IN Volvement of Mitochondrial Potential and Calcium Buffering Capacity in Minocycline Cytoprotective Actions

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Abstract—Minocycline, a semisynthetic derivative of tetracycline, displays beneficial activity in neuroprotective models. Including Parkinson disease, spinal cord injury, amyotrophic lateral sclerosis, Huntington disease and stroke. The mechanisms by which minocycline inhibits apoptosis remain poorly understood. In the present report we have investigated the effects of minocycline in mitochondria, due to their crucial role in apoptotic pathways. In mitochondria isolated suspensions, minocycline failed to block superoxide-induced swelling but was effective in blocking mitochondrial swelling induced by calcium. This latter effect might be mediated through dissipation of mitochondrial transmembrane potential and blockade of mitochondrial calcium uptake. Consistently, minocycline fails to protect SH-SY5Y cell cultures in a size-dependent manner and inhibition of mitochondrial swelling occurs at the higher concentration tested (100 μM) inhibited complex II/III activity. Other members of the minocycline antibiotic family like tetracycline were also analyzed. Minocycline did not modify complex IV activity, and only at the higher concentration inhibited complex II/III activity.

Key words: apoptosis, mitochondria, Parkinson, neurodegeneration, caspases, ROS.

Neurodegenerative diseases, including Alzheimer (AD), Parkinson (PD) diseases, and amyotrophic lateral sclerosis (ALS), are among the main causes of death in Western countries (Murray and Lopez, 1997). In some of these pathologies a decrease in specific neuronal populations has been described including cholinergic neurons in AD or dopaminergic neurons in PD, where apoptotic mechanisms seem to be involved (Jellinger, 2001). In these processes, mitochondria appear to be a key point of convergence of different pathways initiated by several apoptotic stimuli including receptor activation by Fas-ligand (Kavurma and Khachigian, 2003) or glutamate (Stout et al., 1998), or after exposure to neurotoxins such as perioneurin (Jordan et al., 2002) or staurosporine (Tafani et al., 2002). Opening of a permeability transition pore (PTP) leads to mitochondrial swelling and the release of intramitochondrial proteins to the cytoplasm including cytochrome c, apaf-1 and caspase family members, which participate in apoptosis pathways (van Gurp et al., 2003; Joza et al., 2003; Galindo et al., 2003). Indeed, drugs with the ability to block PTP formation are cytoprotective against a variety of toxic stimuli (Loeffler and Kroemer, 2000; Zamzami and Kroemer, 2001; Jordan et al., 1997, 2003a). Among other triggers, calcium uptake into isolated mitochondria induces the collapse of the mitochondrial membrane potential (Δψm), the opening of the PTP and the release of proapoptotic factors (Kristol and Dubinsky, 1997; Li et al., 1997). So, mitochondrial Ca2+ overload would be a side effect of the rise in cytosolic Ca2+ concentration and inhibition of mitochondrial calcium uptake is antia apoptotic in several models of cell death (Jambrina et al., 2003; Reynolds, 1999; Bae et al., 2003; Lee et al., 2002).

Often, drugs used for the treatment of a specific pathology are later proven to be useful in a number of other diseases. Recently, a modified tetracycline antibiotic, minocycline hydrochloride, commonly used in the treatment of moderate to severe acne vulgaris (Dreno et al., 2001), appeared to display beneficial activity in various models of neurodegeneration including, PD, spinal cord injury, amyotrophic lateral sclerosis, Huntington disease and focal ischemic brain injury (Brundula et al., 2002; Kriz et al., 2002; Stirling et al., 2004; Wang et al., 2003). Also, its administration in mice expressing a mutant superoxide dismutase (SOD1(G37R)) at late presymptomatic stage, delayed the onset of motor neuron degeneration and muscle strength decline, and increased the longevity of SOD1(G37R) mice (Kriz et al., 2002). Treatment of patients with minocycline has, therefore, been proposed as a possible therapy for some neurodegenerative diseases including multiple sclerosis (Popovic et al., 2002), ischemia (Arvin et al., 2002), PD and Huntington’s disease (Thomas et al., 2003, 2004), because minocycline crosses the blood–brain barrier regardless of the dose and route of administration (Colovic and Caccia, 2003) and several clinical trials have been performed (Gordon et al., 2004; Thomas and Lee, 2004;
see also Blum et al., 2004; Domercq and Matute, 2004 for reviews). However, it has been recently reported that minocycline may have variable or even deleterious effects in different species and models depending on the mode of administration and the dose (Diguet et al., 2004). Thus minocycline presents deleterious effects in two phenotypic models of PD and HD (Diguet et al., 2003, 2004). Similarly, it exacerbates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopamine damage, and reverses the neuroprotective effect of creatine in nigrostriatal dopaminergic neurons (Yang et al., 2003).

The exact mechanisms by which minocycline plays these neuroprotective effects remain unknown although a reduced expression of cycloxygenase-2, caspase-1 and inducible nitric oxide synthase (iNOS) mRNA have been described (Chen et al., 2000). It has been shown that mitochondria might be involved in minocycline-activated pathways. Although it has been shown that minocycline prevented mitochondrial swelling induced by different stimuli (Zhu et al., 2002; Wang et al., 2003) in a recent report minocycline was able to induce it (Comet et al., 2004). The aim of the present work was to study whether minocycline can interfere with mitochondrial calcium uptake as a plausible mechanism for the cytoprotective action of this antibiotic. We have also analyzed the effect of minocycline on Ca²⁺- and reactive oxygen species-induced mitochondrial swelling and on mitochondrial transmembrane potential. The effect of minocycline on glutathione (GSH) and nicotinamide adenine dinucleotide coenzyme and its derivatives (NAD(P)H) levels was also addressed. All these determinations were performed in brain mitochondria preparations, since they are different from those derived from a variety of other sources (Kristian et al., 2000) and respond differently to diverse stimuli (Andreyev and Fiskum, 1999; Berman et al., 2000).

**EXPERIMENTAL PROCEDURES**

**Measurement of cell viability**

SH-SYSY cultures were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM L-glutamine, penicillin (20 units/ml), streptomycin (5 µg/ml), and 15% (vol/vol) heat-inactivated fetal calf serum (GIBCO, Gaithersburg, MD, USA) as reported previously by Yuste et al. (2002). Cells were grown in a humidified cell incubator at 37 °C under a 5% CO₂ atmosphere. For viability experiments, cells were plated at a density of 4 × 10⁴ cells/cm² and allowed to attach overnight. Cell viability after drugs additions was assessed by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell survival assays. MTT additions was assessed by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Molecular Probes, Leiden, The Netherlands) fluorescence intensity measured in a Perkin-Elmer (luminescence-spectrophotometer LS50B) fluorimeter at room temperature. The excitation and emission wavelengths for TMRE were 549 and 581 nm (slit 3 nm) for emission. Changes in absorbance at 540 nm (A₅₄₀), indicating mitochondrial swelling due to MTT opening, were followed, after addition of different compounds, using a microplate reader (BioRad, Hercules, CA, USA). Initial A₅₄₀ values were 0.8, and minor differences in loading of the wells were compensated by representing the data as the fraction of the initial absorbance determination remaining at a given time. Mitochondrial protein concentrations were quantified spectrophotometrically (Micro BCA Protein Reagent Kit, Pierce, Rockford, IL, USA), with bovine serum albumin used as standard.

**Monitoring of the Δψm**

Δψm was qualitatively assayed by tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, Leiden, The Netherlands) fluorescence intensity measured in a Perkin-Elmer (luminescence-spectrophotometer LS50B) fluorimeter at room temperature. The excitation and emission wavelengths for TMRE were 549 and 581 nm (slit 3 nm) for emission. Dye (250 mM) was added to a medium containing mitochondria (0.5 mg/ml) and 125 mM KCl, 20 mM HEPES, 2 mM KH₂PO₄, 1 µM EGTA, 1 mM MgCl₂, 5 mM malate and 5 mM glutamate with the pH adjusted to 7.08 with KOH. Changes in absorbance at 540 nm (A₅₄₀), indicating mitochondrial swelling due to MTT opening, were followed, after addition of different compounds, using a microplate reader (BioRad, Hercules, CA, USA). Initial A₅₄₀ values were 0.8, and minor differences in loading of the wells were compensated by representing the data as the fraction of the initial absorbance determination remaining at a given time. Mitochondrial protein concentrations were quantified spectrophotometrically (Micro BCA Protein Reagent Kit, Pierce, Rockford, IL, USA), with bovine serum albumin used as standard.

**Assay for NAD(P)H levels**

NAD(P)H fluorescence in isolated mitochondria (1 mg ml⁻¹ at 25 °C) were measured spectrofluorimetrically with excitation and emission wavelengths of 340 nm (slit 3 nm) and 460 nm (slit 5 nm) at room temperature. We therefore refer to NAD(P)H, indicating the signal derived from either NADH or NADPH or both. Mitochondria were resuspended in 125 mM KCl, 20 mM HEPES, 2 mM KH₂PO₄, 1 µM EGTA, 1 mM MgCl₂, 5 mM malate and 5 mM glutamate with the pH adjusted to 7.08 with KOH. Under these conditions, an increase in autofluorescence signal indicates an increase in the reduced state of the pyridine nucleotide, NAD(P)H, and a decrease in autofluorescence signal indicates an increased oxidation to NAD(P)⁺.

**Measurement of GSH**

Levels of GSH were determined by using monochlorobimane (mBCl) fluorescence. GSH is specifically conjugated with mBCl to form a fluorescent bimane-GSH adduct, in a reaction catalyzed by GSH S-transferases (Shrieve et al., 1988). The concentration of...
the bimane-GSH adduct increases during the initial 10–12 min period of this reaction with first order kinetics, before leveling off (Young et al., 1994). Fluorescence levels at 15 min were used as an indication of GSH content, as has been described previously (Shrieve et al., 1988; Nakamura et al., 2000). Culture medium was removed and cells were washed three times with 1 ml PBS (37 °C) and incubated for 30 min at 37 °C in 1 ml fresh PBS containing 80 μM mBCl. After incubation cells were washed twice with ice-cold PBS and scraped in 500 μl 0.2% Triton X-100 in PBS, centrifuged and 300 μl of the extract were used for GSH determination. Mitochondria were resuspended in 125 mM KCl, 20 mM HEPES, 2 mM KH2PO4, 1 μM EGTA, 1 mM MgCl2, 5 mM malate and 5 mM glutamate with the pH adjusted to 7.08 with KOH. Fluorescence was measured at an excitation wavelength of 340 nm and emission wavelength of 460 nm. Protein content was determined by the bicinchoninic acid method (Pierce).

**Determination enzyme activities**

All enzyme activities were determined at room temperature in mitochondria isolated suspensions using a microplate reader (BioRad). The activity of complex II–III (succinate-cytochrome c reductase) was determined following the method of King (1967). Complex IV (cytochrome c oxidase; E.C.1.9.3.1) activity was determined as described by Wharton and Tzagoloff (1967).

**Measurement of mitochondrial Ca2+ uptake**

Extramitochondrial free Ca2+ was monitored in the presence of isolated mitochondria using the hexapotassium salt of Calcium Green-5N (Molecular Probes) (Rajdev and Reynolds, 1993). Isolated mitochondria (1 mg ml−1) were resuspended in media containing 125 mM KCl, 2 mM K2HPO4, 1 mM MgCl2, 20 mM HEPES, 5 μM EGTA, 1 μM Calcium Green-5N and 20 mM succinate pH 7.0. Fluorescence was continuously monitored using a Perkin-Elmer LS-50B fluorescence spectrometer with the excitation at 506 nm and the emission at 531 nm (slit 2.5 nm).

**RESULTS**

**Effects of minocycline on GSH and NAD(P)H levels**

Cells are in constant equilibrium between oxidation and reduction reactions. GSH and NAD(P)H have been proposed to act as direct biological antioxidant agents, for which some cytoprotector compounds act increasing their levels (Kirsch and De Groot, 2001). Therefore, we were interested in analyzing whether minocycline could modify the cellular levels of these two agents. Treatments with minocycline (0.1–100 μM for 24 h) did not modify GSH or NAD(P)H levels in SH-SY5Y cells (data not shown). Further, we analyzed plausible modification of these two agents in isolated mitochondria. As Fig. 1 illustrates, minocycline (1–10 μM) did not significantly modify GSH and NADP(H) levels. Only, at the higher concentration tested, 100 μM, minocycline showed a depletion in reduced NADP(H) levels but not GSH (Fig. 1), perhaps because NADP(H) oxidation is very sensitive to mitochondrial membrane depolarization (Fig. 3). Consistently, the mitochondrial uncoupler, carbonyl cyanide 4-trifluoromethoxyphenyldrazone (FCCP) (1 μM, FCCP) depleted and rotenone, a mitochondrial respiratory chain complex I inhibitor (10 μM, Rot), increased the nucleotide signal (Fig. 1B).

A key step in cellular death programs associated to some pathological situations is PTP formation (Hirsch et al., 1998). In the next set of experiments, we monitored mitochondrial swelling by following 540 nm absorbance (A540) decrease, using CaCl2 and KO2 as inductors. Minocycline was only able to block Ca2+ (75 μM) but not KO2 (5 μM) induced mitochondrial swelling in a concentration-dependent manner. A concentration of up to 10 μM, minocycline did not significantly modify Ca2+ -induced mitochondrial swelling, but at 100 μM minocycline completely blocked Ca2+ -induced swelling (Fig. 2A). Nevertheless, minocycline, at any of the concentrations tested, did not inhibit KO2-induced mitochondrial swelling (Fig. 2B). To further analyze whether minocycline effect was a common effect of this antibiotic family, we tested the effect of tetracycline on mitochondrial swelling. As shown in Fig. 2,
Minocycline induces mitochondrial potential collapse

Alterations of mitochondria’s electric transmembranal potential ($\Delta\psi_m$) have been related to cell death processes (Prehn et al., 1996; Jordan et al., 2003b). In the next set of experiments, we studied whether minocycline was able to modify $\Delta\psi_m$ in isolated mitochondria. We monitored $\Delta\psi_m$ changes by measuring the release of the cationic membrane-permeant fluorescent probe TMRE pre-loaded into isolated mitochondria. Under these conditions, total fluorescence of the mitochondrial suspension will increase if the organelles depolarize. As shown in Fig. 3A, minocycline induced mitochondrial depolarization in a concentration-dependent manner. Concentrations higher than 10 $\mu$M induced a significant $\Delta\psi_m$ collapse. As shown in Fig. 3, tetracycline did not induce $\Delta\psi_m$ collapse at any of the concentrations tested (0.1–100 $\mu$M), and the addition of FCCP (1 $\mu$M) resulted in TMRE release from the mitochondria.

Minocycline blocks mitochondria calcium buffering capacity

As mitochondria participate in the regulation of cytoplasmic free Ca$^{2+}$ levels (Skulachev, 1999) and PTP is triggered by intramitochondrial Ca$^{2+}$ accumulation, we were interested to know whether or not minocycline was able to modify mitochondrial Ca$^{2+}$ buffering capacity. The maximal quantity of Ca$^{2+}$ that can be sequestered by mitochondria can be measured by monitoring the disappearance of extramitochondrial free Ca$^{2+}$ from media following the addition of known pulses of CaCl$_2$, using Calcium Green-5N fluorescence tracing. A fixed amount of CaCl$_2$ (4 nmol) was added repeatedly every 60 s until there was no further evidence of Ca$^{2+}$ uptake. Addition of Ca$^{2+}$ after this point was associated with a further increase in fluorescence indicating that Ca$^{2+}$ is not taken up by isolated mitochondria and reacts with the dye. Fig. 4 shows a typical fluorescence tracing of Ca$^{2+}$ uptake using isolated mitochondria. Untreated mitochondrial preparations were able to retain up to 31.4±2.8 nmol Ca$^{2+}$/mg mitochondrial protein. The presence of minocycline significantly reduced mitochondrial Ca$^{2+}$ buffering capacity in a concentration-dependent manner (Fig. 4), while 0.1 $\mu$M or 1 $\mu$M minocycline did not modify mitochondrial buffering capacity (31.3±2.6 and 30.6±2.7 nmol Ca$^{2+}$/mg mitochondrial protein respectively), at a concentration of 10 $\mu$M. The antibiotic decreased mitochondrial buffering capacity down to 28.0±3.3 nmol Ca$^{2+}$/mg mitochondrial protein ($P<0.05$). A higher minocycline concentration (100 $\mu$M) further decreased mitochondrial buffering capacity to 21.7±3.9 nmol Ca$^{2+}$/mg mitochondrial protein (Fig. 4). Addition of tetracycline (100 $\mu$M; Fig. 4) did not modify the mitochondrial Ca$^{2+}$ buffering capacity.

Effects of minocycline on mitochondrial respiratory chain complex enzymatic activity

The next set of experiments was performed to analyze the possible effects of minocycline on mitochondrial respira-

![Fig. 2. Minocycline prevents Ca$^{2+}$- but not KO$_2$-induced mitochondrial swelling. Changes in absorbance at 540 nm ($A_{540}$), indicating mitochondrial swelling, were followed, after addition of CaCl$_2$ (75 $\mu$M, panel A) and KO$_2$ (5 $\mu$M, panel B)-induced swelling. The effect of minocycline (0.1–100 $\mu$M), tetracycline (100 $\mu$M; dashed line), Ruthenium Red (RR, 5 $\mu$M) and cyclosporin A (CsA, 10 $\mu$M) was measured. Drugs were added 15 min before starting the experiments. Similar data were found in at least five different experiments. Data represent means±S.E.M. of nine experiments.

![Fig. 3. Minocycline induces $\Delta\psi_m$ collapse. $\Delta\psi_m$ Was measured by using TMRE. Minocycline (0.1–100 $\mu$M) or tetracycline (100 $\mu$M) was added. FCCP (1 $\mu$M) was used to ensure that the mitochondria could be depolarized. Data represent means±S.E.M. of six experiments.](image-url)
tory chain complex activity. Under our experimental conditions mitochondrial complex IV was not modified by the treatment with minocycline for 15 min at any of the doses tested (Fig. 5A). However, the complex II–II activity was modified by minocycline. Incubation of brain mitochondria (0.3 g protein) with 100 μM minocycline for 15 min did result in a significant loss in enzymatic activity compared with control mitochondrial suspension while lower concentrations (1 and 10 μM; 15 min) did not alter complex II–III activity (Fig. 5B).

Minocycline effects on cytotoxic models

The effect of minocycline on cell viability was examined in SH-SY5Y cells exposed to different mitochondrial toxins, including malonate and 6-hydroxydopamine (6-OHDA). We have previously shown that both drugs are able to induce cell death in SH-SY5Y cell cultures by mechanisms involving reactive oxygen species formation (Jordan et al., 2004; Fernandez-Gomez et al., 2005). As shown in Fig. 6, minocycline (100 μM) failed to afford cytoprotection to the cell cultures challenged with mitochondrial toxins. By contrast and in line with previous observations, 100 μM minocycline treatment resulted in a cytoprotective effect against staurosporine-induced cell death (Fig. 6).

DISCUSSION

In the present work we have focused our attention on brain mitochondrion as a plausible pharmacological target to explain minocycline’s neuroprotective actions. Minocycline, by inducing collapse of the mitochondrial potential decreased Ca2+ influx into the organelle and prevented mitochondrial swelling. On the other hand, our data exclude the possibility of a non-specific pathway involving increases in cellular antioxidant capacity, since minocycline did not affect NAD(P)H or GSH levels.

Mitochondrial swelling might be a consequence of PTP formation, which leads to a massive water influx into mitochondria and release of intramitochondrial proteins that might activate downstream apoptotic pathways (Hirsch et al., 1998). Thus, the cytoprotective effects of minocycline may rely on its ability to block mitochondrial swelling, as mitochondrial swelling is involved in several death pathways including those induced by 1-methyl-4-phenylpyridinium, staurosporine, veratridine, or N-methyl-D-aspartate (Jordan et al., 2002; Boada et al., 2000; Duan et al., 2003). In fact, drugs able to block PTP formation afford complete or partial neuroprotection against a broad type of insults.

Fig. 4. Minocycline inhibits mitochondrial Ca2+ uptake capacity. Maximal mitochondrial Ca2+ uptake by isolated mitochondria (1 mg/ml in 0.5 ml) was measured as described using Calcium Green-5N as an indicator of the free Ca2+ concentration in the medium. Minocycline (1–100 μM) or tetracycline (100 μM, dashed line) was added at the arrow. Pulses of 4 nmol CaCl2 (100 μM) were added every 60 s. Data are expressed as mean values obtained from one experiment performed in triplicate. Similar data were found in at least four different experiments.

Fig. 5. Effect of minocycline on mitochondrial respiratory chain complexWindow activity. Brain mitochondria suspensions were incubated in the absence (control) or presence of minocycline for 15 min at room temperature. Enzyme activities of complex IV (A) and complex II/III (B) were determined in the mitochondrial suspensions as described in Experimental Procedures. Values are expressed as percentages of control conditions. The mean±S.E.M. values from three different mitochondria preparations. ** P<0.01 versus vehicle conditions using ANOVA, Turkey’s test.
causing apoptotic death (Jordan et al., 2003b; Tatton and Chalmers-Redman, 1998).

Although previous works have shown that mitochondrial swelling by preventing the capacity of mitochondria to accumulate and retain large amounts of Ca^{2+} (Gunter et al., 1994; Nicholls and Akerman, 1982), Although sequestration of Ca^{2+} into mitochondria buffers potentially toxic Ca^{2+} loads, it may also produce deleterious effects (Stout et al., 1998; Castilho et al., 1999). So, increases in mitochondrial Ca^{2+} concentration accelerate electron transport (McCormack et al., 1990) and stimulate reactive oxygen species production (Castilho et al., 1999; Chacon and Acosta, 1991). We provide evidence, for the first time, that minocycline is able to block mitochondrial Ca^{2+} uptake. Consistent with these results, the inhibition of mitochondrial unipporter blocked cell death induced by different agents including curcumin (Bae et al., 2003) and 3-nitropropionic acid (Lee et al., 2002). So, our results are consistent with the hypothesis that Ca^{2+} needs to be taken up by mitochondria to induce their swelling and ultimate cell death. On the other hand, excessive cytoplasmic Ca^{2+} levels may be tolerated by the cells without affecting survival (Stout et al., 1998). Minocycline, by inhibiting Ca^{2+} mitochondrial uptake, might attenuate reactive oxygen species production that has been involved in several neuronal death models (Prehn et al., 1997; Jordan et al., 1995). However, we cannot exclude that minocycline could be blocking cell death by other mechanisms such as inhibition of metalloproteases or iNOS expression (Chen et al., 2000; Sadowski and Steinmeyer, 1998). Consistently, minocycline failed to afford cytoprotection to SH-SY5Y cultures exposed to mitochondrial toxins including malonate and 6-OHDA, which are known to induce cell death through a reactive oxygen species dependent pathway (Fernandez-Gomez et al., 2005; Glinka et al., 1998; Jordan et al., 2004). Moreover, in the line of these results, we...
(Goñi-Allo et al., 2005) and others (Cornet et al., 2004) have demonstrated that minocycline administration does not abrogate malonate-induced striatal lesions and increases motor alterations and striatal impairments induced by the mitochondrial complex II inhibitor 3-nitropropionic acid (Diguet et al., 2003, 2004).

Together with recent results, our data suggest an important role for mitochondria mediating minocycline cytoprotective effects. Hence, minocycline exerts cytoprotection by inducing Δψm dissipation and blocking both mitochondrial Ca2+ accumulation and subsequent PTP opening. Since, PTP might play a key role in the development of a number of neurological disorders including AD, PD and vascular dementia this plausible mechanism requires further attention in the investigation of new drugs used to treat neurodegenerative disorders.

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