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Minocycline Attenuates Iron Neurotoxicity in Cortical Cell Cultures

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Abstract

Iron neurotoxicity may contribute to the pathogenesis of intracerebral hemorrhage (ICH). The tetracycline derivative minocycline is protective in ICH models, due putatively to inhibition of microglial activation. Although minocycline also chelates iron, its effect on iron neurotoxicity has not been reported, and was examined in this study. Cortical cultures treated with 10 μ M ferrous sulfate for 24h sustained loss of most neurons and an increase in malondialdehyde. Minocycline prevented this injury, with near-complete protection at 30 μ M. Two other inhibitors of microglial activation, doxycycline and macrophage/microglia inhibitory factor, were ineffective. Oxidation of isolated culture membranes by iron was also inhibited by minocycline. Consistent with prior observations, minocycline chelated iron in a siderophore colorimetric assay; at concentrations less than 100 μ M, its activity exceeded that of deferoxamine. These results suggest that attenuation of iron neurotoxicity may contribute to the beneficial effect of minocycline in hemorrhagic stroke and other CNS injury models.

Keywords

cell culture; free radical; hemoglobin toxicity; inflammation; intracerebral hemorrhage; mouse; oxidative stress; stroke

Introduction

Tissue iron is increased within one day in the vicinity of an experimental intracerebral hemorrhage [1], and persists for at least three months [2]. A growing body of experimental evidence suggests that this iron may contribute to cell injury. Reducing heme breakdown and iron release with heme oxygenase (HO) inhibitors or HO gene knockout is beneficial in animal models [3,4], and protects neurons from hemoglobin toxicity in cell culture [5]. More specifically, post-hemorrhage treatment with the iron chelator deferoxamine reduces edema, oxidative injury markers, and neuronal loss, and also improves behavioral outcome [2,6].

A peri-hematoma inflammatory infiltrate, consisting of leukocytes and activated microglia, is observed within 24 hours of experimental intracerebral hemorrhage and also may contribute to secondary injury [7]. This inflammation hypothesis has recently been tested using the tetracycline derivative minocycline [8,9,10], which inhibits microglial activation and is

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beneficial in several ischemic stroke models [11], presumably due to its anti-inflammatory effect. However, as described by Grenier et al. [12], minocycline has strong iron-chelating activity, which has been of some clinical relevance. Its absorption after oral administration is greatly reduced when administered with iron or calcium supplements, consistent with its affinity for metal cations [13]. Skin hyperpigmentation, an adverse effect of long-term minocycline therapy, is a consequence of dermal precipitation of a minocycline-iron complex [14]. By depriving bacteria of an essential nutrient, iron chelation may also account in part for its antibiotic effect [12], although evidence supporting the physiologic relevance of this mechanism is limited.

The redox activity of iron is altered in a highly variable manner by chelator binding. Catalysis of hydroxyl radical generation via the Fenton reaction requires at least one of six iron coordination sites to be available, or occupied by a low-affinity ligand such as water [15]. A chelator that occupies fewer than six sites may not prevent oxidative injury, and may even increase it if it mobilizes iron from protein binding sites in a redox-active state [16]. Despite the recent interest in minocycline therapy for hemorrhagic stroke, its effect on iron-mediated oxidative neuronal injury has never been reported. The present study tested that hypothesis that minocycline attenuates the oxidative neurotoxicity of iron in primary cortical cell cultures.

Materials and Methods

Cortical cell cultures

All procedures on animals were reviewed and approved by the Thomas Jefferson University Institutional Animal Care and Use Committee (IACUC). Mixed cortical cell cultures, containing both neurons and glia were prepared from fetal B6129 mice (gestational age 13- to 15-days), as previously described [5]. The dissociated cell suspension was plated on glial feeder cultures (>90% GFAP+, approximately 2% microglia by tomato lectin staining [17]) in 24-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ), using a plating density of 0.12 hemisphere in 0.4 ml medium per well. Plating medium contained Minimal Essential Medium (MEM, Invitrogen, Carlsbad, CA), 5% equine serum (Hyclone, Logan, UT), 5% fetal bovine serum (Hyclone), 23 mM glucose, and 2 mM glutamine. On day 5-6 in vitro, two-thirds of the culture medium was aspirated and replaced with feeding medium, which was similar to plating medium except that it contained 10% equine serum and no fetal bovine serum. This procedure was repeated on day 9 or 10 and then daily beginning on day 11. Glial feeder cultures were prepared from postnatal day 1-3 mice, using plating medium similar to that described above, except that it was supplemented with 10 ng/ml epidermal growth factor (Sigma, St. Louis, MO, Product #E1247), 10% equine serum and 10% fetal bovine serum. Glial culture medium was partially changed twice weekly.

Iron exposure

Experiments were conducted at 12-16 days in vitro. At this time point, neurons are readily distinguished from glial cells in dissociated cultures by their phase-bright cell bodies and processes. Ferrous sulfate (FeSO_4) was used exclusively since heme breakdown by the heme oxygenase enzymes, as occurs after hemorrhagic CNS injuries, releases ferrous iron [18]. Exposure to FeSO_4 alone or with drugs was carried out in MEM containing 10 mM glucose (MEM10) at 37°C in a 5% CO_2 atmosphere. Control cultures were included in each experiment and were subjected to medium exchanges only. Minocycline and doxycycline (both purchased from Sigma) were prepared as 10 mM stock solutions in cell culture-grade water, and were then diluted in MEM10. Macrophage/microglia inhibitory factor (MIF, Thr-Lys-Pro) was purchased from American Peptide Company, Sunnyvale, CA. A 20 mM stock solution was prepared in cell culture-grade water, aliquoted, and stored at -20°C until used. The FeSO_4 concentration used in these experiments was determined from prior concentration-toxicity

studies, which demonstrated that 24 h treatment with 10 μM FeSO_4 killed almost all neurons, without significantly injuring glial cells in this culture system [5,19].

Cell injury assessment

Cell death was quantified by lactate dehydrogenase (LDH) release assay, which correlates well with cell counts after trypan blue staining in mixed cortical cultures [20], as previously described [21]. In order to compare results from experiments using cultures prepared in different platings, which have slightly different neuronal densities, all LDH values were normalized to those in cultures treated with 10 μM FeSO_4 . The mean LDH activity of control sister cultures treated with medium exchange only was subtracted from all values to quantify the signal that was specific to the cytotoxic exposure, following the protocol of Koh and Choi [20].

Malondialdehyde is a sensitive marker of iron-mediated neuronal injury in this system [21]. After sampling for LDH assay, cultures were harvested, proteins were precipitated with 4.5% trichloroacetic acid, and malondialdehyde was quantified as previously described [21]. Protein was assayed by the BCA method (Pierce, Rockford, IL); malondialdehyde values were expressed as nanomoles/milligram protein.

In vitro membrane oxidation assay

Iron-mediated lipid oxidation was assessed in a cell-free system using the method of Sadrzadeh et al. [22]. Glial cultures were used as a source of membranes for this assay due to their greater availability compared with post-mitotic primary neurons. Glial cells were harvested by scraping in physiologic saline, and were then sonicated. Membranes from two culture wells per sample (approximately 50 μg protein) were collected by centrifugation (8000 $\times$ g, 5 min). After the supernatant was discarded, FeSO_4 (50 μM) was added in 375 μL 50 mM Tris buffer (pH 7.4), alone or with tetracyclines. Samples were incubated at room temperature for 1 hour. Malondialdehyde was then quantified as previously described [21].

Iron chelation assay

The universal siderophore assay of Schwyn and Neilands, as modified by Grenier et al. [12], was used to assess iron chelating activity. The indicator for this assay is a chrome azurol S/ Fe^{3+} /hexadecyltrimethylammonium bromide (HDTMA) complex that undergoes color change from blue to orange when iron is extracted by a strong chelator. The assay solution contained 150 μM chrome azurol S, 600 μM HDTMA, and 15 μM FeCl_3 in piperazine buffer (pH 5.6). Dilutions of test compounds were incubated in the assay solution (total volume 0.5 ml) for 1 hour at room temperature; absorbance at 630 nm was then quantified.

Immunoblotting

Cells were lysed in buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, 0.1 % sodium dodecyl sulfate, and 0.1 % Triton X-100. After sonication and centrifugation, the protein concentration of the supernatant was quantified (BCA method, Pierce, Rockford, IL). Samples (20 μg protein each) were then boiled in buffer containing Tris-Cl 60 mM, β -mercaptoethanol 5%, sodium dodecyl sulfate 2%, glycerol 10%, and bromophenol blue 0.05% for 3 min. Proteins were separated on 12 % SDS polyacrylamide gels at 80-100 V. The gels were then soaked in transfer buffer (glycine 39mM, Tris-CL 48mM, SDS 0.037% and methanol 20%), and were transferred to polyvinylidene difluoride membranes with a semidry transfer apparatus for 40 minutes at 20V. Completion of transfer was assessed by observing the transfer of the pre-stained protein marker (Bio-Rad Laboratories, Hercules, CA, Cat. No. 161-0375). After blocking with 5% nonfat dry milk, membranes were exposed overnight at 4°C to rabbit anti-horse spleen ferritin (Sigma, 1:250 dilution) combined with

rabbit anti-actin antibody (1/1500, Sigma) as a gel loading control. After washing, they were then exposed to HRP-conjugated goat anti-rabbit IgG antibody (1:3500) for 1h at room temperature. Immunoreactive proteins were visualized using Super Signal West Femto Reagent (Pierce) and Kodak Gel Logic 2200.

Statistical Analysis

Data were analyzed with one-way analysis of variance. Differences between groups were then assessed with the Bonferroni Multiple Comparisons test.

Results

Minocycline protects cortical neurons from iron

Consistent with prior observations using this model [5,23], cultures treated with 10 μM FeSO_4 for 24 hours sustained widespread neuronal death, without injury to the feeder glial monolayer. Concomitant treatment with equimolar minocycline reduced neuronal death, as measured by LDH release assay, by approximately one-third at 24 hours (Fig 1A). Increasing the minocycline concentration to 30 μM reduced LDH release by $87.4 \pm 1.8\%$, which was similar to the neuroprotection provided by 30 μM deferoxamine ($91.0 \pm 4.4\%$ reduction). Malondialdehyde (MDA) was increased approximately twenty-fold by iron treatment (Fig 1B). Changes in mean MDA values associated with minocycline treatment correlated with injury reduction as assessed by LDH release.

Other microglial activation inhibitors have no effect on iron neurotoxicity

Doxycycline and the peptide Thr-Lys-Pro (microglia inhibitory factor) inhibit microglial activation in primary cultures. At concentrations previously determined to be effective in vitro [24,25], neither significantly reduced neuronal death or malondialdehyde levels in iron-treated cells (Table 1).

Minocycline prevents lipid oxidation in isolated cell membranes

In order to determine if minocycline directly inhibited iron-catalyzed lipid oxidation, its effect on malondialdehyde production in isolated culture membranes was quantified. In samples treated with 50 μM FeSO_4 for 1h, malondialdehyde was increased 4.5-fold compared with control samples incubated with Tris buffer only (Fig. 2). Equimolar minocycline reduced malondialdehyde to control levels, while equimolar doxycycline had no effect.

Minocycline increases ferritin expression in cultures treated with iron

Ferritin expression was increased tenfold in cultures treated with 10 μM FeSO_4 for 24 h, compared with sham-washed controls (Fig. 3). In cultures treated with FeSO_4 plus minocycline for 24h, the mean ferritin level was 17-fold higher than controls, and was significantly increased compared with the mean value in cultures treated with FeSO_4 only. Minocycline treatment alone had no effect on ferritin expression (relative density values: 0.179 ± 0.041 v. 0.183 ± 0.036 for sham-wash). Doxycycline and deferoxamine did not alter ferritin induction by iron.

Comparison of chelating activities of minocycline and deferoxamine

Incubation with minocycline produced a rapid decrease in absorbance at 630 nm of the chrome azurol S-based universal siderophore assay solution (Fig. 4), in agreement with prior observations [12]. Surprisingly, at concentrations below 100 μM , its iron chelating activity as assessed by this assay exceeded that of deferoxamine. Deferoxamine had greater activity at concentrations greater than 100 μM .

Discussion

These results demonstrate that minocycline potently inhibits iron neurotoxicity in cortical cell cultures and iron-catalyzed lipid oxidation in isolated cell membranes. Furthermore, two other inhibitors of microglial activation, doxycycline and macrophage/microglia inhibitory factor (MIF) [24,25], provide no cytoprotection. Since iron neurotoxicity contributes to neuronal loss after experimental ICH [2,6], these data suggest that any therapeutic benefit of minocycline may be due at least in part to direct attenuation of this injury mechanism, rather than to a specific anti-inflammatory effect.

Minocycline concentrations in the low nanomolar range are sufficient to prevent microglial activation [26], which may be mediated by inhibition of poly (ADP-ribose) polymerase-1 (PARP-1) [27]. In contrast, low micromolar concentrations were required for neuroprotection in the present study. The effect of minocycline in ICH models resulted from treatment with 45-90 mg/kg/day [8,9,10]. Published pharmacokinetic data suggest that these high doses are quite likely to produce minocycline levels in the micromolar range. In a rat model of ischemic stroke, peak serum concentrations after intravenous injection of 3 or 10 mg/kg minocycline were 7 μ M and 26 μ M, respectively. Although brain levels were not measured, minocycline readily crosses the blood-brain barrier, and steady state brain concentrations are only slightly lower than those in serum. In uninjured dogs, Barza et al. [28] reported a venous serum concentration of 3.4 μ g/ml (6.9 μ M) and a brain concentration of 2.9 μ g/ml (5.9 μ M) after administration of a 5 mg/kg minocycline loading dose followed by continuous infusion of 1 mg/kg for three hours. Further studies testing minocycline at doses that are likely to produce low nanomolar brain concentrations may help to clarify its mechanism of action.

Doxycycline has also been reported to have iron chelating activity [12], but it provided no cytoprotection at effective minocycline concentrations. Two factors may account for this discrepancy. First, minocycline is considerably more lipid-soluble, with a chloroform-water partition coefficient of 30 at pH 7.4, versus 0.48 for doxycycline and 0.09 for tetracycline [28]. It may therefore accumulate at a higher concentration in cell compartments that are vulnerable to iron-catalyzed lipid oxidation, such as the hydrophobic interior of cell membranes. Second, minocycline also has a direct antioxidant effect, scavenging free radicals in the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay with an IC₅₀ in of 3.1 μ M, compared with 783 μ M for doxycycline and 985 μ M for tetracycline [29].

Contrary to our expectation that iron chelators would reduce ferritin induction by ferrous sulfate, deferoxamine and doxycycline had no effect, while minocycline increased ferritin levels. Ferritin expression in response to iron loading is regulated at the translational level by iron regulatory proteins (IRP's), which bind to an iron responsive element in the 5' untranslated region of ferritin mRNA and inhibit translation [30]. In this culture system and in the intact CNS, IRP2 is the primary regulator [31,32]; it undergoes ubiquitination and proteasomal degradation in iron-replete cells, enabling translation [33]. This process is initiated by iron binding at cysteine residues [34], and is apparently not significantly impaired by a threefold molar excess of deferoxamine or doxycycline. Moreover, it appears to be facilitated by minocycline, which may be due to increased iron delivery by the lipophilic minocycline-iron complex, although other mechanisms related to the efficiency of iron transfer from minocycline to IRP2 cannot be excluded. Since ferritin attenuates iron-mediated neuronal injury [35], its increased expression may also contribute to the neuroprotective effect of minocycline.

Injury to cells surrounding a hematoma may be mediated by a variety of mechanisms, including mechanical injury from clot expansion and retraction, toxicity of hemoglobin and thrombin, and inflammation [36]. It is unlikely that any single drug that specifically targets one mechanism will provide an optimal outcome. Minocycline appears to have multiple beneficial

actions. Since it has been proven to be safe for human administration, it clearly seems worthy of further investigation. However, at doses that are likely to produce micromolar concentrations in the CNS, it has very limited utility as a pharmacologic probe in mechanistic studies. Its efficacy in hemorrhagic stroke models should not be considered compelling evidence that microglial activation plays a role in the pathogenesis of ICH.

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Figure 1.

Minocycline protects cortical neurons from iron. Bars represent mean ($\pm$ S.E.M, n = 6-12/condition) percentage lactate dehydrogenase (LDH) release (A) and malondialdehyde (MDA) levels (B) after 24 hour treatment with 10 μ M ferrous sulfate (Fe) alone or with indicated concentrations (μ M) of minocycline (MN). LDH values are scaled to that in cultures treated with 10 μ M ferrous sulfate, which produces near-100% neuronal death, after subtraction of mean LDH in sister cultures subjected to wash and medium exchange only (SW, sham wash), in order to yield the LDH signal specific for neuronal injury. ***P<0.001. v. ferrous sulfate alone, Bonferroni multiple comparisons test.

Figure 2.

Effect of minocycline and doxycycline on lipid peroxidation in isolated culture membranes. Malondialdehyde (mean $\pm$ S.E.M, $n = 8$ /condition) in membranes incubated for 30 minutes with 50 μ M ferrous sulfate (Fe) alone or with indicated concentrations (μ M) of minocycline (MN) or doxycycline (DX). Control samples were incubated in Tris buffer alone. *** $P < 0.001$. v. ferrous sulfate alone, Bonferroni multiple comparisons test.

Figure 3.

Effect of minocycline, doxycycline, and deferoxamine on ferritin induction by ferrous sulfate. Bars represent mean ferritin band density ($\pm$ S.E.M, $n = 6-9$ /condition) in mixed neuron-glia cultures harvested after 24 hour incubation with ferrous sulfate (Fe) 10 μ M alone or with 30 μ M minocycline (MN), doxycycline (DX), or deferoxamine (DFO). Sham wash (SW) cultures were subjected to washes and medium exchange only. Representative immunoblot was immunostained with anti-horse spleen ferritin and anti-actin (gel loading control). ** $P < 0.01$. v. ferrous sulfate alone.

Figure 4.

Comparison of chelating activities of minocycline and deferoxamine. Absorbance (630 nm, mean $\pm$ S.E.M., $n = 5$ /condition) of chrome azurol S/ Fe^{3+} /hexadecyltrimethylammonium bromide (HDTMA) complex after 1 hour incubation with indicated concentrations (μM) of minocycline or deferoxamine, scaled to absorbance of samples incubated with water vehicle only (=1.0). * $P < 0.05$, *** $P < 0.001$. v. minocycline absorbance.

Table 1

Effect of doxycycline and macrophage/microglia inhibitory factor on iron neurotoxicity

	LDH	MDA
Sham Wash	0.0 ± 4.5	0.4 ± 0.1
Fe 10	100.0 ± 4.4	10.2 ± 0.9
Fe10+DX10	87.1 ± 3.2	7.7 ± 1.7
Fe10+DX30	88.8 ± 4.2	8.2 ± 1.8
Fe10+MIF500	82.9 ± 9.5	9.2 ± 0.5

Percentage LDH release and culture malondialdehyde (MDA) content (nmol/mg protein, mean ± S.E.M, n = 6-12/condition) in cultures after 24 hour treatment with 10 µM ferrous sulfate (Fe) alone or with indicated concentrations (µM) of doxycycline (DX) or macrophage/microglia inhibitory factor (MIF).