Andrographolide Reduces Inflammation-Mediated Dopaminergic Neurodegeneration in Mesencephalic Neuron-Glia Cultures by Inhibiting Microglial Activation

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Received October 6, 2003; accepted November 25, 2003

ABSTRACT

Inflammation plays an important role in the pathogenesis of several neurodegenerative diseases, including Parkinson’s disease. Recent reports have indicated that andrographolide (ANDRO) has an anti-inflammatory effect by modulating macrophage and neutrophil activity. Whereas microglia, the counterpart of macrophages in the brain, are pivotal in the inflammatory process in the central nervous system, the effect of ANDRO on inflammation-mediated neurodegeneration has not been examined. In this study, we show that both pretreatment and post-treatment with ANDRO exhibited a significant protective effect against lipopolysaccharide (LPS)-induced neurotoxicity in mixed neuron-glia cultures, as determined by [3H]dopamine uptake and immunocytochemical analysis. In contrast, ANDRO showed no protective effect on 1-methyl-4-phenyl-pyridine (0.5 μM)-induced neurotoxicity in neuron-enriched cultures. ANDRO significantly attenuated LPS-induced microglial activation and production of reactive oxygen species, tumor necrosis factor-α, nitric oxide, and prostaglandin E2. Furthermore, ANDRO dose-dependently attenuated LPS-induced inducible nitric-oxide synthase and cyclooxygenase-2 protein expression in BV-2 microglia, as determined by Western blot. These findings demonstrate that ANDRO reduces inflammation-mediated dopaminergic neurodegeneration in mesencephalic neuron-glia cultures by inhibiting microglial activation. In addition, these results indicate that ANDRO may have clinical utility for the treatment of inflammation-related neurodegenerative disorders such as Parkinson’s disease.

Inflammation is an important contributor to neuronal damage in neurodegenerative diseases such as Parkinson’s disease (PD), Alzheimer’s disease (AD), multiple sclerosis, and amyotrophic lateral sclerosis (McGeer et al., 1988; Dickson et al., 1993; Raine, 1994; Rogers and Shen, 2000; Liu and Hong, 2003). Microglia, the resident immune cells in the central nervous system, play a key role in the inflammatory reaction (Dickson et al., 1993; Kreutzberg, 1996; Hauss-Wegrzyniak et al., 1998; Aloisi, 1999; Hirsch, 2000; Streit, 2000). Reactive microglia are found in the substantia nigra of PD and AD brains (McGeer et al., 1988). Our laboratory has recently reported that LPS treatment induces neurotoxicity via microglial activation (Liu et al., 2000). Activated microglia produce large amounts of prostanoids, reactive oxygen species (ROS), nitric oxide (NO), and proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1, which, in turn, cause neuronal damage (Boje and Arora, 1992; Merrill et al., 1992; Bronstein et al., 1995; Bagnati et al., 1998; Minghetti and Levi, 1998; Gonzalez-Scarano and Baltuch, 1999; Liu et al., 2002).

Andrographis paniculata is a traditional herb that has been used in South Asia and China for the treatment of inflammation-related diseases such as viral infections and rheumatoid arthritis. It has recently been reported that andrographolide (ANDRO), a diterpenoid and the major component of A. paniculata, exerts its anti-inflammatory effect by inhibiting ROS production in neutrophils and LPS-induced NO production in macrophages (Chiou et al., 1998, 2002).
Because ROS and NO are important players in microglia-involved neurodegenerative diseases, we set out to determine whether ANDRO had any effect on microglial activation, the release of proinflammatory factors, or the subsequent neurotoxicity in LPS-treated mesencephalic neuron-glia cultures.

We found that ANDRO exerted a protective effect against LPS-induced dopaminergic neurodegeneration in mesencephalic neuron-glia cultures through inhibition of microglial activation and production of proinflammatory factors. We also found that ANDRO inhibited LPS-induced expression of cyclooxygenase-2 (COX-2) and inducible nitric-oxide synthase (iNOS) through a post-transcriptional mechanism.

Materials and Methods

Animals. Timed-pregnant Fisher 344 rats were purchased from Charles River Laboratories, Inc. (Raleigh, NC).

Reagents. ANDRO, purchased from Aldrich (Milwaukee, WI), was dissolved in ethanol and then diluted in medium for treatment. The final concentration of ethanol was 0.025%, which showed no effect by itself in any parameters measured in this study. 2′,7′-Dichlorofluorescein (DCFH) diacetate and LPS (E. coli, 0111:B4) were obtained from Calbiochem (San Diego, CA). [3H]Dopamine (DA) (30 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). The tyrosine hydroxylase (TH) antiserum was a gift from Dr. John Reinhart (GlaxoSmithKline, Research Triangle Park, NC). The monoclonal antibody OX-42 was purchased from BD PharMingen (San Diego, CA), and the Vectastain avidin-biotin complex kit and biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA). Tissue culture media, supplements, and horse serum were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from Invitrogen or HyClone Laboratories (Logan, Utah).

Mesencephalic Mixed Neuron-Glia Cultures and Neuron-Enriched Cultures. Primary rat mesencephalic neuron-glia cultures were prepared by following our previously published protocol (Liu et al., 2000). Immunocytochemical analysis indicated that at the time of treatment the cultures were made up of ~12% microglia, 48% astrocytes, and 40% neurons, of which 1 to 2% were TH-immunoreactive (TH-IR) neurons.

Mesencephalic neuron-enriched cultures were prepared following our previously published protocol (Qin et al., 2002). Briefly, mesencephalic neuron-glia seeded at 5 × 10^5/well in 24-well plates were treated with 5 to 10 μM cytosine β-d-arabinofuranoside at 48 h. Two days later, β-d-arabinofuranoside was replaced with fresh complete medium.

Immunocytochemistry. Immunostaining was performed as previously described (Liu et al., 2000). Briefly, after blocking, formaldehyde-fixed cells were incubated overnight at 4°C with antibodies against TH (1:20,000) or OX-42 (5 μg/ml). The bound primary antibodies were visualized by incubation with an appropriate biotinylated secondary antibody, followed by the Vectastain avidin-biotin complex reagents and color development with 3,3′-diaminobenzidine. Images were recorded with a charge-coupled device camera and the MetaMorph software (Universal Imaging Corporation, West Chester, PA).

[3H]DA Uptake Assay. Cultures were incubated for 20 min at 37°C with [3H]DA ([3H]DA plus unlabeled DA, 1 μM final concentration) in Krebs-Ringer buffer. After washing three times with ice-cold Krebs-Ringer buffer, the cells were collected in 1 N NaOH. Radioactivity was determined with a liquid scintillation counter (Liu et al., 2000). Nonspecific uptake was determined in the presence of 1 μM mazindol.

DCFH Oxidation as an Indicator of Intracellular Oxidative Stress. DCFH diacetate enters cells passively and is deacetylated by esterases to form nonfluorescent DCFH. DCFH reacts with ROS to form the fluorescent product 2′,7′-dichlorofluorescein. DCFH diacetate was dissolved in methanol at 10 mM and diluted 500-fold in Hanks′ balanced salts solution (HBSS) to yield a final concentration of 20 μM. Mixed neuron-glia cultures were exposed to DCFH diacetate and corresponding concentrations of ANDRO for 1 h and then treated with HBSS containing LPS (5 ng/ml) for 2 h. The fluorescence was read at 485 nm for excitation and 530 nm for emission on a SPECTRAnmax GEMINI XS fluorescence microplate spectrophotometer ( Molecular Devices, Sunnyvale, CA). The value of the control group was viewed as background, and the increase in value was viewed as an increase of intracellular ROS.

Measurement of Proinflammatory Factors. Superoxide production in enriched microglia cultures after LPS (10 ng/ml) treatment with and without superoxide dismutase (500 U/ml) was measured with the PST-1 assay (Tan and Berridge, 2000). Primary microglia (1 × 10^6/well) were grown overnight in 96-well plates in DMEM containing 10% fetal bovine serum. For the superoxide assay, the cultures were washed twice with HBSS and then maintained in 100 μl/well of phenol red-free HBSS. HBSS (50 μl) was added to each well containing the desired concentrations of ANDRO and immediately followed by 50 μl of 4 mM PST-1 in HBSS. The absorbance at 450 nm was read for a period of 30 min at 37°C with a SPECTRAnmax Plus microplate spectrophotometer (Molecular Devices). The amount of superoxide production was determined as the increase of absorbance in 30 min and expressed as a percentage of the control cultures.

The production of NO was assessed as the accumulation of nitrate in the medium using a colorimetric reaction with the Griess reagent (Green et al., 1982). Briefly, after 24 h of treatment with LPS (5 ng/ml), the culture supernatants were collected and mixed with an equal volume of Griess reagent (0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H₃PO₄). The absorbance was measured at 540 nm.

Prostaglandin E₂ (PGE₂) production after 24 h of LPS treatment in supernatant was measured with a PGE₂ enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI). TNF-α production in supernatant was determined after 6 h of LPS treatment by using an enzyme-linked immunosorbent assay kit from R&D Systems (Minneapolis, MN) according to the manufacturer’s instructions.

mRNA Expression Evaluated by RT-PCR. TRI reagent from Sigma-Aldrich was used for the extraction of total RNA from cells. Yield and purity of RNA preparations were checked spectrophotometrically at 260 and 280 nm. One microgram of total RNA from each sample was used for cDNA synthesis. Reverse transcriptions were performed using the Superscript first-strand synthesis system for RT-PCR (Invitrogen) following the manufacturer’s directions. PCR was performed using Taq DNA polymerase obtained from Invitrogen. The following primers derived from the published cDNA
sequences were used for the PCR amplifications: COX-2 forward, 5'-TTC AAA AGA GTG CTT GGA AAA GGT-3'; COX-2 reverse, 5'-CAT TCT TGC ACG TCT GCT TCT TTT-3'; iNOS forward, 5'-CCC TCG AGT TTT TGG CAG CAG C-3'; iNOS reverse, 5'-GCG TGT CAG AGC TTC GTG GCC GTG-3'; TNF-α forward, 5'-TGT CTT CTA CTT AAC TTT GGG ATC GTT CC-3'; TNF-α reverse, 5'-TGA GAT AGC AAA TCG GCT GAC GGT GTG GG-3'; GAPDH forward, 5'-CAT TGA CCA TCA CAT GAT GCT-3'; and GAPDH reverse, 5'-TGA TCA TAC GAA ATG AGC-3'.

PCR was performed for 1 min at 94°C, 64°C, and 72°C, respectively. To ensure that the amplification was still in the exponential range, reactions were stopped at the following specific cycle numbers for the different primer pairs: COX-2, 27 cycles; iNOS, 26 cycles; TNF-α, 25 cycles; and GAPDH, 23 cycles. Products were inspected visually on 1.5% precast agarose gel with ethidium bromide staining (Invitrogen). Bands were quantified by densitometry. Counts for background were subtracted from counts for the specific bands for the COX-2, iNOS, TNF-α, or GAPDH signals. Ratios were calculated for COX-2, iNOS, and TNF-α signals with the control signals from GAPDH. Averages from these ratios were presented.

**Western Blot.** After treatment with LPS for 18 h, BV-2 cells were collected and lysed for Western blot for COX-2 and iNOS. Protein concentration was determined with the bicinchoninic acid assay (Pierce Chemical, Rockford, IL) following the manufacturer’s guide. Equal amounts of protein (20 μg per lane) were separated by NuPAGE gel (Novex, San Diego, CA) and transferred to polyvinylidene difluoride membranes (Novex). Membranes were blocked with 10% nonfat milk and incubated with polyclonal anti-COX-2 antibody (1: 3000; Cayman) or polyclonal anti-iNOS (1:5000; Transduction Laboratories, Lexington, KY) for 1 h at 25°C. Peroxidase-linked anti-rabbit IgG (1:5000; 1 h at 25°C) and ECL Plus reagents (Amersham Biosciences Inc., Piscataway, NJ) were used as a detection system. Purified COX-2 and iNOS were used as positive controls. The optical density of the bands was measured with a model GS-700 imaging densitometer (Bio-Rad, Hercules, CA).

**Effect of ANDRO on the COX-2 Stability.** To determine whether ANDRO had any effect on the protein stability of COX-2, BV-2 cells were treated with LPS (5 ng/ml) to produce COX-2. Twelve hours later, LPS was washed off three times with HBSS. Then 10 μM cycloheximide (Sigma-Aldrich) was added to block further protein synthesis. Thirty minutes later, ANDRO was added to cultures for an extra 12 h of incubation, and COX-2 immunoreactivity was determined by Western blot analysis.

**Cell Viability Assay.** The effect of various agents on the viability of microglia was analyzed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (Liu et al., 2001). After treatment with LPS and corresponding concentrations of ANDRO for 48 h, 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium was added to the BV-2 microglia cultures. Cells were then incubated for another 2 h. The supernatant was then removed, and 100 μl of dimethyl sulfoxide was added to dissolve the formed formazan. Absorbance was read at 420 nm.

**Statistical Analysis.** Data were expressed as mean ± S.E.M. Statistical significance was determined using an analysis of variance, followed by Bonferroni’s t test using the StatView program (Abacus Concepts, Berkeley, CA). A value of p < 0.05 was considered statistically significant.

**Results**

**Pretreatment by ANDRO Dose-Dependently Protected Dopaminergic Neurons against LPS-Induced Neurotoxicity.** We evaluated the effect of ANDRO on LPS-induced dopaminergic neurodegeneration by using the rat ventral mesencephalic mixed neuron-glia cultures. Neuron-glia cultures were treated for 7 days with vehicle or 5 ng/ml LPS. To evaluate the protective effect of ANDRO, cultures were pretreated for 30 min with desired concentrations of ANDRO before LPS treatment. The extent of the degeneration of dopaminergic neurons was assessed by [3H]DA uptake and immunocytochemical analysis. As shown in Fig. 1A, LPS treatment decreased DA uptake significantly. Pretreatment with ANDRO (0.5–5 μM) attenuated the LPS-induced decrease in DA uptake in a dose-dependent manner. Treatment
with ANDRO alone (5 μM) had no effect on DA uptake compared with control cultures (Fig. 1A).

Immunocytochemical analysis for TH-IR neurons demonstrated that LPS-induced degeneration (5 ng/ml; 7 days) of dopaminergic neurons involved a significant decrease in the number of TH-IR neurons and shortening of TH-IR dendrites. Similar to that observed with DA uptake, ANDRO (1–5 μM) effectively reduced the loss of TH-IR neurons and shortened TH-IR dendrites (Fig. 1, B and C).

ANDRO Showed No Protective Effect on 1-Methyl-4-phenyl-pyridine-Induced Neurotoxicity in Neuron-Enriched Cultures. To determine whether the protective effect of ANDRO was due to its direct effect on neurons, we evaluated the effect of ANDRO on 1-methyl-4-phenyl-pyridine (MPP⁺)-induced dopaminergic neurotoxicity in rat mesencephalic neuron-enriched cultures. Cultures were treated for 7 days with vehicle or 0.5 μM MPP⁺. To evaluate the effect of ANDRO, cultures were pretreated with desired concentrations of the agent for 30 min before LPS treatment. The extent of the degeneration of dopaminergic neurons was assessed by [3H]DA uptake. As shown in Fig. 2, MPP⁺ treatment decreased DA uptake significantly. Pretreatment with ANDRO (0.5–5 μM) failed to affect MPP⁺-induced decrease in DA uptake.

Effect of ANDRO on Microglial Activation and Production of Proinflammatory and Neurotoxic Factors by LPS-Treated Neuron-Glia Cultures. Microglial activation and the subsequent release of proinflammatory factors underlie the LPS-induced neurotoxicity in neuron-glia cultures. To investigate whether ANDRO protected neurons by inhibiting microglial activation, we looked at the effect of ANDRO on microglial activation and the production of proinflammatory factors, including ROS, TNF-α, NO, and PGE₂ from neuron-glia culture stimulated with LPS.

We measured the morphological changes of microglial activation in LPS-treated neuron-glia culture by immunocytochemical assay. As shown in Fig. 3, LPS treatment (5 ng/ml; 24 h) induced a significant increase in OX-42 immunoreactivity. Pretreatment with ANDRO significantly and dose-dependently attenuated the LPS-induced microglial activation. Measurement of superoxide production demonstrated that treatment with ANDRO (0.5–5 μM) dose-dependently attenuated LPS-induced superoxide production in microglia-enriched cultures (Fig. 4A). A moderate reduction in the release of TNF-α by LPS-treated neuron-glia cultures was observed for ANDRO at 6 h (Fig. 4B). Measurement of the levels of nitrite, an indicator of NO production, and PGE₂ indicates that ANDRO (0.1–5 μM) had a potent and dose-dependent inhibitory effect on LPS-induced production of NO (Fig. 4C) and PGE₂ (Fig. 4D) in neuron-glia cultures at 24 h. Because intracellular ROS may act as second messengers in regulating LPS-stimulated TNF-α and PGE₂ production (Wang et al., 2003b; Qin et al., 2003), we also determined intracellular ROS production in neuron-glia cultures by using DCFH diacetate. The results showed that treatment with ANDRO (0.5–5 μM) dose-dependently attenuated LPS-induced intracellular ROS production in neuron-glia cultures at 2 h (Fig. 4E).

Post-Treatment by ANDRO Significantly Attenuated LPS-Induced Release of Proinflammatory Factors and Protected Dopaminergic Neurons against LPS-Induced Neurotoxicity. In addition to pretreatment, we also evaluated the effect of post-treatment with ANDRO on the LPS-induced dopaminergic neurodegeneration. Neuron-glia cultures were treated for 7 days with vehicle or 5 ng/ml LPS. ANDRO (2.5 μM) was given at indicated time points after the start of LPS treatment. The extent of dopaminergic neuron degeneration was assessed by [3H]DA uptake. As shown in Fig. 5A, LPS treatment significantly decreased DA uptake. Post-treatment with ANDRO up to 8 h after LPS treatment attenuated LPS-induced reduction in DA uptake by 20 to 60%. Post-treatment with ANDRO at 20 h after LPS treatment showed no neuroprotective effect.

Similar patterns were observed for the effect of post-treatment with ANDRO on the LPS-induced NO (Fig. 5B) and PGE₂ (Fig. 5C) production. When the levels of NO and PGE₂ released into supernatant at 48 h after LPS treatment were determined, the addition of ANDRO at 8 h after LPS treatment still exhibited an inhibitory effect on LPS-induced PGE₂ (60%) and NO (65%) production. The addition of ANDRO at 20 h after LPS treatment showed no inhibitory effect on either PGE₂ or NO production.
ANDRO Inhibited LPS-Induced Expression of Proinflammatory Factors in Microglia. Microglia are the main source of LPS-induced proinflammatory factors (Boje and Arora, 1992; Bronstein et al., 1995; Minghetti and Levi, 1998; Liu et al., 2002). To determine the mechanism that underlies the inhibitory effect of ANDRO on LPS-induced production of proinflammatory factors, we used the BV-2 microglia cell (a microglial cell line from the mouse that is used extensively for studying the regulation of proinflammatory factors) to analyze the effect of ANDRO on LPS-induced COX-2, iNOS, and TNF-α by RT-PCR and/or Western blot.

Using semiquantitative RT-PCR, we found that LPS induced mRNAs for COX-2, iNOS, and TNF-α at 4 h. Pretreatment with ANDRO did not significantly affect iNOS mRNA expression but slightly attenuated COX-2 mRNA (30%) and TNF-α mRNA (20%) (Fig. 6A).

An examination of the levels of COX-2 or iNOS protein indicated that LPS treatment (5 ng/ml; 24 h) induced COX-2 and iNOS protein expression (Fig. 6B). Pretreatment with ANDRO decreased LPS-induced COX-2 and iNOS protein expression in a dose-dependent manner. The magnitude of inhibition of protein expression was positively correlated with the degree of reduction in LPS-induced production of PGE2 and NO in neuron-glia cultures.

ANDRO Enhanced Protein Degradation of COX-2. To determine whether ANDRO had any effect on the stability of the expressed COX-2 protein, BV-2 cells were treated with LPS (5 ng/ml) to produce COX-2. After 12h, LPS was washed off with HBSS, and cycloheximide was added to prevent further protein synthesis. Thirty minutes later, ANDRO was added to cultures for another 12 h, and COX-2 immunoreactivity was determined by Western blot analysis. Results showed that ANDRO dose-dependently decreased the immunoreactivity of COX-2 (Fig. 7), which indicates that ANDRO enhanced the degradation of the COX-2 enzyme.

Discussion

It has become increasingly evident that inflammation plays an important role in a variety of neurodegenerative disorders. However, therapies using specific inhibitors to prevent the production of individual proinflammatory and neurotoxic factors have not been particularly successful. Two possible reasons may be responsible for this slow progress in developing therapies for neurodegenerative diseases. First, patients recruited in clinical studies might be in such advanced stages of neurodegeneration that anti-inflammatory drugs could not reverse the course of the degenerative process. Second, a multitude of proinflammatory factors have been found to contribute to the pathogenesis of neurodegenerative disorders (Boje and Arora, 1992; Merrill et al., 1992;
Bronstein et al., 1995; Banati et al., 1998; Minghetti and Levi, 1998; Gonzalez-Scarano and Baltuch, 1999; Liu et al., 2002). Therefore, inhibition of only one or two factors by selective inhibitors may not be sufficient to halt the degenerative process. Thus, searching for drugs that have wide-spectrum anti-inflammatory effects and that remain effective after the initiation of the inflammatory process may be most promising. In this study, we reported that ANDRO exhibited a neuroprotective effect in both pretreatment and post-treatment schemes and was effective in attenuating LPS-induced production of several proinflammatory factors, including superoxide, TNF-α, NO, and PGE2.

First, we found that treatment with ANDRO significantly protected dopaminergic neurons from LPS-induced neurotoxicity, as determined by DA uptake assay and TH-IR immunocytochemical staining. MPP+-induces dopaminergic neurotoxicity by acting directly on dopaminergic neurons. The fact that ANDRO showed no protective effect on MPP+-induced dopaminergic neuronal death suggests that the protective effect of ANDRO was glia-dependent. In agreement with the observation that microglial activation and the subsequent release of proinflammatory factors play pivotal roles in inflammatory-mediated neurotoxicity (Boje and Arora, 1992; Merrill et al., 1992; Bronstein et al., 1995; Banati et al., 1998; Minghetti and Levi, 1998; Gonzalez-Scarano and Baltuch, 1999; Liu et al., 2002), we showed that ANDRO inhibited LPS-induced microglial activation, hence affording neuroprotection against inflammation-mediated neurotoxicity.

Second, we found that ANDRO showed a moderate inhibitory effect on LPS-induced production of superoxide, intracellular ROS, and TNF-α but a very potent inhibitory effect on LPS-induced production of NO and PGE2. These findings agree with previous reports that state that ANDRO had an inhibitory effect on LPS-induced NO production in macrophages and fMLP-induced ROS production in neutrophils (Chiou et al., 1998, 2000; Shen et al., 2000, 2002). ROS and NO produced by activated microglia are two factors that have been implicated in the mediation of inflammation-induced neurotoxicity (Boje and Arora, 1992; Farber, 1994). ROS, including superoxide anions, hydroxyl radicals, lipid hydroperoxides and their byproducts (e.g., hydrogen peroxide), are toxic to neurons by inducing lipid peroxidation, DNA fragmentation, and protein oxidation (Farber, 1994). Furthermore, peroxynitrite produced by the combination of NO and superoxide has been indicated to be even more toxic to neurons than either factor alone by inducing DNA strand breaks, lipid peroxidation, and protein nitration (Liu et al., 2002). Recent reports have also suggested that, through the activation of NADPH oxidase, LPS increases the levels of intracellular ROS that serve as second messengers to enhance LPS-induced expression of genes encoding a variety of proinflammatory factors (Sanlioglu et al., 2001; Qin et al., 2004; Wang et al., 2004b). We have found that treatment with ROS scavengers superoxide dismutase/catalase or their mimetic scavenger Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin provides neuroprotection by inhibiting LPS-induced microglial activation (Wang et al., 2004a). As for PGE2 production by activated microglia, it is known to be a major product of arachidonic acid, catalyzed by the rate-limiting enzyme COX. One of the two isoforms of COX, COX-2, is inducible and plays an important role in inflammation and mitogenesis (Hla and Neilson, 1992; Lee et al., 1992; Smith et al., 1996). A number of epidemiological studies have pointed to the beneficial effects of nonsteroidal anti-inflammatory drugs, which are inhibitors of COX, in delaying the clinical progression of AD or PD (McGeer et al., 1996; Chen et al., 2003). Our laboratory has recently reported that COX-2 knockout mice are more resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurode-
Fig. 6. Effect of ANDRO on LPS-induced iNOS, TNF-α, and COX-2 expression. BV-2 cells were pretreated with and without ANDRO for 30 min prior to treatment with LPS (5 ng/ml). After 4 h, mRNA expression was analyzed by RT-PCR (A). After 24 h, protein products were determined by Western blot analysis (B). The intensity of the bands was measured by densitometry, and the value for LPS-treated groups was taken as 100%. Means ± S.E.M. of the percentages obtained in three independent experiments are shown; p < 0.05 (*) and p < 0.01 (**) compared with LPS-treated cultures. Images from one experiment, representative of three, are shown.
terns of ANDRO were also observed in LPS-induced PGE2 generation than are wild-type mice (Feng et al., 2002). Thus, it is likely that COX-2 and its products also play important roles in inflammation-related neurodegeneration.

As inflammation may well have developed in most PD patients with clinical symptoms, it is desirable to have a therapeutic agent for PD that can suppress the ongoing inflammatory process and halt the progression of disease states. For this purpose, we tested to determine whether post-treatment with ANDRO was still neuroprotective in LPS-induced neurotoxicity. Notably, post-treatment with ANDRO was protective against LPS-induced neurotoxicity. Treatment with ANDRO at 1 and 3 h post-LPS treatment exhibited similar degrees of neuroprotection compared with that observed with pretreatment. The same inhibitory patterns of ANDRO were also observed in LPS-induced PGE2 and NO production. Therefore, it is likely that the attenuation of LPS-stimulated PGE2 and NO production is at least partially responsible for the observed neuroprotective effect of ANDRO.

Among the proinflammatory factors affected by ANDRO, PGE2 and NO were the most severely inhibited; 5 μM ANDRO almost completely inhibited LPS-induced release of these two factors (Fig. 4, C and D). To search for the underlying mechanism for this potent effect of ANDRO, we examined its effects on the transcriptional and translational levels of both COX and iNOS enzymes. RT-PCR analysis revealed that ANDRO did not affect the LPS-elicted increase in the expression of iNOS mRNA level and showed moderate decrease of COX-2 mRNA (Fig. 6A). Thus, the potent inhibition of PGE2 and NO release by ANDRO could not be explained on the level of transcriptional regulation. Further Western blot analysis showed greater inhibition of both COX-2 and iNOS immunoreactivities, which paralleled the effects on the release of PGE2 and NO (Fig. 6B). These results indicate that the main effect of ANDRO on the reduction of PGE2 and NO was on the translational level. To distinguish whether the reduction in the expression of enzyme proteins was due to a disruption in protein translation or an increase in the instability of the proteins, we determined the effect of ANDRO on the COX-2 immunoreactivity by adding ANDRO to the cultures 12 h after LPS treatment and observed the stability of this protein. Western blot analysis showed that ANDRO decreased COX-2 immunoreactivity in a dose-dependent manner, which indicates that enhanced COX-2 protein degradation is at least one of the explanations for the inhibition of PGE2 release by ANDRO (Fig. 7). This observation is consistent with the report by Chiou et al. (2000), which indicates that ANDRO increases the instability of iNOS.

Our findings indicate that ANDRO can effectively attenuate LPS-induced microglial activation and subsequent dopaminergic neurotoxicity. The inhibitory effects of ANDRO on LPS-induced microglial activation may depend on two mechanisms. One of these mechanisms is the inhibitory effect of ANDRO on the production of ROS, the latter of which may serve as secondary messengers to induce the production of other inflammatory factors such as PGE2 and TNF-α; the other mechanism is the ability of ANDRO to enhance protein degradation in the same way as COX-2 and iNOS, which results in the reduced production of inflammatory factors. These mechanisms may underlie the two important observations that should be underscored. First, ANDRO had a wide-spectrum inhibitory effect on microglial release of proinflammatory factors, including ROS, prostaglandins, NO, and TNF-α. Second, post-treatment with ANDRO also exhibited a significant protective effect on LPS-induced dopaminergic neurotoxicity. These observations suggest that ANDRO may be a potential therapeutic agent for the treatment of inflammatory-related neurodegenerative disorders such as Parkinson’s disease.

**Fig. 7.** Effect of ANDRO on the protein stability of COX-2. BV-2 cells were treated with or without LPS (5 ng/ml). After 12 h, LPS was washed off with HBSS three times. Then 1 μM cycloheximide was added to block further protein synthesis. ANDRO was added to cultures 30 min later. After another 12 h, protein products were determined by Western blot analysis. The intensity of the bands was measured by densitometry, and the value for LPS-treated groups was taken as 100%. Means ± S.E.M. of the percentages obtained in three independent experiments are shown; *, p < 0.05 compared with LPS-treated cultures. Images from one experiment, representative of three, are shown.

References


Andrographolide and Microglia-Mediated Neurodegeneration


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