In vivo studies on the protective role of minocycline against excitotoxicity caused by malonate or \( N \)-methyl-\( \alpha \)-aspartate

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Abstract

Minocycline has been shown to exert neuroprotection against a wide variety of toxic insults both in vitro and in vivo. However, contradictory results have recently been reported. We now report that minocycline affords no protection against the neurotoxicity caused by malonate or \( N \)-methyl-\( \alpha \)-aspartate (NMDA). Rats were treated with minocycline (45 mg/kg i.p. \( \times \) 7) every 12 h. Thirty minutes after the second dose of minocycline, an intrastriatal stereotaxic injection of malonate (1.5 \( \mu \)mol) or NMDA (0.1 \( \mu \)mol) was administered. Seven days later, the rats were killed, and lesion volumes were quantified using two different methods [triphenyltetrazolium chloride (TTC) staining or cytochrome oxidase histochemistry]. Our results show that minocycline does not prevent the lesions caused by either malonate or by NMDA. On the contrary, the putative NMDA receptor antagonist, MK-801, blocked the toxicity caused by both toxins indicating that, although by different mechanisms, excitotoxicity is mediating neuronal death. We conclude that minocycline, at least under our experimental conditions, is not neuroprotective against excitotoxicity caused by either malonate or NMDA.

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Introduction

Excitotoxicity plays an important role in many central nervous system diseases (e.g., Beal, 1995; Sattler and Tymiaski, 2001). Toxins that affect mitochondria are being used as pharmacological tools to mimic different neurodegenerative diseases (Browne and Beal, 2002). Among these drugs is malonate. Stereotaxic injection of malonate has been shown to cause dose-dependent neurotoxicity by inhibition of succinate dehydrogenase and depletion of striatal ATP, which results in neuronal depolarization and secondary excitotoxicity (Beal et al., 1993; Greene and Greenamyre, 1995, 1996a; Greene et al., 1993; Henshaw et al., 1994). Curiously, histological characterization of lesions produced by malonate has revealed striking similarities to “excitotoxic” lesions observed in some neurological diseases, such as focal ischemia and HD (for review, see Browne and Beal, 2002, 2004; Browne et al., 1999; Greene and Greenamyre, 1996b).

On the other hand, minocycline has been shown to protect against excitotoxicity caused by \( N \)-methyl-\( \alpha \)-aspartate (NMDA) in vitro (Tikka and Koistinaho, 2001; Tikka et al., 2001) to protect against focal and global ischemia (Wang et al., 2002, 2003a,b; Xu et al., 2004; Yrjanheikki et al., 1998, 1999) as well as to delay mortality in the R6/2 transgenic mouse model of HD (Chen et al., 2000; Wang et al., 2003a,b). However, more recently, minocycline has
clearly been shown to have variable effects in different species and models of neurological disorders (Diguet et al., 2004a,b; Smith et al., 2003; Stefanova et al., 2004; Tsuji et al., 2004; Yang et al., 2003). Based on these previous findings, we analyzed whether minocycline would protect against excitotoxicity caused by intrastriatal administration of malonate or N-methyl-D-aspartate (NMDA). Our results show that minocycline does not protect against excitotoxicity caused by either excitotoxin.

Material and methods

Animals and treatments

Male Wistar rats (280–300 g) were housed in plastic cages in a temperature controlled room (22°C ± 1°C) with free access to food and water and maintained on a 12/12-h light/dark cycle (lights on at 07:00 h) during the whole course of the experiments. Rats received a total of seven injections of saline or minocycline (45 mg/kg i.p.) every 12 h. Dosage regimen of minocycline was chosen based on previous reports showing its neuroprotective effects against both focal and global ischemia (Yrjanheikki et al., 1998, 1999). Thirty minutes after the second injection of minocycline, rats were anesthetized with pentobarbital (60 mg/kg i.p.) and placed in a Kopf stereotaxic frame, with the incisor bar set at 3.3 mm below the interaural line. The skull was exposed, and two holes were drilled at coordinates: +0.6 mm anteroposterior (AP), ±2.8 mm mediolateral (ML) relative to bregma according to the atlas of Paxinos and Watson (1997). Animals were injected in both striata with 2 µl of solution (0.5 µl/min) using a 10-µl Hamilton syringe with a 26-gauge needle lowered 5.5 mm from the skull. After injection, the needle was left in place for 5 min to allow complete diffusion of the injected volume. Solutions injected included malonate (750 mM) or NMDA (50 mM). One week after toxin injections, rats were killed, and lesion volume was analyzed by two different methods (see below). In a different set of experiments, animals received MK-801 (5 mg/kg i.p.) 30 min before and 210 min after the injection of malonate or NMDA. MK-801 administration protocol was chosen based on a previous report showing that it does not affect body temperature of animals (Greene and Greenamyre, 1995).

Fig. 1. Striatal lesion volume following injection of malonate or NMDA alone or in combination with minocycline. Each bar represents mean ± SEM of 10 rats (malonate) or 7 rats (NMDA), calculated from TTC-stained sections. No statistically significant difference was found between saline- and minocycline-treated animals. Two-tailed unpaired t test: malonate versus minocycline + malonate, t (9) = –0.972, P > 0.05; NMDA versus minocycline + NMDA, t (6) = –0.943, P > 0.05.

Fig. 2. Digitized images of representative TTC-stained sections from brains of rats injected with 1.5 µmol of malonate or with 0.1 µmol of NMDA plus saline or minocycline. Note the lack of protection by minocycline against the toxicity induced by malonate or NMDA.

Fig. 3. Striatal lesion volume following injection of malonate or NMDA alone or in combination with minocycline or MK-801. Each bar represents mean ± SEM of 12 rats (panel A) or 6 rats (panel B), calculated from cytochrome oxidase-stained sections. Note the marked neuroprotective effect of MK-801. The data were statistically evaluated using a one-way ANOVA followed by Tukey post hoc test. Panel A: treatment F(2,35) = 18.695, P < 0.001. *P < 0.05 versus malonate. Panel B: treatment F(2, 17) = 25.497, P < 0.001. *P < 0.05 versus NMDA.
All the procedures followed in the present work were in compliance with the European Community Council Directive of November 24, 1986 (86/609/EEC), and were approved by the Ethical Committee of the University of Navarra.

**Quantification of volume lesion using the TTC method**

Seven days after malonate or NMDA injections with and without minocycline, rats were killed, and their brains were quickly removed and placed in ice-cold 0.9% saline solution. Brains were sectioned at 2-mm intervals throughout the rostrocaudal axis of the striatum. Slices were then placed posterior-side down in 2% 2,3,5-triphenyltetrazolium chloride (TTC). Slices were stained in the dark at room temperature for 30 min and then removed and placed in 4% paraformaldehyde, pH 7.3. Lesions noted by pale staining were outlined, and the area quantified using NIH image 6.1 software by an individual blinded to the experimental conditions. Because this method is not too accurate and subtle differences can be underestimated, we also used the following method in order to be able to measure the lesion volume more precisely.

**Cytochrome oxidase histochemistry and analysis of lesion size**

For these experiments, animals received malonate or NMDA into one striatum only. Seven days after toxin injections with and without minocycline or MK-801, rats were killed, and brains were frozen immediately on dry ice and then sectioned (50 μm) on a cryostat. Every second section throughout the extent of the lesion was mounted on a polylysine-coated slide. Incubation medium consisted of 5 mg cytochrome c and 30 mg 3,3′-diaminobenzidine in 50 ml of 0.1 M phosphate buffer, pH 7.4. Slides were incubated for 90 min at 37°C and then removed to 4% neutral, buffered paraformaldehyde for 10 min. Sections were rinsed with distilled water, dehydrated, cleared in xylene, and coverslipped (Greene and Greenamyre, 1996a). The lesioned area on each section was quantified using a video-based MCID image analysis system (Imaging Research, St. Catherines, Ontario, Canada). Area measurements were summed and multiplied by intersectional distance (100 μm) to determine lesion volume.

**Results**

**Effect of minocycline on striatal lesions caused by malonate or NMDA**

To investigate the neuroprotective effects of minocycline against different excitotoxins-induced neuronal death in vivo, we treated rats with minocycline (45 mg/kg i.p., × 7, every 12 h) starting 12 h before striatal stereotaxic injections of 1.5 μmol of malonate or 0.1 μmol of NMDA. Seven days later, rats were killed and lesion volumes were analyzed in 2-mm brain slices stained with TTC. As shown in Fig. 1, both toxins produced a large striatal lesion, which was not prevented by minocycline administration (Fig. 2).

In a different set of experiments, rats were treated with minocycline or MK-801 in combination with malonate or NMDA. In these animals, the lesion volume was calculated from cytochrome oxidase-stained sections. As it can be seen in Fig. 3, minocycline did not afford any protection against (A) malonate or (B) NMDA toxicity, while the NMDA receptor antagonist, MK-801, caused an almost complete protection (Fig. 4).

**Discussion**

Since it was first reported that minocycline, a second-generation tetracycline, protects neurons against both focal and global ischemia (Yrjanheikki et al., 1998, 1999), many
other research groups have tested the beneficial effects of this tetracycline in different in vitro and in vivo models of neurodegeneration. Thus, minocycline has been reported to delay disease progression and prolong the life-span of transgenic mouse models of amyotrophic lateral sclerosis (Kriz et al., 2002; Van Den Bosch et al., 2002; Zhu et al., 2002) and Huntington disease (Chen et al., 2000; Wang et al., 2003a,b). Minocycline also protects neurons against excitotoxic damage in vitro (Tikka and Koistinaho, 2001; Tikka et al., 2001) and nigral cell loss induced by 6-hydroxydopamine and MPTP in mice (Du et al., 2001; He et al., 2001; Wu et al., 2002).

There is a large body of evidence indicating that, independently of its antimicrobial effects, minocycline has anti-inflammatory properties, an effect mediated by the inhibition of microglia activation (He et al., 2001; Tikka et al., 2001; Wu et al., 2002; Yang et al., 2003; Yrjanheikki et al., 1998). Several other mechanisms have also been suggested to explain the neuroprotective properties of minocycline, including inhibition of caspase-1, caspase-3, inducible nitric oxide synthase (iNOS) expression, the mitochondrial permeability transition pore, mitochondrial swelling, and cytochrome c release and inhibition of p38 MAP kinase (Amin et al., 1996; Chen et al., 2000; Du et al., 2001; He et al., 2001; Lin et al., 2001; Tikka et al., 2001; Yrjanheikki et al., 1998, 1999; Zhu et al., 2002).

However, although all these later studies demonstrate the anti-inflammatory and beneficial properties of minocycline, it is worth noting that very recent evidences show variable and even contradictory (beneficial or detrimental) effects of minocycline in different animal models of neurodegeneration. Thus, in a recent study by Smith et al. (2003), no evidence for minocycline to delay the disease course in a transgenic mouse model of Huntington disease was found. Furthermore, it has been shown that minocycline worsens motor symptoms and increases cell loss in the MPTP-intoxicated nonhuman primate model of PD and the 3-nitropropionic acid (3-NP)-intoxicated mouse model of HD (Diguet et al., 2003, 2004a,b). Similarly, it has also been reported that minocycline enhances rather than prevents MPTP toxicity to dopaminergic neurons in mice (Yang et al., 2003). Finally, although different studies have reported beneficial effects of minocycline in adult animal models of ischemic injury (Wang et al., 2002, 2003a,b; Xu et al., 2004; Yrjanheikki et al., 1998, 1999) and against hypoxic-ischemic brain damage in neonates (Arvin et al., 2002), the opposite has also been shown (Tsuji et al., 2004). Thus, minocycline appears to have variable and even deleterious effects in different species and models.

A question that remains unresolved is why there is such discrepancy and inconsistency regarding the neuroprotective effects of minocycline. As discussed by others (Diguet et al., 2004a,b; Fagan et al., 2004; Smith et al., 2003; Tsuji et al., 2004; Yang et al., 2003), this may be a matter of drug origin, dosing regimen (route, dose, and number of administrations), time at which minocycline is given (pretoxic, at the time of toxic insult, or posttoxic insult), and species. As no data are available to date using minocycline in combination with malonate or NMDA in vivo, we used the same administration protocol as that shown to prevent focal ischemic lesions in rats (Yrjanheikki et al., 1999). Despite this consideration, our results show that minocycline affords no protection against malonate or NMDA toxicity. These results are in agreement with those previously reported in the 3-nitropropionic acid (3-NP) mouse model of HD (Diguet et al., 2004a,b) and again raise doubts on the beneficial effects of minocycline (Diguet et al., 2003, 2004a,b; Smith et al., 2003; Stefanova et al., 2004; Tsuji et al., 2004).

Finally, and taking into account all the negative results already published and our own, it seems clear that further experiments should be conducted in order to clarify what makes minocycline to behave as a neuroprotective or deleterious drug.

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