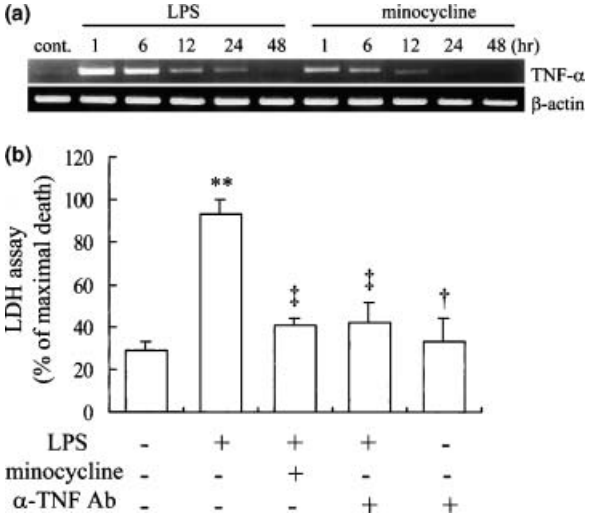

Fig. 5 The effect of minocycline on TNF-α mRNA expression and cytotoxicity in mixed neuron/glia co-cultures after LPS treatment. Cells were pre-treated with minocycline (1 nM) and TNF-α-neutralizing antibody (5 ng/mL) before LPS stimulation (10 μg/mL). After incubation, total RNA was prepared and analyzed by RT-PCR for levels of TNF-α and β-actin mRNA levels. The result shows that minocycline treatment significantly reduced TNF-α mRNA expression produced by LPS treatment (a). The gel shown is representative of results form three separate experiments. After 48 h, cytotoxicity was determined by LDH assay. The result shows that minocycline and TNF-α-neutralizing antibody reduced cell death by LPS-induced toxicity (b). $^{**}p < 0.005$ vs. control; $†p < 0.05$, $‡p < 0.005$ vs. LPS-treated.

L-NMMA and the iNOS inhibitor SMT, 1 h prior to LPS treatment. As shown in Fig. 4, L-NMMA and SMT inhibited NO production and LDH release compared to the release from the control (61.5 ± 14.4% of maximal cell death for SMT; 57.8 ± 15.6 for L-NMMA). This suggests LPS-induced neurotoxicity was mediated via iNOS-produced NO. Furthermore, pre-treatment with minocycline significantly inhibited NO production and LDH release (Fig. 4). These results indicate that minocycline inhibits neuronal cell death by attenuating iNOS expression and NO production after LPS treatment in neuron/glia co-cultures.

Minocycline also inhibits LPS-induced TNF-α expression

It has been reported that LPS increases the production of proinflammatory cytokines, such as TNF-α (Coyle 1996; Bhat *et al*. 1999). Here, we examined the effect of minocycline on the expression of TNF-α after LPS treatment in neuron/glia co-cultures. As shown in Fig. 5(a), expression of TNF-α mRNA rapidly increased, peaked 1 h after LPS treatment, and gradually decreased in a time-dependent manner. Interestingly, pre-treatment with minocycline significantly inhibited the expression of TNF-α.

The neuroprotective effect of minocycline was executed by inhibition of TNF-α-mediated iNOS/NO signaling cascades after LPS treatment

As previous findings have indicated that TNF-α contributes to neuronal cell death via the iNOS/NO signaling cascade (Zujovic *et al*. 2000; Nicholas *et al*. 2001), we examined the role of TNF-α in iNOS/NO signaling cascades and neuronal cell death using neutralizing antibody to TNF-α in neuron/glia co-cultures after LPS treatment. As shown in Fig. 5(b), treatment with TNF-α-neutralizing antibody or minocycline significantly reduced LDH release, and inhibited iNOS expression and NO production (Figs 6a,b). For example, treatment with anti-TNF-α antibody (5 ng/mL) inhibited neuronal cell death to levels about 68% of those achieved with LPS, and also inhibited NO production (12.4 μM ± 0.6) more than LPS did (24 μM ± 1.2). To further investigate whether TNF-α mediates iNOS/NO signaling cascades after LPS treatment, we examined the co-localization of TNF-α and iNOS by immunocytochemical double-staining in glial cultures. As shown in Fig. 7(a), iNOS-positive cells were detected in microglia as well as astrocyte cell cultures by immunocytochemistry. By double-immunostaining, iNOS-

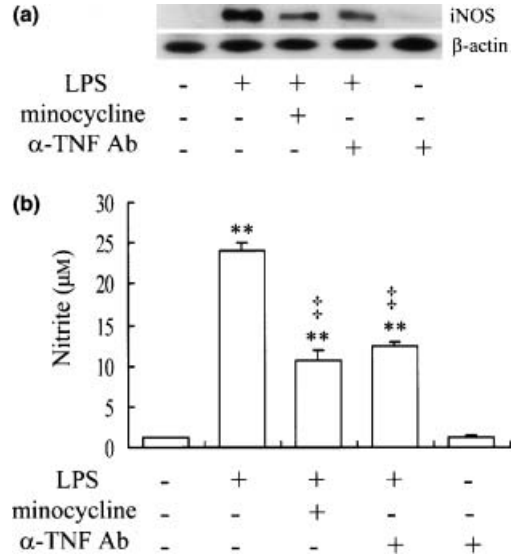

Fig. 6 The effect of TNF-α-neutralizing antibody on NO production in mixed neuron/glia co-cultures. Cells were incubated with 1 nM of minocycline and TNF-α-neutralizing antibody (5 ng/mL) 1 h before LPS treatment (10 μg/mL). At 48 h, iNOS expression in cell lysate was determined by western blotting (a) and NO production was determined by Griess reagent (b). Note that minocycline and TNF-α-neutralizing antibody inhibited iNOS expression and NO production by LPS-induced toxicity. Values are expressed as mean ± SD. $^{**}p < 0.005$ vs. control; $‡p < 0.005$ vs. LPS-treated.

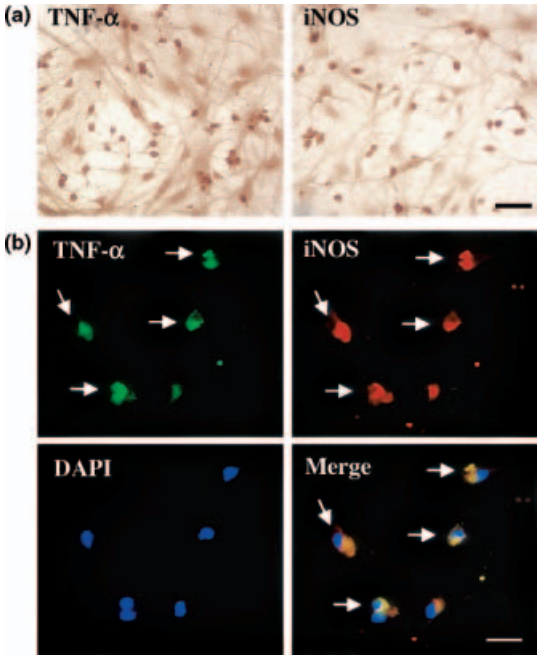

Fig. 7 Immunocytochemistry of iNOS and TNF-α expression in glial cells. After treatment of 10 μg/mL of LPS for 12 h in astrocytes, the cells were fixed in 4% paraformaldehyde and then immunostained with iNOS and TNF-α antibody (a). Scale bar is 50 μm. Note that iNOS and TNF-α have immunoreactivity specific to both astrocyte and microglia by LPS treatment. By double immunostaining for TNF-α and iNOS, iNOS immunoreactivity was observed in TNF-α-positive cells in microglia (b). Scale bar indicates 25 μm.

positive cells were also co-localized with TNF-α in astrocytes (data not shown) and microglia (Fig. 7b). These results suggest that TNF-α mediates iNOS/NO signaling cascades, and minocycline inhibits neuronal cell death by reducing TNF-α expression after LPS treatment.

Discussion

In this study, we demonstrated the neuroprotective effect of minocycline and its mechanisms against LPS-induced neurotoxicity in primary neuron/glia co-cultures. Our data showed that LPS-induced apoptotic neuronal cell death was mediated by NO produced by iNOS induction, and that minocycline significantly reduced neuronal cell death by inhibiting NO production and iNOS induction after LPS-treatment. Most notably, we determined that iNOS/NO signaling cascades were mediated by TNF-α which was rapidly produced by LPS treatment, and that the neuroprotective effects of minocycline were mechanistically dependent on the inhibition of TNF-α production.

Minocycline is a tetracycline derivative that has both antimicrobial effects and anti-inflammatory effects (Smilack 1999). This drug is tolerated well clinically and currently used for treatment of chronic inflammatory diseases such as rheumatoid arthritis (Tilley *et al*. 1995; Mantovani *et al*. 1997). Many reports, including our previous report, have verified the existence of neuroprotective effects of minocycline during *in vivo* and *in vitro* studies. However, the neuroprotective mechanisms of minocycline had not, until now, been explained sufficiently. Here we attempted to ascertain the neuroprotective effects and mechanisms of minocycline in inflammatory-mediated neurotoxicity using primary neuron/glia co-cultures treated with LPS. As in previous reports (Kim *et al*. 2002), our data indicated that LPS treatment induced neuronal cell death in primary mixed neuron/glia co-cultures. In addition, minocycline significantly protected neuronal cells from death by inhibiting caspase-3 activation after LPS treatment (Figs 1–3). These results correlate with several previous reports. For example, Chen *et al*. (2000) reported that minocylcine inhibited caspase-1 and caspase-3 activation and delayed mortality in a mouse model of Huntington's disease. Zhu *et al*. (2002) also reported that minocycline inhibited mitochondrial permeability-transition-mediated cytochrome *c* release and subsequently extended survivals in ALS. Furthermore, we previously reported that minocycline reduced caspase-3 activity, and improved functional recovery after SCI (Lee *et al*. 2003).

In this study, 1 nM of minocycline showed the highest neuroprotective effect in LPS-induced toxicity in neuron/glia

co-cultures. By stark contrast, previous reports have shown that 10 nM to 20 nM of minocycline exerted neuroprotective effects (Lin *et al*. 2001; Tikka *et al*. 2001a,b; Lin *et al*. 2003). For example, the report by Tikka *et al*. (2001a) showed that 10 nM to 2 μM of minocycline decreased glutamate-induced LDH release, but 1 nM of minocycline was not in primary spinal cord cultures. Also, Lin *et al*. (2001) reported that 10 μM of minocycline attenuated H_2O_2-induced toxicity and NO-induced neurotoxicity in cerebellar granule neurons (CGN). These are very high concentrations when compared to those we found to be efficacious. Although it is difficult to explain why there are different effects of minocycline as a matter of concentration among different culture conditions, we suggest that perhaps differences in the cell populations of glia might be a critical factor regulating this sensitivity. This may not be valid, however, as our data showed that 10–20 nM of minocycline also had neuroprotective effects in our culture systems.

LPS binds to a CD14-like receptor in glia (Galea *et al*. 1996), which increases the expression of iNOS and generates NO (Boje and Arora 1992; Galea *et al*. 1992; Simmons and Murphy 1992; Murphy *et al*. 1993). It has been proposed that excessive NO production participates in neurotoxicity. In brief, NO reacts with proteins directly and modulates their normal functions. Such proteins include heme-containing enzymes and cyclooxygenase. NO also reacts with lipids in membranes inducing lipid peroxidation (Liu *et al*. 2002). Indirectly, NO reacts with superoxide anions and forms peroxynitrite which can act as a markedly neurotoxic molecule inducing DNA breaks, lipid peroxidation, and protein nitration (Beckman 1996). As previous reports have confirmed (Jeohn *et al*. 2000c), our data showed that LPS induced high levels of iNOS expression and NO production in neuron/glia co-cultures and glial cultures (Fig. 3), which in turn caused LPS-induced apoptosis. Furthermore, minocycline significantly reduced iNOS induction and NO production (Fig. 3). This result coincides with the report by Chen *et al*. (2000) that minocycline reduces expression of iNOS and delays progression in mouse models of Huntington's disease, although experimental situations differed from our own. In addition, some reports have also suggested that minocycline inhibited iNOS expression in both microglia and astrocytes in global ischemia (Yrjänheikki *et al*. 1998), and in a model of Parkinson's disease (Du *et al*. 1998).

An inflammatory response, mediated by activation of glia, is a key event in neurodegenerative diseases. As shown in Fig. 3(d), NO was produced only in glial cells, not neuronal cells, which were attenuated by minocycline. Also in concurrence with previous reports, our data suggested that the neuroprotective mechanisms of minocycline might be involved in the suppression of glial activation after LPS treatment. Some reports have also shown that minocycline protects CNS neurons by inhibiting microglial activation against MPTP, 6-hydroxydopamine- or glutamate-induced toxicity *in vivo* and *in vitro* (Yrjänheikki *et al*. 1998, 1999; He *et al*. 2001; Tikka and Koistinaho 2001; Wu *et al*. 2002). Additionally, recent reports have shown that the neuroprotective effects of minocycline were associated with reduced activation of microglia through the inhibition of p38 MAPK activation (Tikka *et al*. 2001a; Tikka and Koistinaho 2001), and reduced numbers of reactive astrocytes after SCI (Teng *et al*. 2004). In addition, previous reports have shown that the neuroprotection of minocycline was associated with reduced activation of microglia and with inhibition of induction of IL-1β-converting enzyme (ICE) mRNA, and release IL-1β and NO (Yrjänheikki *et al*. 1998, 1999; Du *et al*. 1998; Tikka and Koistinaho 2001; Tikka *et al*. 2001a). These observations support our results that glial cells are inactivated by minocycline.

As previous reports have shown, the induction of iNOS expression/NO production in LPS-induced toxicity has been emphasized in glia-related neuronal cell death (Boje and Arora 1992; Chao *et al*. 1992; Kim *et al*. 2002; Liu *et al*. 2002). Although the iNOS inhibitor, SMT, and NOS inhibitor, L-NMMA, blocked LPS-induced neurotoxicity, these compounds were less effective as compared to the effect of minocycline (Fig. 4). These findings suggest that additional mechanisms may be involved in the neuroprotective effects of minocycline. Previous reports showed that LPS-stimulated glial cells produce such proinflammatory cytokines as TNF-α and IL-1β, which contribute to neuronal cell death by LPS-induced cytotoxicity (Hetier *et al*. 1990; Glauser *et al*. 1991; Raitz *et al*. 1991; Boje and Arora 1992; Chao *et al*. 1992; Kong *et al*. 1996; McGeer *et al*. 1988). Yrjänheikki *et al*. (1998, 1999), especially, suggested that the neuroprotection of minocycline was associated with inhibition of induction ICE mRNA, release of IL-1β, and COX-2 expression. In addition, the report by Tikka *et al*. (2001a) showed that minocycline reduced IL-1β release, NO release and inhibited p38 MAPK activation in microglia by NMDA-induced toxicity. As such, it is likely that minocycline has neuroprotective effects at multiple levels and on multiple targets.

It has been reported that LPS stimulates glia and increases the production of proinflammatory cytokines, such as TNF-α, contributing to neuronal cell death by LPS-induced cytotoxicity (McGeer *et al*. 1988; Hetier *et al*. 1990; Glauser *et al*. 1991; Raitz *et al*. 1991; Boje and Arora 1992; Chao *et al*. 1992; Kong *et al*. 1996). It has also been reported that TNF-α, expressed in neurons and/or glial cells, causes apoptotic cell death, in part, through iNOS/NO expression after injury (Streit *et al*. 1998). Other reports have shown that certain protein kinases, including p38 MAPK and MAPK, are involved in iNOS/NO expression in astrocytes and microglia as intracellular signaling pathways (Feinstein *et al*. 1994; Simmons and Murphy 1994; Kong *et al*. 1996; Bhat *et al*. 1998; Tikka and Koistinaho 2001; Tikka *et al*. 2001a). In addition, we reported that apoptosis induced by TNF-α after SCI may be mediated, in part, via NO production through

prior induction of iNOS (Yune *et al*. 2003). Therefore, we attempted to discern whether TNF-α expression was induced by LPS-mediated neurotoxicity. In our results, LPS induced the expression of TNF-α, which was significantly inhibited by pre-treatment with minocycline (Fig. 5a); pre-treatment with TNF-α-neutralizing antibody significantly decreased LDH release, iNOS induction, and NO production by LPS (Figs 5 and 6). In addition, we ascertained, by double immunocytochemical staining that TNF-α was colocalized with iNOS in astrocytes and microglia (Fig. 7). These results suggest LPS-mediated TNF-α induction is responsible for neuronal cell death through the mediation of iNOS/NO signaling in primary neuron/glia co-cultures.

The mechanism by which TNF-α-mediated iNOS/NO signaling cascades occur in glia is not fully understood. Many studies have suggested that multiple regulations, including transcriptional and translational regulation (Lowenstein *et al*. 1993; Xie *et al*. 1993), might be involved in this pathway. Transcriptional regulation of TNF-α and iNOS is very complicated, involving a number of transcriptional factors, such as NF-κB, AP-1, C/EBP, CREB, and the STAT family (Lowenstein *et al*. 1993; Xie *et al*. 1993; Jongeneel 1995). It has been suggested that co-stimulation of IFN-γ and LPS, IFN-γ and TNF-α was induced in iNOS gene expression through the activity of MAPK in macrophages (Nathan and Xie 1994; Chan *et al*. 1999), and that IL-1β and TNF-α treatments induced expression of iNOS in astrocytes, through the p38 MAPK pathway (Da Silva *et al*. 1997; Kopnisky *et al*. 1997). Interestingly, Ogura *et al*. (1997) reported that TNF-α-mediated iNOS expression in human neuroblastoma cell lines involved NF-κB activation. However, it is still in dispute whether endogeous TNF-α regulates the transcriptional expression of iNOS directly or indirectly. Although we did not fully explain the mechanism of TNF-α-induced iNOS/NO signaling, and further study might be required, our data suggest the direct involvement of TNF-α in this signaling by LPS in glia. Needless to say, our data and a further understanding of the mechanisms underlying the neuroprotective effects of minocycline may provide insights into potential therapeutic interventions for inflammation-related neurodegenerative diseases.

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