Minocycline Protects Dopaminergic Neurons Against Long-Term Rotenone Toxicity

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ABSTRACT: Background: In Parkinson’s disease, most of current therapies only provide symptomatic treatment and so far there is no drug which directly affects the disease process. Objectives: To investigate the neuroprotective effects of minocycline against long-term rotenone toxicity in primary dopaminergic cell cultures. Methods: Embryonic mice of 14-days-old were used for preparation of primary dopaminergic cell cultures. On the 6th day in vitro, prepared cultures were treated both with minocycline alone (1, 5, 10 and 20 μM) and concomitantly with rotenone (5 and 20 nM) and minocycline. Cultures were incubated at 37°C for six consecutive days. On Day 12 in vitro culture medium was aspirated and used for measuring lactate dehydrogenase. Cultured cells were fixed in 4% paraformaldehyde and stained immunohistochemically against tyrosine hydroxylase. Results: Treatment of cultures with 5 and 20 nM of rotenone significantly decreased the survival of tyrosine hydroxylase immunoreactive neurons by 27 and 31% and increased the release of lactate dehydrogenase into the culture medium by 31 and 236%, respectively compared to untreated controls. Minocycline (1, 5, 10 μM) significantly protected tyrosine hydroxylase immunoreactive neurons by 17, 15 and 19% and 13, 22 and 23% against 5 and 20 nM of rotenone, respectively compared to rotenone-treated cultures. Minocycline (only at 10 μM) significantly decreased the release of lactate dehydrogenase by 79% and 133% against 5 and 20 nM of rotenone, respectively. Conclusion: Minocycline has neuroprotective potential against the progressive loss of tyrosine hydroxylase immunoreactive neurons induced by long-term rotenone toxicity in primary dopaminergic cultures.

RÉSUMÉ: La minocycline protège les neurones dopaminergiques contre la toxicité à long terme du roténone. Contexte : Dans la maladie de Parkinson, la plupart des traitements actuels ne sont que symptomatiques et il n’existe toujours pas de médicament qui influence directement le processus morbide. Objectifs : Le but de l’étude était d’examiner les effets neuroprotecteurs de la minocycline sur des cellules dopaminergiques en culture primaire contre la toxicité à long terme du roténone. Méthodes : Des cellules dopaminergiques ont été prélevées chez des souris âgées de 14 jours et mises en culture primaire. Au sixième jour in vitro, les cultures ont été traitées soit par la minocycline seule (1, 5, 10 et 20 μM) ou par le roténone (5 et 20 nM) et la minocycline. Les cultures ont été incubées à 37°C pendant six jours consécutifs. Au douzième jour in vitro, le milieu de culture a été aspiré et utilisé pour mesurer la déshydrogénase lactique (LDH). Les cellules cultivées étaient alors fixées dans la paraformaldéhyde à 4% et colorée par immunohistochimie pour révéler la tyrosine-hydroxylase. Résultats : Le traitement des cellules par le roténone à 5 et 20 nM a diminué significativement la survie des neurones contenant de la tyrosine-hydroxylase, soit une diminution de 27% et de 31% respectivement et a augmenté la libération de LDH dans le milieu de culture de 31% et 236% respectivement par rapport aux cultures témoins. La minocycline (1, 5, 10 μM) a protégé de façon significative les neurones contenant de la tyrosine-hydroxylase, soit une protection de 17%, 15% et 19% et de 13%, 22% et 23% contre le roténone à 5 et à 20 nM respectivement. La minocycline (seulement à 10 μM) a diminué significativement la libération de la LDH de 79% et 133% dans les cultures traitées par le roténone à 5 et à 20 nM respectivement. Conclusion : La minocycline a eu un effet neuroprotecteur contre la perte progressive de neurones contenant de la tyrosine-hydroxylase induite par la toxicité à long terme du roténone dans les cultures primaires de cellules dopaminergiques.


Parkinson's disease (PD) is a progressive neurodegenerative disease affecting about 1% of people over the age of 60 years. The disease is characterized clinically by bradykinesia, resting tremors, rigidity and disturbance in posture and gait and pathologically by loss of the dopaminergic neurons in substantia nigra pars compacta. Degeneration of dopamine neurons in PD either occurs due to exposure to some environmental factors (idiopathic) or as the result of mutations in some specific genes (familial). In idiopathic PD, there is much epidemiological evidence suggesting that exposure to pesticides may have a role in degeneration of dopamine neurons. Dhillon et al demonstrated that exposure to organic pesticides such as rotenone in past years increased the risk of PD.
Among neurodegenerative disorders, idiopathic PD remains the only one to respond to symptomatic therapy using levodopa. Whereas levodopa treatment continues to be the gold standard for efficacy, its chronic use is associated with potentially disabling motor complications and does not inhibit the progressive degeneration of DA neurons in substantia nigra. Moreover, there are controversial findings about possible toxic actions of levodopa on remaining dopaminergic neurons. Therefore, researchers have spent an enormous effort to develop neuroprotectants for PD patients for the last 20 years. However, although a number of these drug candidates showed the ability to protect DA neurons in different PD models either in vivo or in vitro, no drug has yet been found to slow the disease progression and prevent the progressive loss of dopaminergic neurons in PD patients.

Minocycline, a semi-synthetic second-generation tetracycline, easily crosses the blood brain barrier and is reported to reduce neurodegeneration in animal models of ischemia, amyotrophic lateral sclerosis, Huntington's disease and Parkinson's disease. Minocycline counteracts neurodegeneration by its anti-inflammatory action which appears to be independent and different from its antimicrobial action. In this context, Du et al. reported that minocycline prevented microglial activation and expression of IL-1 (Interleukin-1) and iNOS (inducible nitric oxide synthase) in the MPTP-induced mouse model of PD. Moreover, minocycline was reported to have antioxidant and radical scavenging activities in hydrogen peroxide (H$_2$O$_2$)-treated cortical cultures.

Rotenone is a classical, well-characterized and high affinity specific inhibitor of mitochondrial b-nicotinamide adenine dinucleotide (NADH) dehydrogenase (complex I). It is widely used as a model substance for PD after the report of Betarbet et al. about its selective nigrostriatal damage in rats.

The present study was carried out to investigate the neuroprotective potential of minocycline against long-term rotenone toxicity in primary dopaminergic cell culture, the more reliable in vitro model mimicking long-lasting neurodegeneration in PD.

**Materials and Methods**

Pregnant mice (OF1/SPF, Himberg, Austria) were cared for and handled in accordance with the guidelines of the European Union Council (86/609/EU) for the use of laboratory animals. At gestation Day 14, uterine horns were dissected and embryos were collected in Petri dishes containing sterile Dulbecco's phosphate buffered saline (DPBS, Invitrogen, Germany). Under a stereoscope (Nikon SMZ-1B, 100x magnification), brains were dissected, ventral mesencephala excised and primary cultures were prepared according to Radad et al. Briefly, mesencephala were cut into small pieces in DPBS and transferred into a sterile test tube containing 2 ml of 0.1% trypsin (Invitrogen, Germany) and 2 ml 0.02% DNase I (Roche, Germany) in DPBS. The tube was incubated in a water bath at 37°C for seven min. Then, 2 ml of trypsin inhibitor (0.125 mg/ml in DPBS) (Invitrogen, Germany) was added and the tissue was centrifuged (Hettich, ROTIXA/AP) at 100g for four min. The tissue pellets were triturated with a fire-polished Pasteur pipette in Dulbecco’s modified Eagle’s medium (DMEM, Sigma, Germany) containing 0.02% DNase I. Dissociated cells were collected in DMEM supplemented with Heps 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%) (Sigma, Germany). The cell suspension was plated into four-well multidishes (Nunclon, Germany) pre-coated with poly-D-lysine (50 μg/ml) (Sigma, Germany). Cultures were grown at 37°C in an atmosphere of 5% CO$_2$/95% air and 100% relative humidity. The medium was exchanged on the 1st day in vitro (DIV) and on the 3rd DIV. On the 5th DIV half of the medium was replaced with serum-free DMEM containing 0.02 ml B-27/ml (Invitrogen, Germany). Serum-free supplemented DMEM was used for feeding from the 6th DIV and subsequently replaced every 2nd day.

**Treatment of cultures with minocycline**

A stock solution of minocycline (10 mM) was prepared by dissolving 4.94 mg in 1 ml of dimethyl sulfoxide (DMSO). Final concentrations of minocycline were prepared in DMEM. The concentration of DMSO in the culture medium did not exceed 0.01%. To investigate the effect of minocycline on the survival of dopaminergic neurons, cultures were treated with minocycline (1, 5, 10 and 20 μM) on the 6th DIV for six consecutive days. During the treatment period, culture medium was changed every two days with the same concentrations of minocycline.

**Treatment of cultures with rotenone and minocycline**

To investigate the neuroprotective potential of minocycline against long-term rotenone toxicity, cultures were concomitantly treated with rotenone (5 and 10 nM), dissolved in DMSO. Minocycline (1, 5, 10 and 20 μM) was added with each
concentration of rotenone on the 6th DIV for six consecutive days. The culture medium was changed every two days with the same concentrations of rotenone and minocycline.

**Lactate dehydrogenase release assay**

Cellular injury was quantitatively assessed by measuring the activity of lactate dehydrogenase (LDH) released from damaged cells into the culture medium. The reaction was initiated by mixing 0.2 ml of cell-free supernatant (diluted 1:1 with aqua dest.) with potassium phosphate buffer containing NADH and sodium pyruvate (0.18 and 0.62 mM in potassium phosphate buffer, respectively) in a final volume of 0.5 ml in 1 ml cuvettes. The decrease of NADH was spectrophotometrically (NOVASPEC II) monitored. Reagent blanks were subtracted. Lactate dehydrogenase activity was calculated from the slope of the decrease in optical density at 334 nm over a 3 min-time period. The LDH release is proportional to the number of damaged or destroyed cells [16].

**Identification of tyrosine hydroxylase immunoreactive (THir) neurons**

On day 12 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 minutes at room temperature. Cultures were washed three times with PBS and incubated with 5% horse serum (Vectastain ABC Kit) for 90 minutes 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 minutes 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 (H$_2$O$_2$). THir neurons were counted in ten randomly selected fields at 100× magnification with a Nikon inverted microscope.

**Statistics**

Data were obtained from 12 wells (three repeats) for each treatment condition. Data were expressed as mean ± standard error of mean (SEM). Statistical significance was calculated using the Kruskal-Wallis (H)-test followed by Chi$^2$-test. Calculations were performed using Statview software. A value of p<0.05 was considered statistically significant.

**RESULTS**

**Effect of minocycline on primary dopaminergic cell culture**

Treatment of cultures with minocycline alone (1, 5 and 10 μM) on the 6th DIV for six consecutive days did not affect the survival and the morphology of THir neurons (data not shown) and LDH release into the culture medium. On the other hand, minocycline (20 μM) decreased the number of THir neurons by 15% and increased LDH release by 17% compared to untreated control cultures (Figure 1).
Effect of long-term rotenone toxicity on primary dopaminergic cell culture

Long-term treatment of cultures with rotenone (5 and 20 nM) on the 6th DIV for six consecutive days significantly decreased the number of THir neurons by 27% and 31%, respectively, compared to untreated control cultures (Figure 2). Both concentrations of rotenone were also found to alter the morphology of THir neurons which showed shrinkage and neurite loss (Figure 3). Moreover, the similar rotenone treatment increased the release of LDH into the culture medium by 31% and 236%, respectively (Figure 4).

Effect of minocycline on rotenone-treated cultures

Concomitant treatment of cultures with minocycline and rotenone on the 6th DIV for six consecutive days resulted in higher numbers of THir neurons compared to rotenone-treated cultures. Minocycline (1, 5 and 10 μM) rescued about 13, 22 and 23% of THir neurons against 5 nM of rotenone compared to rotenone-treated cultures, respectively (Figure 2). Against 20 nM of rotenone, minocycline (1, 5 and 10 μM) rescued about 17, 15 and 19% of THir neurons by 79 and 133% against 5 and 20 nM of rotenone, respectively compared to rotenone-treated cultures (Figure 4). Minocycline also protected the morphology of THir neurons compared to rotenone-treated cultures. The remaining neurons appeared numerous and had longer and many neurites compared to rotenone-treated cells (Figure 3). Also, concomitant treatment of cultures with minocycline (only at 10 μM) and rotenone decreased the release of LDH into the culture medium by 79 and 133% against 5 and 20 nM of rotenone, respectively compared to rotenone-treated cultures (Figure 4).

DISCUSSION

In spite of the failure to find neuroprotective therapies for PD, researchers go to great efforts to find drugs that can slow, stop or reverse the progressive death of dopaminergic neurons in PD. In accordance, our present study used primary dopaminergic cultures to investigate the neuroprotective potential of minocycline against long-term rotenone toxicity relevant to PD. Minocycline has been focused on as a neuroprotective agent for neurodegenerative diseases since the demonstration of its neuroprotective actions in animal models of ischemic injury. Moreover, minocycline was reported to produce neuroprotective effects in animal models for PD, Alzheimer's disease and Huntington's disease.

Our present data showed that minocycline alone did not affect the survival of THir neurons and the release of LDH into the culture medium in primary dopaminergic culture. It did not inhibit aging-induced cell death of dopamine neurons. This may be related to an inability of minocycline to prevent mitochondrial dysfunction and subsequent oxidative stress, the two early events in the process of dopamine cell death according to Bueler. Consistent with this data, Fernandez-Gomez et al. and Domico et al. reported that minocycline failed to block ROS production which is independent of microglial activation in cerebellar granule cells and mesencephalic cells, respectively. However, Gieseler et al. found that minocycline decreased calcium deregulation as the result of the opening of mitochondrial permeability transition pore (mPTP) and showed antioxidant activity against H2O2-induced oxidative stress in rotenone-treated cortical neurons. On the other hand, only treatment of cultures with 20 μM of minocycline decreased the number of THir neurons and increased the LDH release into the culture medium demonstrating a toxic effect at high concentrations.

Since Betarbet and his colleagues have reported the vulnerability of rat dopaminergic neurons to the pesticide rotenone, it was later widely used to reproduce the features of PD in different in vivo and in vitro models. In most in vitro models, particularly in primary dopaminergic cultures, rotenone was used for short duration ranging from 24 - 48 h. In the present study, we used our modified rotenone model where primary dopaminergic cultures were exposed to nanomolar concentrations of rotenone from the 6th DIV for six consecutive days. Similarly, such long-term exposure of primary dopaminergic cell culture to 5 and 20 nM of rotenone produced progressive loss of THir and increased the release of LDH into the culture medium. Long-term exposure of primary dopaminergic cultures to rotenone seems to be a more reliable model than short-term exposure to test neuroprotective drugs relevant to PD. This is due to primary dopaminergic culture exposed to nanomolar concentrations of rotenone over a long period seemed to better reflect the slow progressive neuronal degeneration in PD.

Against rotenone, minocycline was found to produce significant neuroprotection against long-term rotenone exposure in primary dopaminergic cultures. It rescued significant numbers of THir neurons in rotenone-exposed primary dopaminergic cell culture. Moreover, it decreased the release of LDH into the culture medium compared to rotenone-treated cultures. In parallel, it was reported that minocycline blocked MPTP-induced degeneration of dopamine neurons in substantia nigra.
pars compacta and preventing the loss of striatal dopamine and its metabolites in mice. Du and his colleagues showed that the neuroprotective effects of minocycline against MPTP neurotoxicity in mice depend on inhibiting MPTP/MPP+-induced glial iNOS expression and NO toxicity. Consistently counteracting activation and proliferation of microglia is a particular event in neuroprotection by minocycline in neuron/glia co-culture.

In conclusion, our present study demonstrated the neuroprotective effect of minocycline against the progressive loss of dopamine neurons in primary dopaminergic cell culture as the result of long-term rotenone toxicity, the more reliable in vitro model for PD.

REFERENCES


