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Lack of evidence of direct mitochondrial involvement in the neuroprotective effect of minocycline

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Abstract

Minocycline has been reported to exert neuroprotection through inhibition of inflammatory processes and of mitochondrial cell death pathway. To further characterize the neuroprotective effect of minocycline, we determined its efficacy in different neuronal damage paradigms involving inflammation or mitochondrial dysfunction. In transient global ischaemia in gerbils, minocycline reduced hippocampal neuronal damage measured by peripheral type benzodiazepine binding sites density, a marker of microglial activation. The antiinflammatory properties of minocycline were confirmed on the model of carrageenan-induced paw oedema in rats. The use of two experimental animal models involving administration of mitochondrial toxins inhibiting a different complex of the mitochondrial respiratory chain permitted the exploration of the mitochondrial impact of minocycline. Although minocycline exhibited a marked efficacy in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP; complex I inhibitor)-induced neurotoxicity in mice, it was ineffective in malonate (complex II inhibitor)-induced striatal lesion in rats. In vitro investigations on energized mitochondria isolated from rat liver showed that minocycline (1 μM) did not inhibit the swelling induced by MPP^+(1-methyl-4-phenylpyridinium). Moreover, higher concentrations of minocycline induced swelling. From these experiments, the neuroprotective activity of minocycline appears more related to its antiinflammatory activity than to a direct beneficial action on mitochondria.

Keywords: Minocycline; Neuroprotection; Mitochondria; Inflammation

1. Introduction

Minocycline is a semisynthetic second-generation tetracycline, a group of broad-spectrum antimicrobial agents. In addition, tetracyclines exhibit significant antiinflammatory properties in a variety of rheumatologic and dermatologic conditions. Minocycline, which effectively crosses the blood–brain barrier (Aronson, 1980), has also been shown to possess neuroprotective effects independent of its antimicrobial action. The first neuroprotective efficacy of minocycline has been observed in experimental models of acute neurological disease, such as global and focal cerebral ischaemia (Yrjanheikki et al., 1998, 1999) and traumatic brain injury (Sanchez Mejia et al., 2001). Furthermore, minocycline also proved to be efficient in models of more chronic neurodegenerative diseases. Indeed, minocycline delays disease progression in transgenic mouse models of Huntington's disease (R6/2; Chen et al., 2000) and of amyotrophic lateral sclerosis (transgenic superoxide dismutase; Kriz et al., 2002; Van Den et al., 2002). In addition, minocycline had been shown to provide protection in the 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) mouse model (Du et al., 2001; Wu et al., 2002) and in the 6-hydroxydopamine rat model (He et al., 2001) of Parkinson's disease and in a multiple sclerosis rat model (Popovic et al., 2002).

Minocycline possesses antiinflammatory and antioxidative properties, including depression of oxygen radical

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release from polymorphonuclear neutrophils (Gabler et al., 1992), scavenging of peroxinitrite (Whiteman and Halliwell, 1997), inhibition of matrix metalloproteases (Golub et al., 1999) and inhibition of inductible nitric oxide synthase (iNOS; Amin et al., 1996, 1997; Ryan and Ashley, 1998). It was thus observed that minocycline-mediated neuroprotection was associated with inhibition of inflammatory processes and microglial activation (He et al., 2001; Kriz et al., 2002; Popovic et al., 2002; Wu et al., 2002; Yrjanheikki et al., 1998, 1999). A direct inhibitory effect of minocycline on microglial activation and proliferation induced by excitotoxics has been also demonstrated (Tikka et al., 2001). Moreover, the 1-methyl-4-phenylpyridinium (MPP^+) neurotoxicity on cerebellar granules neurons was only inhibited by minocycline in presence of glia (Du et al., 2001). Minocycline also inhibited the up-regulation and activation of iNOS, cyclooxygenase-2, caspase-1 and caspase-3 and p38-mitogen-activated protein kinase (Chen et al., 2000; Popovic et al., 2002; Sanchez Mejia et al., 2001; Yrjanheikki et al., 1998, 1999).

More recently, minocycline has been reported to exert neuroprotection through a mitochondrial-dependent pathway (Wang et al., 2003; Zhu et al., 2002). Opening of high conductance permeability transition pores in mitochondria initiates the release of apoptosis activating factor-1 and cytochrome *c*, which is followed by the activation of caspases (caspase-9, caspase-3), nuclear fragmentation and cell death. Zhu et al. (2002) have shown that treatment with minocycline inhibited the release of cytochrome *c* in the spinal cord of transgenic superoxide dismutase mice. They also observed that the swelling and cytochrome *c* release induced by calcium in isolated brain mitochondria were inhibited in vitro by minocycline. Similarly, in a model of Huntington disease, Wang et al. (2003) observed an inhibition of the release of cytochrome *c* and apoptosis-inducing factor in brain of minocycline-treated R6/2 mice. Furthermore, minocycline blocked the release of mitochondrial cell death mediators, such as cytochrome *c* in polyglutamine-induced cell death in a striatal neurone cell line and influenced the swelling induced by calcium, tert-butyl hydroperoxide and phenylarsine oxide in isolated rat liver mitochondria.

As inflammation and mitochondrial signaling of apoptosis appear to be important pathway in neuronal death, a better understanding of the action mechanism of minocycline is important for the development of novel, mechanism-based therapeutic modalities. In this study, we investigated the neuroprotective efficacy of minocycline in different neuronal damage paradigms involving inflammation or mitochondrial dysfunction. The effect of minocycline was firstly tested in a model of transient global cerebral ischaemia in gerbils, in which the neuronal loss subsequent to a cerebral ischaemic insult is followed by an important increase in the density of peripheral type benzodiazepine binding sites (PTBBS) measured using [^{3}H] PK11195 as a specific radioligand. This increase is an expression of the glial reaction and macrophage invasion of the ischaemic brain tissue (Benavides et al., 1990; Demerle-Pallardy et al., 1991; Kirino, 1982; Lin et al., 1990). Then the protective activity of minocycline was evaluated in in vivo experimental models using toxins acting on complex I (MPTP/MPP^+; Nicklas et al., 1985) and complex II (malonate; Greene et al., 1993) of the mitochondrial respiratory chain. Finally, the direct mitochondrial impact of minocycline was assessed on isolated mitochondria.

2. Materials and methods

2.1. Animals

Animals used in the studies were housed under controlled conditions of temperature (22.0±2.0 °C), relative humidity (55±10%) and illumination (artificial 12-h light cycle starting at 7 a.m.) at least 2 days before experiments. All experiments were carried out in accordance with the recommendations of the European Union regarding care and use of laboratory animals.

2.2. Drugs

Minocycline (Sigma) was dissolved in saline, and pH was adjusted to 7.4 with NaOH. For in vivo studies, minocycline was injected intraperitoneally (i.p.) as a bolus. MPTP (Fluka), carrageenan-λ-type IV (Sigma) and ibuprofen (Sigma) were dissolved in saline. Malonate (malonic acid, Sigma) was dissolved in phosphate buffer salt. MPP^+(RBI) was dissolved in mitochondrial buffer.

2.3. Transient global ischaemia in gerbils

In gerbil, the bilateral carotid occlusion causes complete forebrain ischaemia leading in a consistent and reproducible damage of the CA1 of the hippocampus. An indirect index of neuronal damage was used by the measurement of the total peripheral type benzodiazepine binding sites densities (PTBBS) with a specific ligand [^{3}H] PK11195 (Benavides et al., 1990; Demerle-Pallardy et al., 1991). The viability of this approach is based on the fact that glial reaction and macrophage invasion (cell types that, in contrast to neurons, are richly endowed with PTBBS) are the unavoidable consequences of neuronal injury.

Male Mongolian gerbils (Tumblebrook Farm, West Brookfied, USA), weighing 60–80 g were subjected to a transient forebrain ischaemia induced by bilateral occlusion of the common carotid arteries. Animals were anaesthetised with isoflurane (2% in O_2). Both carotid arteries were isolated via a ventral midline cervical incision. An atraumatic clasp was loosely placed around each artery and tightened for 10 min. At the end of the ischaemic period, blood flow was restored, and the patency of the carotid arteries checked by direct visualisation. The animals

were allowed to survive for 7 days. Sham-operated animals were subjected to simple exposure of the carotid arteries. The animals were kept warm at 38 °C for the first 5 h using an overhead lamp. Gerbils were treated with either vehicle (saline) or minocycline administered 5 min and 5 h after bilateral occlusion of the common carotid artery at the dose of 90 mg/kg followed by the dose of 45 mg/kg at 24 and 48 h. Seven days postocclusion, the brain of the gerbils were rapidly removed, and neuronal damage was assessed by measurement of peripheral type benzodiazepine binding sites (PTBBS) levels. The specific ligand [3H] PK11195 [*N*-methyl-N-(1-methylpropyl)-1-(2-chloro-phenyl)-isoquinoline-3-carboxamide] was used for labelling PTBBS in homogenates. Hippocampi were dissected out, weighed, frozen and stored at −80 °C until binding studies. Hippocampi were polytron homogenized in 1 ml of 50 mM Tris–HCl buffer (pH 7.4). Aliquots of 50 μl of homogenate were incubated with 2 nM of [3H] PK11195 (75 Ci/mmol) in 1 ml Tris–saline buffer for 1 h at 25 °C. Bound radioactivity was recovered by vacuum filtration through glass fibre filters (Watman, GF/B) followed by three washes and counted by liquid scintillation spectrometry. Nonspecific binding was defined by 1 μM PK11195. Binding was measured in triplicate. Results are expressed as Mean±S.E.M. of PTBBS density in fmol of [^{3}H] PK11195/mg of tissue for each group of gerbils. The drug-induced percentage changes in PTBBS density were calculated as follows: ΔPTBBS%= [(1−(treated ischaemic−treated sham)/(vehicle ischaemic−vehicle sham)]×100.

2.4. MPTP model in mice

The MPTP neurotoxicity is due to 1-methyl-4-phenyl-pyridinum ion (MPP+), its principal active metabolite, which is formed by the action of monoamine oxidase B. MPP+ is then recognized by the dopamine transporter and selectively taken up by dopaminergic neurons where it causes mitochondrial impairment by inhibiting complex I of electron transport chain. This leads to an extensive and quite selective destruction of dopaminergic neurons in the substantia nigra pars compacta and a dramatic decrease of dopamine content in the striatum. Striatal dopamine content is therefore used to estimate the neuronal striatal damage. Male C57BL6 mice, 8 weeks old (Charles River, France), received three intraperitoneal injections of 20 mg/kg MPTP hydrochloride at 2-h intervals. Mice were treated with either vehicle or minocycline (60, 90 and 120 mg/kg) administered 30 min before each administration of MPTP, 90 min after the last administration of MPTP and 24 h after the first injection of vehicle or minocycline. Twenty-four hours after the last MPTP injection, mice were decapitated, the brains removed, and the striatum were dissected, frozen on solid CO_2 and stored at −80 °C. Dopamine was extracted by adding homogenization solution containing L-cysteine, $Na_2S_2O_5$ and $HClO_4$ to the striatum (40 mg/1 ml). The striatum were then homogenized and centrifuged for 10 min at 2000 *g* at 4 °C. The supernatant was again centrifuged at 15,000 *g* for 1 min and then analyzed by high-performance liquid chromatography coupled to electrochemical detection (ANTEC Leyden) The mobile phase consisted in 90% of 70 mM KH_2PO_4, 0.1 mM EDTA, 2.1 mM TEA (ethanamine,N,N,diethyl) and 1.25 mM sodium octane sulfate, pH 3.7, and 10% methanol.

2.5. Malonate-induced striatal lesion in rats

The neurotoxin, malonate, reversibly inhibits the enzyme succinate dehydrogenase in both the tricarboxylic acid cycle and respiratory chain (complex II), and it also may block mitochondrial anion transport. In adult rat, direct intrastriatal injection of micromolar quantities of malonate elicits focal necrotic lesion. The volume of lesion is characterized by absence of TTC-staining (2,3,5-triphenyl-tetrazolium chloride), an indicator of mitochondrial viability.

Male Sprague Dawley rats (Iffa Credo France) weighing 380–400 g were anaesthetised with chloral hydrate (400 mg/kg/5 ml i.p.) and positioned in a David Kopf stereotaxic instrument. Intrastriatal injection of malonate was made with a 10 μl Hamilton syringe fitted with a 26-gauze blunt-tipped needle in the left striatum at coordinates 0.8 mm anterior to Bregma, 3 mm lateral to the midline and 6.5 mm ventral. Injection volume was 1 μl (2 μmol malonate/1 μl). Injections were made over 1 min, and the needle was left in place for an additional 1 min before being slowly withdrawn. Rats were treated with either vehicle or minocycline (45, 90 and 180 mg/kg) administered 1 h before and 5 h after intrastriatal administration of malonate. Forty-eight hours after malonate injection, animals were deeply anaesthetised with isoflurane (1% in O_2) and decapitated. Brains were removed quickly and coronally sectioned into six 2-mm-thick slices (from rostral to caudal). The brain slices were incubated for 20 min in a 2% solution of TTC (ACROS) at room temperature and fixed by immersion in 10% buffered formalin solution. The volume of cerebral lesion was measured using an image analysis program (Biocom). Each TTC-stained coronal section was digitalised by a camera (Sony). The unstained area of the fixed brain section was defined as lesion. The lesion volume was calculated by summing the lesion area of each coronal section and multiplying by the thickness of the slices. All values are expressed as means±S.E.M. of lesion volumes.

2.6. Carrageenan paw oedema model in rats

Carrageenan-induced paw oedema represent the most commonly used experimental model to assess the antiinflammatory properties of agents. The study was performed on male Sprague–Dawley rats (Charles River, France) weighing 175–202 g. Minocycline (45, 90 and 180 mg/kg) or vehicle were administered intraperitoneally 30 min before a subplantar injection of carrageenan (1 mg/0.1 ml) in the rat right hind paw. The paw volume (ml) was

monitored with a plethysmometer (Water Plethysmometer 7150 Ugo Basile) before treatment (baseline) and 3 h after carrageenan injection. Results are expressed as means of percentage of protection of volume oedema (±S.E.M.) where % protection corresponded to 100(% inflammation in control group−% inflammation in minocycline group)/% inflammation in control group. Percentage of inflammation was defined as 100(paw volume at 3 h−paw volume at 0 h(baseline))/paw volume at 0 h.

2.7. In vitro measurement of mitochondrial swelling

Mitochondrial swelling due to the opening of the mitochondrial permeability transition pore is one of the initial step of mitochondrial dysfunction leading to activation of caspases and apoptosis. Mitochondria were prepared from the livers of fasted overnight male Sprague–Dawley rats (220–270 g). The peritoneal cavity of isoflurane (2% in O_2)-anaesthetised rats was opened, and the livers were rapidly excised and homogenized on ice and placed in ice cold extraction buffer containing (225 mM mannitol; 75 mM sucrose; 0.2 mM EDTA; 5 mM TRIS–HCl [Tris Hydroxymethylaminoethane–HCl]; pH 7.4 at 4 °C). The homogenate was centrifuged for 5 min at 1.085×*g*. The resulting supernatant was centrifuged at 17,000×*g* for 10 min. Then the pellet was resuspended in extraction buffer by stirring gently with a glass rod, and the suspension was centrifuged at 17,000×*g* for 10 min. The resulting pellet was resuspended in 1 ml of extraction buffer at 4 °C. Mitochondrial protein was determined by the method of Lowry (BioRad Dc protein assay). The mitochondrial suspension was stored on ice and used within 3 h. Mitochondria (1 mg/ml of protein) were incubated in buffer (225 mM Mannitol, 70 mM sucrose, 5 mM Hepes, 5 mM/0.5 mM glutamate/malate), pH 7.4, at 25 °C.

Determination of mitochondrial swelling were performed in a spectrophotometer by measuring the decrease in light scattering at 540 nm. Mitochondria were preincubated for 5 min in the assay buffer at 25 °C with 1 mM MPP^+ or with 1 mM MPP^+ and minocycline (1 μM). Five minutes later, 50 μM Ca^{2+} was added followed 2 min later by the addition of 300 μM Pi (*t*=0 of the recording) according to Cassarino et al. (1999). In another set of experiments, minocycline (1, 5, 10 and 25 μM) was added to the assay buffer, and the mitochondrial swelling was recorded.

3. Results

3.1. Transient global ischaemia in gerbils

Global cerebral ischaemia followed by recirculation induced 7 days later a neuronal damage as shown by a significant increase of 86.4% in [^{3}H]PK11195 binding, reflecting PTBBS density in the hippocampus (185.57±7.75 versus 99.57±3.72 fmol/mg tissue, *P*=0.0001; Fig. 1). Posttreatment with minocycline (successive doses of 90 and 45 mg/kg i.p.) reduced significantly the increase in PTBBS by 36% (159.39±4.77 versus 185.57±7.75 fmol/mg tissue, *P*=0.0092).

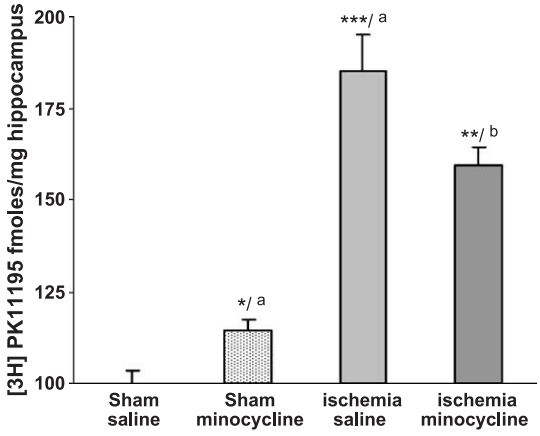

Fig. 1. Effect of minocycline 7 days after 10 min of transient global ischaemia in gerbils. Neuronal damage was indirectly evaluated by the measurement of PTBBS in hippocampus. The specific ligand [^{3}H] PK11195 was used for labelling PTBBS homogenates. Sham-operated animals (*n*=4–5) were subjected to simple exposure of carotid arteries. In ischaemic groups, *n*=13–14. Minocycline or saline was administered i.p. 5 min and 5 h after ischaemia at the dose of 90 mg/kg, followed by a dose of 45 mg/kg at 24 and 48 h. Results are expressed as means±S.E.M. (a)—indicates comparison with sham saline group (Student's test [two-tailed]). (b)—indicates comparison with ischaemia saline group (Student's test [two-tailed]). Statistical significant difference, **P*<0.05, ***P*<0.01 and ****P*<0.001.

In sham-operated gerbils, minocycline at the same dose increased slightly but significantly the basal level of PTBBS by 14.7% in comparison with saline-treated sham-operated gerbils (*P*=0.0250).

3.2. MPTP model in mice

Twenty-four hours after the last MPTP injection, MPTP-mice exhibited a significant marked depletion of 58% in striatal dopamine level in comparison to control mice (3.06±0.39 versus 12.3±0.61 ng/mg of tissue, *P*=0.0001; Fig. 2).

Treatment with minocycline 60 and 90 mg/kg (i.p.) dose dependently reduced MPTP-induced dopamine depletion in striatum by 13% and by 75%, respectively (4.24±0.16 ng/mg of tissue and 10.02±1.17 ng/mg of tissue, respectively); this latter effect was statistically significant (*P*<0.0000). Higher dose of minocycline (120 mg/kg) resulted in the death of all animals following the last administration of minocycline.

In another set of experiments, the dose of 90 mg/kg minocycline has been evaluated on dopamine level of naïve mice, as well as of MPTP-treated mice. Minocycline did not significantly modify basal striatal dopamine level of naïve mice (12.5±0.5 versus 13.7±0.3 ng/mg of tissue for minocycline-treated and control mice, respectively; *P*=0.0615) and reduced by 69% (11.7±0.8 versus 7.2±1.3

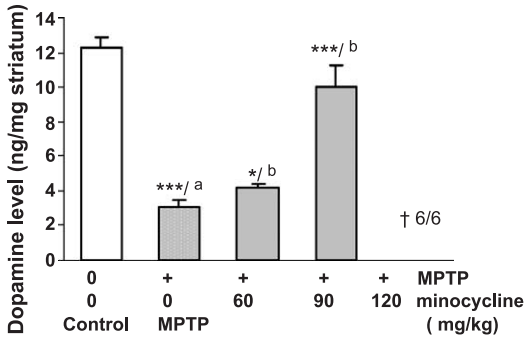

Fig. 2. Effect–dose of minocycline on dopamine level reduction induced by MPTP in striatum of C57BL/6 mice. Mice were injected (+) or not (0) with MPTP (3*20mg/kg, i.p. at 2-h intervals). Minocycline or saline was administered 30 min before each injection of MPTP, 90 min after the last injection of MPTP and 24 h after the first injection of saline or minocycline. The second group treated with saline is the control MPTP group. The three other groups were injected with different doses of minocycline (60, 90 and 120 mg/kg, i.p.). Results are expressed as means±S.E.M (n=6). The symbol (†) noted the number of dead mice. (a)—indicates comparison with control group (Student's test [two-tailed]). (b)—indicates comparison with MPTP group (Dunnett's test [two-tailed]). Statistical significant difference, *P<0.05 and ***P<0.001.

ng/mg of tissue, P=0.023) the MPTP-induced dopamine depletion (7.2±1.3 versus 13.7±1.3 ng/mg of tissue, P=0.0007).

3.3. Malonate-induced striatal lesion in rats

Forty-eight hours after intrastriatal injection of 2 μmol of malonate, the volume lesion was of 53±7.6 mm^3 in the striatum of control rats (n=7).

Pretreatment with minocycline (45, 90 and 120 mg/kg) was ineffective to reduce the malonate-induced striatal lesion (Fig. 3).

3.4. Carrageenan paw oedema model in rats

Subplantar injection of carrageenan in the right hind paw of control rats resulted 180 min later in an increase in the paw volume (2.87±0.08 versus 1.65±0.0.3 ml; n=8; Fig. 4).

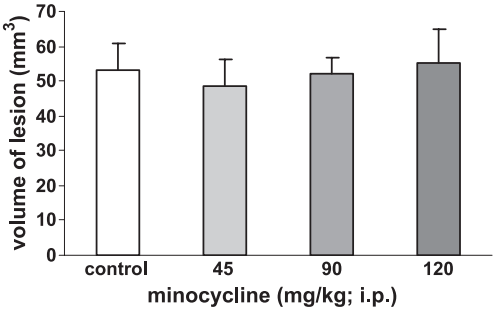

Fig. 3. Dose–effect of minocycline on striatal lesion 48 h after intrastrial injection of malonate (2 μmol) in rats. Minocycline (45, 90, 120 mg/kg, i.p.) or saline was administered 1 h before and 5 h after malonate injection. The volume of lesion was measured after TTC staining (2%). Results are expressed as means±S.E.M (n=8). ***P<0.001 significantly different from saline group (one-way analysis of variance).

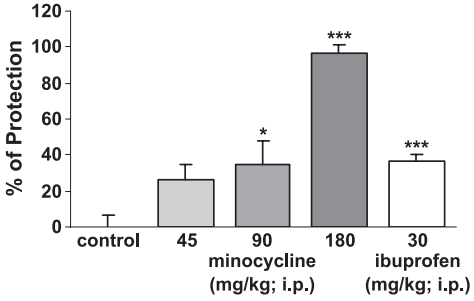

Fig. 4. Effect of minocycline 180 min after carrageenan-induced paw oedema in rat. Oedema was measured with a plethysmometer. Minocycline (45, 90 and 180 mg/kg, i.p.) or saline (control group) was administered 30 min before the carrageenan injection. Ibuprofen (30mg/kg, i.p.), a reference molecule for this model, was tested in same conditions. Results are expressed as means of percentage of protection±S.E.M. Results are expressed as means of percentage of protection of volume oedema (±S.E.M.) were % protection corresponded to 100(% inflammation in control group−% inflammation in treated group) / % inflammation in control group. Percentage of inflammation was defined as 100(paw volume at 3-h baseline paw volume) / baseline paw volume. *P<0.1, **P<0.01 and ***P<0.001 significantly different from control group (Student's test [two-tailed] and Dunnet's test [two-tailed] for ibuprofen and minocycline, respectively).

Pretreatment with minocycline elicited a dose dependent antiinflammatory effect in comparison with control group. Minocycline 45, 90 and 180 mg/kg reduced paw oedema formation by 26.6±8.5%, 34.3±13.3% and 96.5±4.9%, respectively (P=0.0985, P=0.0256 and P=0.0000, respectively). In the same conditions, pretreatment with ibuprofen (a reference antiinflammatory agent) at 30 mg/kg (i.p.) limited significantly the carrageenan-induced paw inflammation in comparison with saline group (36.4±4.0% of protection; P=0.0003).

3.5. In vitro measurement of mitochondrial swelling

To assess the direct effect of minocycline on mitochondria, we evaluated the effect of minocycline on energized mitochondria (with oxidative substrates) and MPP^+-induced mitochondrial swelling.

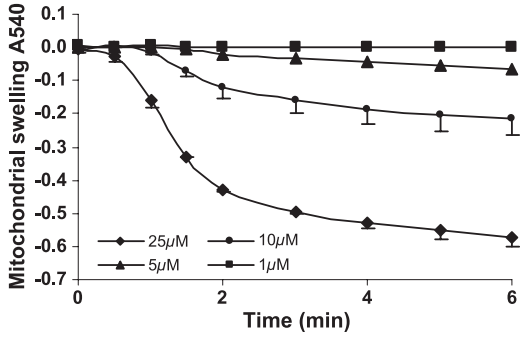

Fig. 5. Dose–effect of minocycline alone on mitochondria isolated from rat liver. The mitochondrial impact of minocycline was evaluated by the measurement of mitochondrial swelling in spectrophotometry at 540 nm. Different doses of minocycline were tested (1, 5, 10 and 25 μM). Results are expressed as means±S.E.M (experiment has been made at least on two rats).

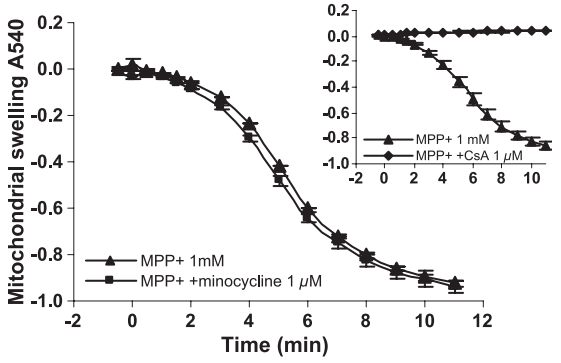

Fig. 6. Effect of minocycline on mitochondrial swelling induced by MPP^+. Maximal swelling was measured for 11 min after addition of MPP^+(1 mM). To test minocycline, the dose of 1 μM, which did not induce mitochondrial swelling, was chosen. Mitochondria were preincubated for 5 min with or without minocycline and MPP+(1mM) (A). Results are expressed as means±S.E.M (experiment has been made on at least two rats). Cyclosporine (CsA) (1μM), the reference molecule, was tested in the same conditions on MPP^+-induced swelling (see insert).

Mitochondrial dysfunction initiates a sequence of events including mitochondrial depolarisation, osmotic swelling and the release of mitochondrial proteins with the potential to activate the initial steps of apoptosis. Such cascade of events may be triggered by neurotoxin like MPP^+, a complex I inhibitor of mitochondrial respiratory chain.

In a first set of experiment, minocycline was tested on isolated liver mitochondria. Addition of minocycline (1, 5, 10 and 25 μM) to suspension of isolated liver mitochondria resulted in a dose-dependent induction of mitochondrial swelling by rapid decreases in absorbance indicating mitochondrial dysfunction (Fig. 5). The ineffective concentration of 1 μM of minocycline has subsequently been tested on the mitochondrial swelling induced by MPP^+(1 mM). This concentration of minocycline was unable to reduce mitochondrial swelling induced by MPP^+, whereas in the same conditions, the inhibitor of mitochondrial transition pore cyclosporine A (CsA) prevented MPP^+-induced opening of mitochondrial permeability transition pore (Fig. 6).

4. Discussion

Numerous studies have demonstrated the neuroprotective benefit of minocycline in experimental stroke and trauma models, as well as in models of Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (Chen et al., 2000; Du et al., 2001; He et al., 2001; Kriz et al., 2002; Sanchez Mejia et al., 2001; Van Den et al., 2002; Wu et al., 2002; Yrjanheikki et al., 1998, 1999). The study herein confirms the neuroprotective potential of this compound. Pioneer experiments showing neuroprotection afforded by minocycline has been observed in a model of global transient ischaemia in gerbils (Yrjanheikki et al., 1998). In this model, the ischaemic insult mainly results in a delayed neuronal death associated with an inflammatory reaction consisting in a glial activation and a macrophage invasion. The authors showed that treatment with minocycline increased the number of surviving neurons and completely prevented the activation of microglia assessed by immunohistochemistry. Using a similar model of global transient ischaemia in gerbils and a similar posttreatment protocol (90 mg/kg followed with 45 mg/kg), we also found beneficial effects of minocycline which reduced neuronal damage measured by PTBBS density, a marker of microglial activation. Moreover, in our study, the beneficial effect of minocycline may be underestimated since minocycline slightly but significantly increased basal PTBBS density. These results point up the interaction of minocycline with microglial activation. Minocycline can inhibit microglial activation, p38-kinase activation, mRNA induction of interleukin-1β-converting enzyme (ICE), expression of iNOS mRNA, cyclooxygenase-2 expression and prostaglandin E2 production (Tikka et al., 2001; Yrjanheikki et al., 1998, 1999). The antiinflammatory properties of minocycline have been highlighted in our study by the potent protective effect of minocycline observed on oedema induced by carrageenan injection in the rat paw, a classical model of inflammation (Morris, 2003). Both antiinflammatory and neuroprotective properties of minocycline occurred in the same dose range.

To assess the possible involvement of the mitochondrial pathway in neuroprotective properties of minocycline, we measured its effects in experimental animal models involving mitochondrial toxins administration, such as MPTP and malonate, which inhibit complex I and II of the mitochondrial respiratory chain, respectively (Greene et al., 1993; Nicklas et al., 1985).

Minocycline exhibited a clear protective effect in the model of systemic MPTP intoxication, whereas no effect was observed in the intrastriatal malonate administration models, suggesting that minocycline is unlikely to be a direct and ubiquitous mitochondrial protective agent. It may be therefore postulated that the discrepancies of the results may be due to the specific features and the complexity of the two models under experimentation.

In the MPTP mice model beyond energy failure due to mitochondrial respiration blockade, microglial reaction has been already observed using interleukin-6 immunoreactivity (Kohutnicka et al., 1998) or the PTBBS density marker [3H]-PK11195 (Kuhlmann and Guilarte, 1999). A time course study of MPTP neurotoxicity in mice has shown that MPTP administration induced a robust gliosis (microglia and astroglia) in the substantia nigra and in the striatum that preceded or paralleled dopaminergic degeneration (Liberatore et al., 1999). Microglial activation was evident 12 h after MPTP administration, peaked at 24–48 h and was no longer detected after 7 days. These studies indicate that microglial activation largely contribute to the neuronal damage following systemic MPTP administration

in mice and may explain the protective effect of minocycline that we and others observed using striatal dopamine measurement or histological approach in this MPTP model of Parkinson's disease (Du et al., 2001; Wu et al., 2002). Conversely, a more recent study from Yang et al. (2003) has shown that despite inhibition of microglial activation, minocycline treatment exacerbated MPTP-induced dopamine depletion and damage to dopamine neurons in mice. The authors suggested that schedule of administration, doses or drug concentrations could account for such differences with the previous studies. In line with this, it should be emphasized that in our experimental conditions, the higher dosage of minocycline (120 mg/kg) resulted in the death of all animals following the last administration of minocycline. This toxicity may be attributed to an increased toxicity of MPTP by minocycline or to the fact that this high dose reaches the minocycline toxicity limits. Nevertheless, as in our treatment paradigm, the maximal protective effect was obtained with the dose of 90 mg/kg. It should be assumed that minocycline displays a limited therapeutic index to induce neuroprotection in this model of Parkinson's disease.

While there is accumulative evidence suggesting the implication of microglial activation in the MPTP mice model, such information are lacking for the murine model of malonate intoxication. Malonate, a reversible mitochondrial toxin of succinate dehydrogenase/complex II injected in striatum of rat, induced a large striatal lesion 72 h later with marked loss of neurons, apparent preservation of axon bundles passing through the lesion and sparing of glial cells (Greene et al., 1993). Malonate toxicity induces impairment of oxidative metabolism that leads to excitotoxicity and overactivation of excitatory amino acids receptors, as well as their second messenger, such as nitric oxide from neuronal nitric oxide synthase (Connop et al., 1997; Greene et al., 1993; Greene and Greenamyre, 1995; Matthews et al., 1997). In our experimental conditions, intrastriatal injection of malonate to rats induced 48 h later a large lesion volume which was unaffected by pretreatment with minocycline. These results tail with the recent work performed by Diguet et al. (2003) reporting a lack of protective effect of minocycline in a mouse model of mitochondrial intoxication using the irreversible inhibitor of succinate dehydrogenase 3-nitropropionic acid. Moreover, a deleterious effect of minocycline has been observed in this mice model of Huntington's disease, stressing the importance to evaluate the tolerance of minocycline in these models of neuroprotection. To our knowledge, the protective effect of antiinflammatory agent in a rat model of mitochondrial intoxication by an inhibitor of complex II has not been reported, whereas such an effect has been observed with cyclosporine A (CsA), a reference compound for mitochondrial protection (Leventhal et al., 2000).

Collectively, our in vivo results evidenced the neuroprotective properties of minocycline but indicated that these properties were only manifest in experimental animal models involving clear inflammation and microglial activation and not direct mitochondrial poisoning. To ascertain this point, we investigated the effect of minocycline in energized mitochondria isolated from rat liver and treated by 1-methyl-4-phenylpyridinium (MPP^+, 1 mM, in the presence of $Ca^{2+}$and Pi) that leads to impaired ATP synthesis and to toxic free radical production. This causes the mitochondria to undergo an irreversible permeability transition with subsequent large amplitude swelling and cytochrome *c* release (Cassarino et al., 1999). MPP^+ has been chosen since it is the active metabolite of MPTP, the mitochondrial toxin we have used in our animal model to study the neuroprotective effect of minocycline. To conduct this experiment, we firstly examined the effect per se of minocycline on isolated mitochondria. Addition of minocycline at relatively low concentrations (10–25 μM) to energize mitochondria resulted in an unexpected mitochondrial swelling. Moreover, minocycline tested at a concentration (1 μM) that did not induce swelling was unable to reduce mitochondrial swelling induced by MPP^+. In this same experimental condition, CsA prevented MPP^+-induced opening of mitochondrial permeability transition pore, as well as the subsequent release of cytochrome *c* (data not shown). This result which confirms the lack of direct mitochondrial protective action of minocycline is in disagreement with the assumption that minocycline exerts neuroprotection through a mitochondrial dependent pathway (Wang et al., 2003; Zhu et al., 2002). Although in these two studies, the swelling has also been performed on rat liver mitochondria, the minocycline-mediated inhibition of mitochondrial swelling has been assessed using inducers different to MPP^+ and concentration of minocycline up to 120 μM. Moreover, a concentration-related effect of minocycline on control mitochondria has not been examined.

In summary, the study confirms the neuroprotective potential of minocycline and emphasizes the role of the antiinflammatory properties of this compound in this effect. Minocycline exhibited no efficacy in malonate-induced striatal lesion in rats and a marked efficacy in MPTP-induced neurotoxicity. These heterogeneous results suggest that minocycline does not directly protect mitochondria from specific toxins. This is confirmed by in vitro results using isolated mitochondria showing the absence of effect of minocycline on swelling induced by MPP^+. Moreover, we observed a deleterious effect of minocycline, which induced mitochondrial swelling at relatively low concentrations cautioning the possible toxic effect of minocycline on mitochondria. Taking account of the fact that mitochondrial dysfunction is a key effector of neuronal death, these results would suggest that, in certain conditions, minocycline may aggravate instead to protect neurodegeneration. Indeed, it has been already reported that minocycline enhanced MPTP toxicity in mice (Yang et al., 2003) and elicited deleterious effects in the model of 3-nitropropionic acid-induced striatal cell loss in mice (Diguet et al., 2003).

Finally, a limited safety margin of minocycline as it was observed in our model of MPTP intoxication in mice advocate that dose escalation using minocycline should be performed cautiously.

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