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Ginsenoside Rd attenuates neuroinflammation of dopaminergic cells in culture

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Summary In Parkinson's disease clinical and experimental evidence suggest that neuroinflammatory changes in cytokines caused by microglial activation contribute to neuronal death. Experimentally, neuroinflammation of dopaminergic neurons can be evoked by lipopolysaccharide (LPS) exposure. In mesencephalic primary cultures LPS (100 μg/ml) resulted in 30–50% loss of dendritic processes, changes in the perikarya, cellular atrophy and neuronal cell loss of TH-immunoreactive (TH^+) cells. iNOS activity was increased dose dependently as well as prostaglandin E_2 concentrations. Ginsenosides, as the active compounds responsible for ginseng action, are reported to have antioxidant and anti-inflammatory effects. Here ginsenoside Rd was used to counteract LPS neurodegeneration. Partial reduction of LPS neurotoxic action was seen in dopaminergic neurons. Cell death by LPS as well as neuroprotective action by ginsenoside Rd was not selective for dopaminergic neurons. Neuronal losses as well as cytoprotective effects were similar when counting NeuN identified neurons. The anti-inflammatory effect of ginsenoside Rd could equally be demonstrated by a reduction of NO-formation and PGE_2 synthesis. Thus, protective mechanisms of ginsenoside Rd may involve interference with iNOS and COX-2 expression.

Keywords: Dopaminergic, lipopolysaccharide, ginsenoside Rd, neuroinflammation

Introduction

Parkinson's disease (PD) as an age-related, progressive neurodegenerative disorder is characterized by massive depletion of striatal dopamine as a result of the degeneration of dopaminergic neurons in the substantia nigra. Clinical and neuropathological data indicate that chronic inflammatory processes could be responsible for this cell death. Particularly, activation of microglia seems to be of importance, as their proliferation and invasion into neuronal tissue involve the release of inflammatory cytokines. The formation of neurotoxic factors such as nitric oxide (NO) and reactive oxygen species equally participates (Ruano et al., 2006). Therefore, the term neuroinflammation, referring to changes characteristic of immune activation, regardless of their origin, regulation or consequence has been applied by Hunot and Hirsch (2003).

Inside nervous tissue, as well as experimentally in neuronal culture systems lipopolysaccharides (LPS) as the active components of the cell wall of Gram negative bacteria trigger the synthesis and release of cytokines and NO. Primary cell cultures of mesencephalic neurons, when exposed to LPS undergo molecular inflammatory processes that have been well documented (Chock and Giffard, 2005; Minghetti et al., 2005). NO is a diatomic free radical generated from conversion of L-arginine to L-citrulline by inducible nitric oxide synthase (iNOS). NO plays important roles as an immune regulator, vasodilator and neurotransmitter in a variety of tissues, and as a mediator under inflammatory conditions. Exceedingly high levels of NO produced by iNOS, however, are considered to be cytotoxic in inflammation and endotoxemia. Here, nitric oxide can react with superoxide to produce peroxynitrite and other oxygen radicals (Beckman and Crow, 1993). These reactive agents, in turn, can produce extensive cellular damage by oxidizing DNA, proteins and lipids.

Therefore, an effective anti-inflammatory therapy should not only alleviate the disease-associated symptoms, but also interfere with glial reactions, an increase in inflammatory factors and progressive dopaminergic cell death. The neuroprotection conferred by preventing iNOS expression may thus come from a reduction in oxygen free radicals and related NO reaction products. Anti-inflammatory compounds have been tested for their ability to counteract these processes. Non steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, the non-selective cyclooxygenase (COX-1/COX-2) inhibitor indomethacin and the selective COX-2

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inhibitor NS-398 may reduce inflammatory mediators and neurotoxicity by inhibiting COX induction (Kyrkanides et al., 2002), decreasing iNOS mRNA expression (Heneka et al., 1999), or down-regulation of NF-κB activation (Yin et al., 1998).

However, in clinical practice the long term use of NSAIDs may cause gastrointestinal complications and even result in potentially fatal peptic ulceration and bleeding, next to liver and kidney damage as long term effects (Langford, 2006). Ginsenosides, as the active ingredients of ginseng root (Panax ginseng) with a saponin structure, are known for their antioxidant, anti-inflammatory and anti-apoptotic properties. Min et al. (2006) found that ginsenoside Rg3 prevented human endothelial cells apoptosis via inhibition of the mitochondrial apoptotic signaling pathway. Experimentally ginsenosides scavenge free radicals, and counteract glutamate excitotoxicity in dopaminergic neuronal cultures (Radad et al., 2004). Ginsenosides Rb_1 and Rg_1 decreased tumour necrosis factor-α (TNF-α) production by macrophages (Cho et al., 1998), pre-treatment with ginsenoside Rg_3 abrogated COX-2 expression in response to 12-O-tetradecanoylphorbol-13-acetate in mouse skin (Keum et al., 2003), and ginsenoside Rh_2 inhibited the expression of COX-2, pro-inflammatory TNF-α and interleukin-1β (IL-1β) in BV-2 cells induced by LPS/IFN-γ (Bae et al., 2006). However ginsenosides appear to exert different potencies of their anti-inflammatory action. Particularly the minor ginsenoside Rd, formed by hydrolyzing and removing a sugar moiety from the major ginsenosides Rb_1, Rb_2 and Rc, has been shown to protect neural systems by attenuating NO overproduction (Choi et al., 2003). The pharmaceutical property of ginseng in protecting neurons from neurotoxic kainic acid is attributed mostly to ginsenoside Rd (Lee et al., 2003). Therefore, the potential of ginsenoside Rd to interfere with LPS neurotoxicity was tested in mesencephalic dopaminergic cultures.

Materials and methods

Materials

Pregnant OF1/SPF mice were purchased from the Institute for Laboratory Zoology and Veterinary Genetics in Himberg (Austria). Dulbecco's Modified Eagles Medium (DMEM), fetal calf serum (FCS), diaminobenzidine, L-glutamic acid (monosodium salt), sulfanilamide, N-1-naphthylyethylenediamine dihydrochloride, paraformaldehyde and LPS (*E. coli*, L8274) were obtained from Sigma (Germany). Penicillin-streptomycin, anti-tyrosine hydroxylase antibody (anti-TH antibody) and DNase I were obtained from Roche Molecular Biochemicals (Germany). The Vectastain ABC Elite Kit (Mouse IgG) was purchased from Vector Laboratories (USA). B-27 (without antioxidants), trypsin-EDTA, soybean trypsin inhibitor, Dulbecco's PBS (w/o Ca^{2+}, Mg^{2+} and Na^{+} bicarbonate) and Hanks' balanced salt solution (w/o Ca^{2+} and Mg^{2+}, HBSS) were ordered from Invitrogen (Germany).

Preparation of mouse primary neuron-glia cultures

Primary mesencephalic neuron-glia cultures were prepared from embryonic mouse brains at gestation day 14. Embryos were carefully removed under aseptic conditions and collected in buffered saline (DPBS) at room temperature. Brains were dissected under a stereoscope (Nikon SMZ-1B, 10× magnification) and the ventral mesencephala excised. Primary cultures were prepared according to Radad et al. (2006). Briefly, after careful removal of the meninges, tissues were mechanically cut into small pieces in DPBS and transferred into a sterile test tube containing 2 ml 0.1% trypsin and 2 ml 0.02% DNase I in DPBS. The tubes were incubated in a water bath at 37°C for 7 min. Then, 2 ml of trypsin inhibitor (0.125 mg/ml in DPBS) were added, the tissue was centrifuged (Hettich, ROTIXA/AP, Germany) at 800 rpm for 4 min and the supernatant was aspirated. The tissue pellets were triturated with a fire-polished Pasteur pipette in DMEM containing 0.02% DNase I. Dissociated cells were collected in DMEM supplemented with HEPES buffer (25 mM), glucose (30 mM), glutamine (2 mM), penicillin-streptomycin (10 U/ml and 0.1 mg/ml, respectively) and heat-inactivated fetal calf serum (FCS, 10%). The cell suspension was plated into 4-well multi-dishes (Nunclon, Germany) pre-coated with poly-D-lysine (50 μg/ml). Cultures were grown at 37°C in an atmosphere of 5% CO_2/95% air and 100% relative humidity.

Treatment with LPS and ginsenoside Rd

LPS was diluted to the desired final concentration in medium, containing 2% FCS, HEPES buffer (25 mM), glucose (30 mM), glutamine (2 mM), penicillin-streptomycin (10 U/ml and 0.1 mg/ml, respectively) and 2% B-27 medium. Ginsenoside Rd was freshly prepared in DMEM before diluting to the desired final concentrations (1, 10, 50 μM) with medium. The seven-day-old cultures were pretreated with vehicle or ginsenoside Rd for 2 h at 37°C before treatment with LPS (100 μg/ml). The culture medium was changed every 3 days containing the above compounds. Experiments were run at least in triplicate with three wells for each treatment condition.

Identification of TH^+ neurons

On the 13th DIV cultures were rinsed carefully with PBS (pH 7.2) and fixed in 4% paraformaldehyde for 45 min at 4°C. Fixed cells were permeabilized with 0.4% Triton X-100 for 30 min at room temperature. Cultures were washed 3 times with PBS and incubated with 5% horse serum (Vectastain ABC Kit) for 90 min to block non-specific binding sites. Cells were sequentially incubated with anti-TH antibody overnight at 4°C, biotinylated secondary antibody (Vectastain) and avidin-biotin-horseradish peroxidase complex (Vectastain) for 90 min at room temperature and washed with PBS between incubations. The reaction product was developed in a solution of diaminobenzidine (1.4 mM) in PBS containing 3.3 mM hydrogen peroxide. The numbers of TH^+ neurons were counted at 100× magnification with a Nikon inverted microscope. To measure length changes of neuronal processes in different groups, the pictures of 10 randomly selected neurons per well were taken and lengths of the processes measured with Scion® Image software.

Nitrite assay

The NO levels in the supernatants were indirectly assayed by quantitating the nitrite concentrations by the Griess reaction. Briefly, Griess reagent [0.5% sulfanilamide, 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride, and 2.5% phosphoric acid] was added to an equal volume of supernatant and incubated at room temperature for 10 min. The absorbance at 550 nm was measured with a semi-auto biochemical analyzer (SBA-860, Sunostik, China), and the nitrite concentrations were calculated using sodium nitrite in culture medium as a standard (1–100 μM). Fresh culture medium served as a blank.

PGE_2 assay

PGE_2 concentrations were quantified using a commercial PGE_2 assay kit (R&D Systems, USA) on 96 well polystyrene microplates coated with a goat anti-mouse polyclonal antibody following the manufacturer instructions. One-hundred microlitres of supernatants (3- or 10-fold dilution) and standards were added to the wells. The optical density of each well was determined within 30 min, using a microplate reader set to 450 nm.

Statistical analyses

Data were expressed as mean ± standard error of mean (SEM). Significant differences between different groups were calculated by using One-way ANOVA assay and subsequent Student-Newman-Keuls test. Differences with $p < 0.05$ were considered statistically significant.

Results

LPS had dose dependent toxicity to TH^+ neurons

Dopaminergic neurons in primary culture undergo degeneration when exposed to LPS. Cultures were treated for 6 days with different concentrations of LPS (50–400 μg/ml) from the 7th DIV. Cell numbers of dopaminergic cells and other cell populations were decreased dose dependently. Changes in morphology were manifested in losses of dendritic processes and branching and deterioration of cell shape and nuclei (Fig. 1). Our results indicated that LPS destroyed TH^+ neurons in a dose-dependent manner. LD_{50} values in such culture systems were in the range of 100–200 μg LPS/ml medium (Fig. 2).

LPS equally affects other neuronal populations

Neuron-specific nuclear protein (NeuN) is expressed in the nuclei and cell bodies of most neuronal cells. It has been used successfully as a neuronal marker in cell cultures. Dopaminergic cultures exposed to LPS were stained by NeuN immunocytochemistry and the numbers of $NeuN^+$ cells were counted. A high amount of cell loss is evident with 400 μg/ml LPS (Fig. 3). This decrease was dose-dependent. The numbers of $NeuN^+$ cells were reduced by about 24, 41, 65, 92% in the 50, 100, 200, 400 μg/ml LPS-treated groups, respectively (Fig. 4).

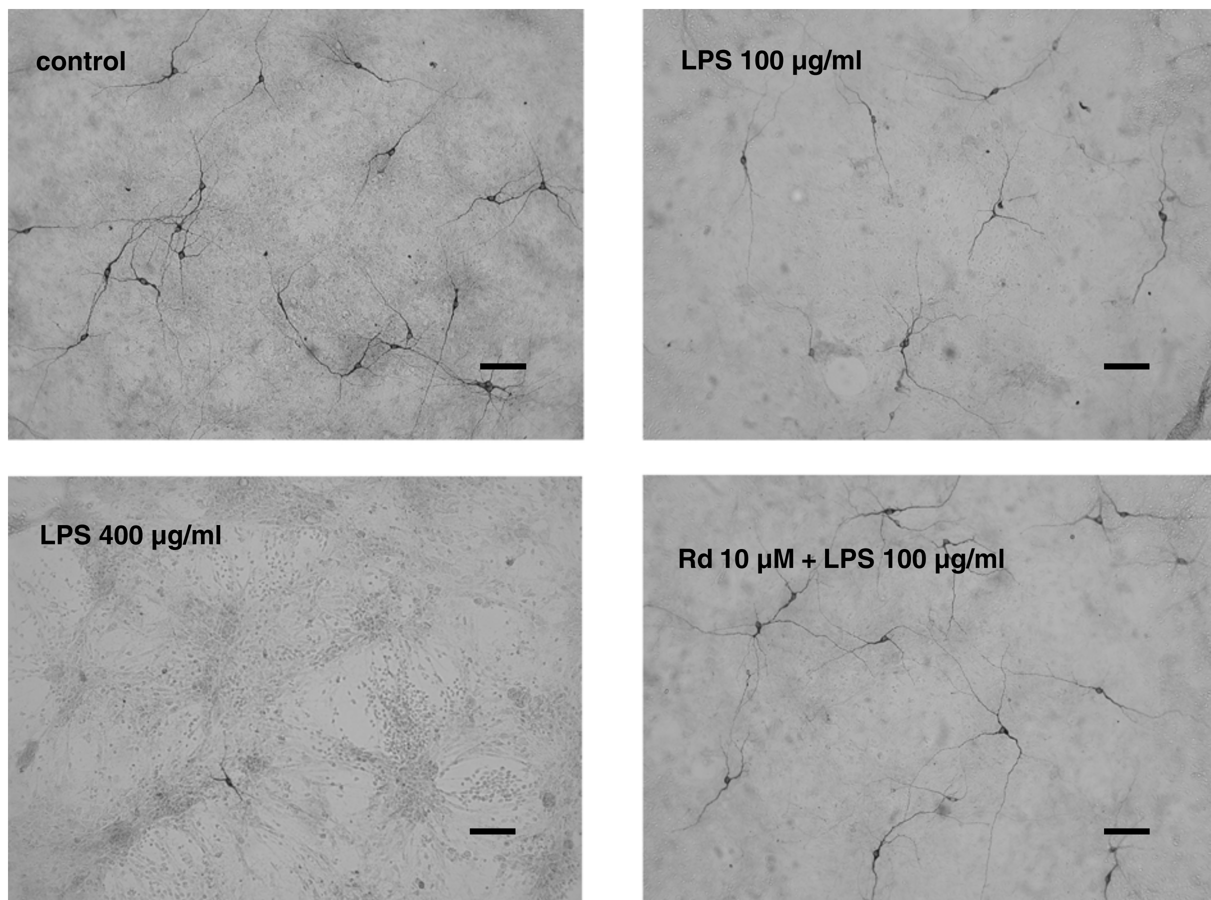

Fig. 1. Degenerative changes in TH^+ cells by LPS. Note the shrinkage and loss of dendritic processes and changes in the perikarya in the LPS (100 μg/ml) group. A high extent of cell loss is evident at 400 μg/ml LPS (bar = 100 μm)

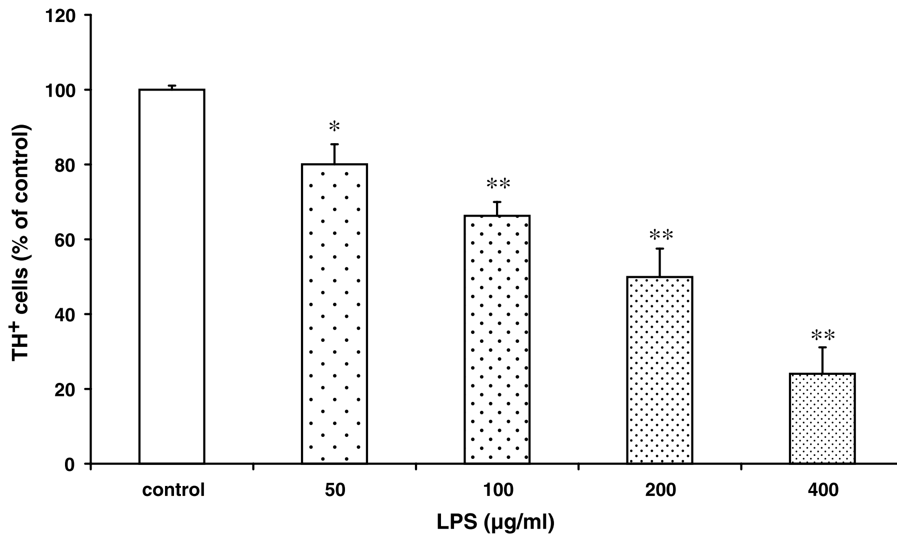

Fig. 2. Effects of LPS on TH^+ cells in the primary neuron-glia cultures. The cultures were treated with different concentration of LPS for 6 days and stained for TH. The results are the mean ± SEM of three experiments. $^*p < 0.05$, $^{**}p < 0.01$ versus control (without LPS)

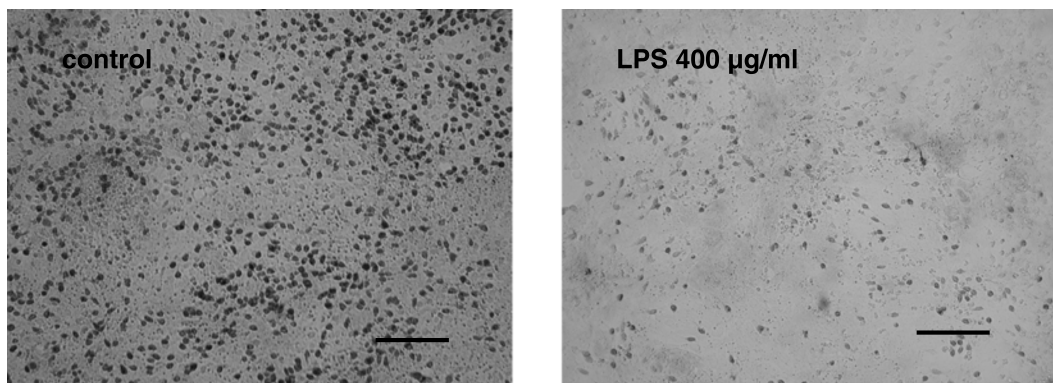

Fig. 3. LPS leads to cell loss of $NeuN^+$ cells in mesencephalic cultures. Dramatic loss of $NeuN^+$ cells and decreased staining intensity is evident at 400 μg/ml (bar = 100 μm)

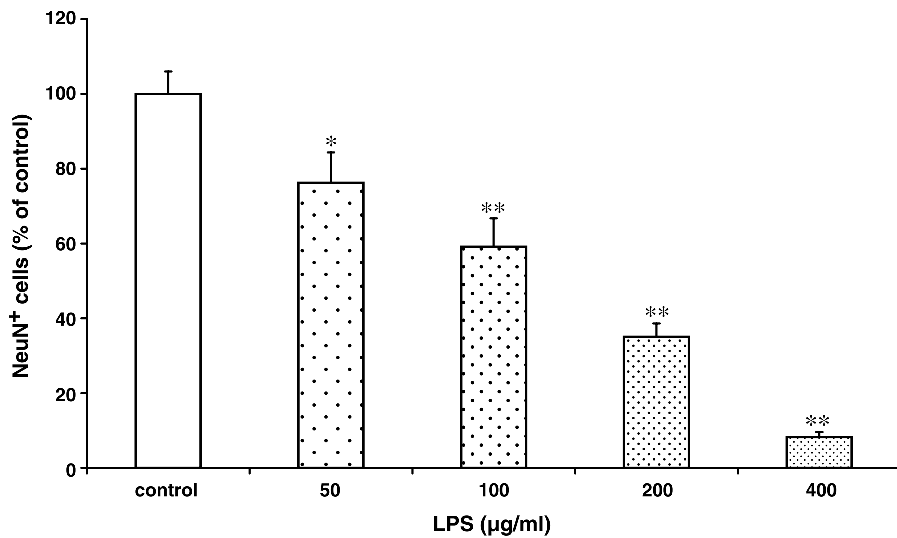

Fig. 4. Effect of LPS on $NeuN^+$ cells in mesencephalic cultures. The cells were treated with different concentration of LPS for 6 days and stained for NeuN. The results are the mean ± SEM of three experiments. $^*p < 0.05$, $^{**}p < 0.01$ versus control (without LPS)

LPS increased NO formation

Supernatants from cultures were collected to assay NO formation following incubation with different concentrations of LPS. Basal NO formation was about 5 μM in control cultures. There was a dose-dependent increase in NO formation with LPS concentrations ranging from 6.25 to

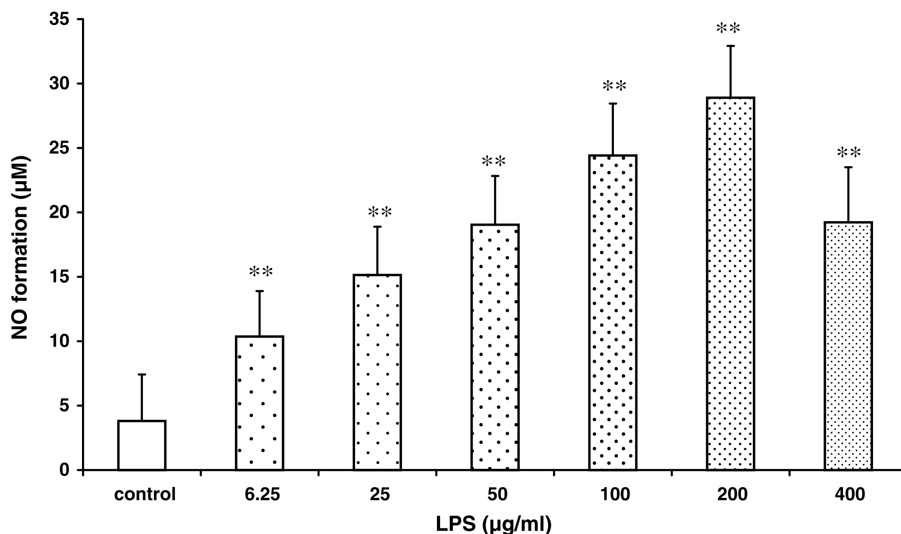

Fig. 5. LPS induced NO formation. Mesencephalic cultures were treated with different concentrations of LPS on the 7th DIV, incubated for 3 days, supernatants were assayed by the Griess reaction. Data represent the mean ± SEM of three experiments. $^{**}p < 0.01$ versus control (without LPS)

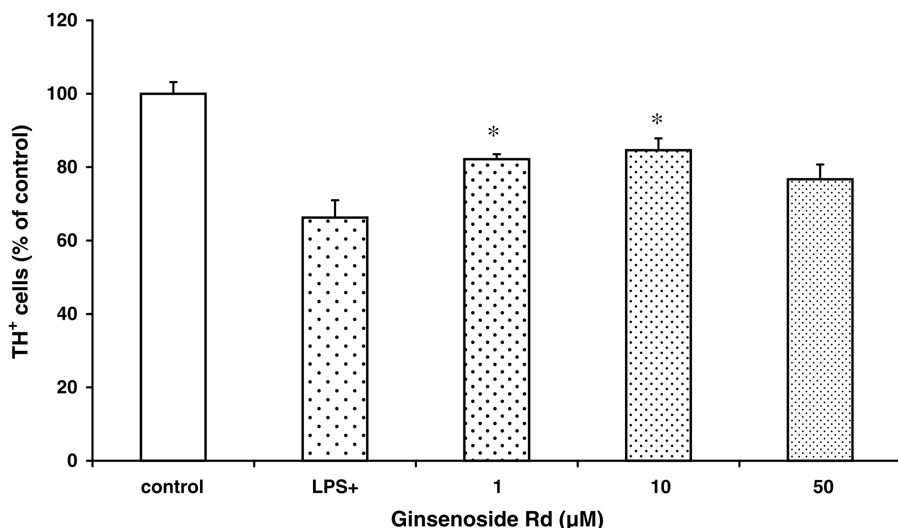

Fig. 6. Effect of ginsenoside Rd on TH^+ cells after exposure to LPS. The cultures were pre-treated with ginsenoside Rd for 2 h before adding 100 μg/ml LPS, and stained for TH after six days incubation. The results are the mean ± SEM of three experiments. $^{*}p < 0.05$ in comparison with the cultures treated with LPS alone

200 μg/ml that reached a peak with 200 μg/ml of LPS (8-fold above basal level, Fig. 5). Above that reduced NO formation at 400 μg/ml possibly reflected the generalized toxic effect of LPS on all cells.

Ginsenoside Rd protected dopaminergic neurons against LPS-neurotoxicity

Significant dopaminergic cell loss underlies as the primary pathology of PD. Therefore, we tested the effects of ginsenoside Rd on LPS-induced dopaminergic cell loss. The mesencephalic cultures were pretreated with ginsenoside Rd (1–50 μM) before including LPS (100 μg/ml, approx. LD_{50}). At the end of the 6 day treatment period, TH-immunostained neurons were determined. As shown in Fig. 6, ginsenoside Rd (1–50 μM) exerted a significant neuroprotective effect on dopaminergic neurons (123.1, 127.7% with 1 and 10 μM, respectively) compared to cultures treated with LPS.

Effect of ginsenoside Rd on LPS-induced $NeuN^+$ cytotoxicity

Mesencephalic cultures exposed to 100 μg/ml LPS for 6 days were stained for NeuN immunoreactivity. As shown in the Fig. 7, the results demonstrate that 41% NeuN immunoreactive ($NeuN^+$) cells were lost. Ginsenoside Rd attenuated the LPS-induced reduction in the number of $NeuN^+$ cells similar to TH^+ cells (Fig. 7). In the 1 μM ginsenoside Rd-treated group, the $NeuN^+$ cells were increased by 16% ($p < 0.05$). In the 10 μM ginsenoside Rd-treated group, the cell counts of $NeuN^+$ cells were higher by 24% compared to the LPS group ($p < 0.05$).

Inhibition of LPS-induced NO formation by ginsenoside Rd

To test whether ginsenoside Rd has inhibitory effects on LPS-induced NO formation, we assayed nitrite production

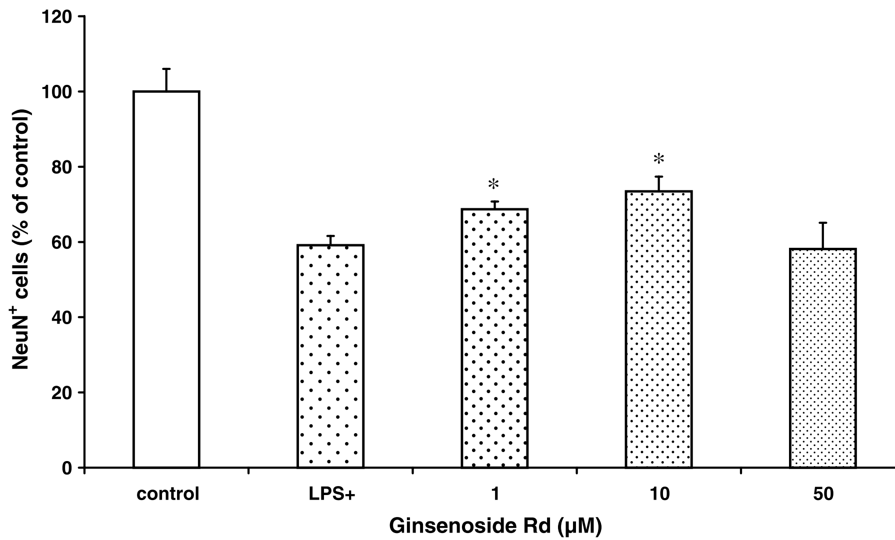

Fig. 7. Effect of ginsenoside Rd on $NeuN^+$ cells of LPS-induced cytotoxicity. The results are the mean ± SEM of 10 counted fields for each condition, given as percentage of controls. $^*p < 0.05$ in comparison with the cultures treated with LPS

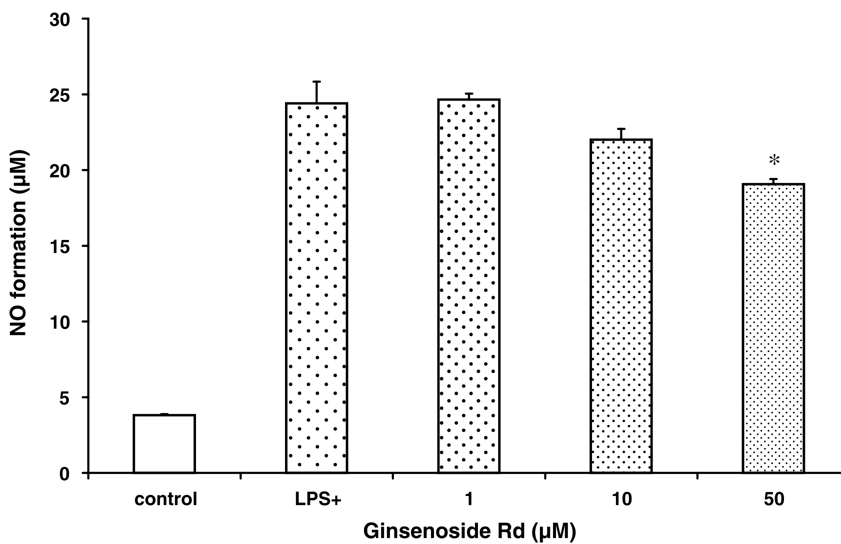

Fig. 8. Inhibition of LPS-induced NO formation by ginsenoside Rd. The cultures were pre-treated with Rd for 2 h before treated with 100 µg/ml LPS, supernatants were collected at 3 days and assayed by the Griess reaction. The results are the mean ± SEM of three experiments. $^*p < 0.05$ in comparison with the cultures treated with LPS

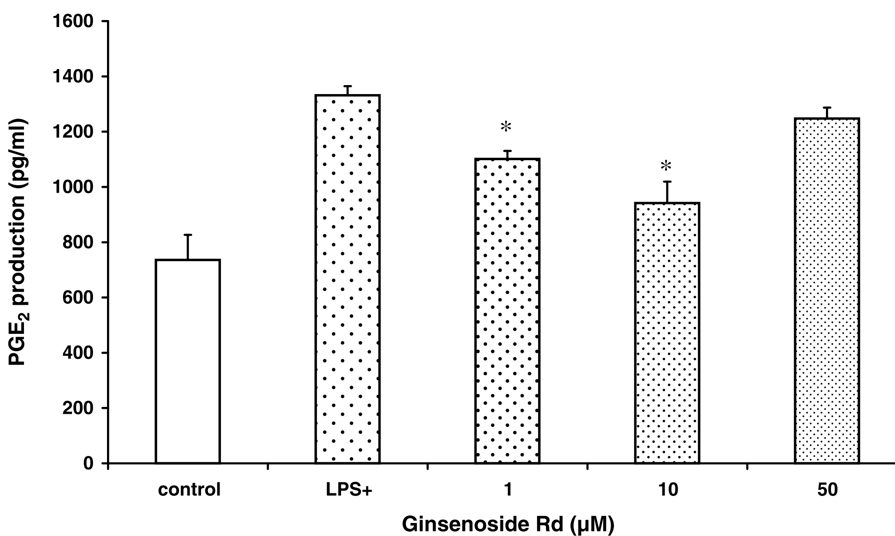

Fig. 9. Effect of ginsenoside Rd on LPS-induced PGE_2 production. Data represent the mean ± SEM of three independent determinations. $^*p < 0.05$ in comparison with the cultures treated with LPS

in the culture medium of neuron-glia cultures. As shown in Fig. 8, co-incubation of ginsenoside Rd with LPS could inhibit the production of NO at 50 μM ginsenoside Rd by about 20%.

Effect of ginsenoside Rd on LPS-induced PGE_2 production

The influence of different concentrations of ginsenoside Rd on PGE_2 production was investigated. Basal conditions are characterized by PGE_2 of about 750 pg/ml (Fig. 9). LPS caused a 2-fold increase in the biosynthesis of PGE_2 as compared to controls. However, when challenged with LPS in the presence of ginsenoside Rd significant reductions at 1 and 10 μM ginsenoside Rd were observed.

Discussion

Over the last years, LPS has been used experimentally either in vivo or in neuronal cultures as this model compound provides a tentative mechanistic link between the occurrence of inflammation in the brain and dopaminergic neurodegeneration. LPS significantly reduced the number of TH^+ neurons compared to control cultures and affected cells had fewer dendrites, shorter or even truncated axons (Li et al., 2005). In our study 100 μg/ml LPS (approx. LD_{50}) caused neuronal loss in primary mesencephalic cultures. LPS led to death of TH^+ cells in a dose dependent manner, however fairly high concentrations of LPS were required. Similar findings have been shown by Gayle et al. (2001) with different approaches. From their data TH^+ cells were progressively decreased by LPS concentrations ranging from 10 to 320 μg/ml. Other authors showed that much lower LPS concentrations (10 ng/ml) induced the same extent of TH^+ cell loss (Wang et al., 2004). This disparity is likely due to various sources of LPS used or different cell culture condition applied, as e.g. LPS from Salmonella typhimurium displayed similar higher toxicities in our hands (data not shown).

Cytotoxicity of LPS however was not selective for dopaminergic neurons, as other neurons were similarly affected. NeuN is expressed in nuclei and cell bodies of most neuronal cell types (Mullen et al., 1992) but not by glial cells. In our hands LPS equally affected $NeuN^+$ cells and the loss of TH^+ neurons correlated with the disappearance of $NeuN^+$ cells. Increases in NO and PGE_2 levels may both contribute to elevated TH^+ cell death. Our results are supportive to previous studies where massive neuronal death was induced by excessive NO formation in LPS-treated cultures (Jeong et al., 2003). In addition, NO has been shown to inhibit mitochondrial respiration and react with superoxide to produce peroxynitrite ($ONOO^-$), which is a highly toxic oxidant (Bal-Price et al., 2002). 200 μg/ml LPS led to the accumulation of about 30 μM NO and there was a dramatic decrease in the number of TH^+ cells when compared with controls. A similar aspect holds true for affecting PGE_2 increases which are derived from the metabolism of arachidonic acid by COX and PGE synthesizing enzymes. Our study demonstrated that stimulation of the neural cultures with LPS increased the synthesis of PGE_2. Takadera and Oyashiki (2006) showed that PGE_2 directly stimulated several signaling pathways via EP receptors, resulting in changes in cAMP and cellular levels of phosphoinositides (Narumiya et al., 1999) that induced apoptosis in hippocampal neurons. LPS treatment also induced production of other pro-inflammatory and neurotoxic factors, such as TNF-α, IL-1β and reactive oxygen species (ROS) (Liu and Hong, 2003). The precise mechanisms of LPS-induced neurodegeneration in mesencephalic dopaminergic culture thus may be even more complex.

Ginsenoside Rd could partially prevent the toxic action of LPS. This has not been shown for dopaminergic cells so far, however anti-inflammatory action of ginsenosides has been observed in different non-neuronal systems. For example, ginsenoside Rb_1 can inhibit LPS-induced expression of the proinflammatory cytokine TNF-α in vitro (Cho et al., 1998), ginsenoside Rb_1 inhibited LPS-induced IL-6 and/or TNF-α production in murine macrophages (Smolinski and Pestka, 2003) and compound K, the Rb_1 metabolite by intestinal bacteria, potently inhibited the production of NO and PGE_2, reduced the expression levels of the iNOS, COX-2 proteins, and prevented the activation of NF-κB (Park et al., 2005). Our study indicates that the neuroprotective effect of ginsenoside Rd against LPS toxicity involves anti-inflammatory mechanisms. Ginsenoside Rd (10 μM) significantly increased the survival rate of dopaminergic cells in the primary neuron-glia cultures and caused a (though small) reduction of NO levels (at 50 μM). In this context, ginsenoside Rd also exerted inhibitory action against NO production induced by LPS plus TNF-α in C6 glioma cells (Choi et al., 2003). However, also other protective mechanisms should be considered. The neuroprotective effect of ginsenoside Rd on survival of dopaminergic cells may be mediated through improving energy metabolism and preserving the structural integrity of neurons (Jiang and Qian, 1995). Induction of antioxidant enzymes by ginsenosides which are important for maintaining cell viability may equally contribute by lowering free radicals (Nishiyama et al., 1994). On the other hand, ginsenoside Rd could inhibit lipid peroxidation. That was explained by its intervention with the GSH/GSSG redox status (Yokozawa et al.,

2004). Additional modes of action of ginsenoside as an interference with NMDA-receptors and calcium metabolism (Kim and Rhim, 2004) as well as stabilizing cellular membrance fluidity (Li and Zhang, 1997) could as well contribute to its neuroprotective action.

Conclusions

Mesencephalic dopaminergic cultures exposed to LPS undergo degeneration caused by microglial activation and a concomitant increase of NO and PGE_2. A partial reversal of dopaminergic cell death by ginsenoside Rd indicates that anti-inflammatory actions of this compound are of relevance. Beyond the pharmacological action of this natural compound in cell culture, chronic treatment in animal models of experimental inflammation should help to validate its actual therapeutic value for degenerative aging diseases.

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