Chlortetracycline and Demeclocycline Inhibit Calpains and Protect Mouse Neurons against Glutamate Toxicity and Cerebral Ischemia*

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Minocycline is a potent neuroprotective tetracycline in animal models of cerebral ischemia. We examined the protective properties of chlortetracycline (CTC) and demeclocycline (DMC) and showed that these two tetracyclines were also potent neuroprotective against glutamate-induced neuronal death in vitro and cerebral ischemia in vivo. However, CTC and DMC appeared to confer neuroprotection through a unique mechanism compared with minocycline. Rather than inhibiting microglial activation and caspase, CTC and DMC suppressed calpain activities. In addition, CTC and DMC only weakly antagonized 

N-methyl-D-aspartate (NMDA) receptor activities causing 16 and 14%, respectively, inhibition of NMDA-activated whole cell currents and partially blocked NMDA-induced Ca2+ influx, commonly regarded as the major trigger of neuronal death. In vitro and in vivo experiments demonstrated that the two compounds selectively inhibited the activities of calpain I and II activated following glutamate treatment and cerebral ischemia. In contrast, minocycline did not significantly inhibit calpain activity. Taken together, these results suggested that CTC and DMC provide neuroprotection through suppression of a rise in intracellular Ca2+ and inhibition of calpains.

Stroke is one of the most common life-threatening neurological diseases. Despite significant advances in the understanding of the molecular events following cerebral ischemia, there are still no potent neuroprotective therapies against stroke-induced brain damage (1–3). The ischemia-induced excessive release of neurotransmitter glutamate causes excitotoxicity, which is believed to be the major cause of toxicity to neurons (1, 4, 5). Glutamate overactivates NMDA2 receptors, causing increased intracellular Ca2+ influx leading to the accumulation of toxic levels of intracellular calcium ions (4, 5). Elevation in intracellular Ca2+-dependent proteases, such as calpains,
Experimental Procedures

Materials

All chemicals and reagents, unless stated otherwise, were purchased from Sigma (Burlington, Ontario, Canada). 5-(6)-Carboxyfluorescein diacetate (CFDA) was purchased from Molecular Probes (Eugene, OR). DAKO® fluorescent mounting medium was from Dako Corp. (Carpinteria, CA). Hoechst 33258 was from Aldrich Chemicals (Mississauga, Ontario, Canada). Protein size markers were obtained from Bio-Rad (Mississauga, Ontario, Canada). Antibodies to spectrin breakdown products were gifts from Dr. J. Durkin as described previously (11), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Advanced Immunochemicals (Long Beach, CA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG and donkey anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Detection of Western blots utilized ECL-Plus reagents from Amersham Biosciences. Minocycline hydrochloride, CTC hydrochloride, and DMC hydrochloride were purchased from Fluka Chemie (Burlington, Ontario, Canada).

Primary Cultures of Cerebellar Granule Neurons (CGNs)

Primary cultures of mouse (C57/B6) CGNs were prepared from 6- to 9-day-old postnatal mice as described previously (34, 35). Briefly, cerebella were explanted and cleaned free of meninges. Mechanical and enzymatic dissociation in a 0.025% w/v trypsin solution for 25 min followed. A trypsin inhibitor was then added to block the enzyme, and 0.05% w/v DNase was added to break DNAs from dead cells. A series of trituration and mild centrifugation steps were included to disperse the debris prior to plating in Eagle’s minimum essential medium containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 10 mM HEPES, 3 mM glucose, pH 7.4. The perfusion solution also contained 1 μM tetrodotoxin, 30 μM glycine, and 1 μM strychnine. Patch pipettes (2–4 MΩ) were constructed from 1.5 mm outer diameter/1.0 mm inner diameter Pyrex 7740 glass (Corning, Big Flats, MN). A modified DAD-12 perfusion system (ALA Scientific Instruments, Westbury, NY) was used to rapidly apply NMDA (2-s duration) followed by co-application of NMDA and the test compound (5-s duration). The pipette solution contained 140 mM CsCl, 1.1 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP at pH 7.2. Whole-cell currents were acquired using an Axopatch 1-D amplifier equipped with a CV-4 head stage with a 1 GΩ feedback resistor (Axon Instruments, Foster City, CA). Voltage command and current acquisition were accomplished using a Digidata 1200 interface and pClamp 6.0 software (Axon Inst). Neurons were held at a membrane potential of −60 mV. The fractional block of NMDA-evoked currents was calculated according to the formula: B = 1 – Ig/I, where I is the steady-state current evoked by NMDA, and Ig is the current evoked by NMDA in the presence of the test compound at the end of the co-application.

Intracellular Ca²⁺ Measurement

Fluo-4 Measurement—Intracellular calcium concentration was measured as described previously (37). Briefly, culture medium in the 24-well plate was replaced with a calcium sensitive dye Fluo-4 (4.5 μM) in a balanced salt solution. After 30-min incubation, the dye was removed and cells were incubated with the original medium with or without the compound at 37 °C for 15 min. Fluorescent intensities were quantified using a Cytofluor™ 2350 Fluorescence Measurement System (Millipore) at λem = 485 nm and λex = 530 nm. NMDA (50 μM) was then added to the wells and changes in fluorescence were recorded after 5, 10, 20, 30, and 40 min. The fold increase in Ca²⁺ was calculated by subtracting the initial reading from each reading divided by the initial reading.

Ratiometric Measurement of [Ca²⁺] / Using Fura-2—To quantitatively determine the effect of CTC or DMC on glutamate-induced changes in intracellular Ca²⁺ ([Ca²⁺]) level, ratiometric measurement of [Ca²⁺], was performed using fura-2 AM. Briefly, mouse CGNs at 7 days in vitro on glass coverslips were loaded with 5 μM fura-2 AM (Molecular Probes) plus 0.02% pluronic (Molecular Probes) for 30 min at 37 °C. After rinsing with PSS Mg²⁺-free buffer containing 2 mM HEPES (pH 7.2), 140 mM NaCl, 5 mM KCl, 2.3 mM CaCl₂, and 10 mM glucose, and stabilized in the same buffer for 5 min, fura-2 intensities were measured using a Northern Eclipse Digital Ratio Image System (Empix, Mississauga, Ontario, Canada) with Axiovert 200 camera and light source (Zeiss, Thornwood, NY). Fura-2 fluorescence was measured at 510 nm emission with 340/380 nm dual excitation selected by a DG-5 system (Sutter Instrument Co., Novato, CA). Changes in [Ca²⁺], concentration was measured by converting the 340/380 ratio of fura-2 fluorescence (after correction for background) to approximate [Ca²⁺], using the method as described by Grynkiewicz et al. (38) and Young et al. (39). The 340– to 380 nm fluorescence ratio (R340/380) for 20 cells in one field of each coverslip was averaged. The minimal and maximal fluorescence ratios (Rmin and Rmax) were obtained from a sample set of CGNs using 5 μM ionomycin plus 6 mM EGTA and by 10 mM CaCl₂, respectively. The Kᵢ for fura-2 was assumed to be 224 nM as described by Young et al. (39). The basal level of [Ca²⁺], was recorded for 10 s, followed by the application of the compound dissolved in PSS buffer, which also contains 10 μM glycine for another 40 s, and finally glutamate (50 μM) was applied, and the recording was continued for another 200 s. All measurements were repeated for at least three times. The data were analyzed using Microsoft Excel.
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Animal Ischemia Surgery

All procedures using animals were approved by the Institute of Behavioral Science Animal Care Committee following the guidelines established by the Canadian Council on Animal Care. C57B/6 mice (20–23 g) were obtained from Charles River and bred locally. Under temporary isofluorane anesthesia, mice were subjected to MCAO using an intraluminal filament as previously described (40, 41). After 1 h of MCAO, the filament was withdrawn, blood flow restored to normal by laser Doppler flowmetry, and wounds sutured. Mice were injected with CTC or DMC intraperitoneal at 90 mg/kg body weight 4 h before ischemia, followed by injection twice per day. The control groups included no treatment or vehicle in which animals were injected with the same volume of saline. Brains were removed after 24-h reperfusion, and the brain infarction was measured as described as follows.

Infarct Size Measurement

Infarct size was measured by a colorometric staining method using 2,3,5-triphenyltetrazolium chloride (TTC) as described previously (40, 41). Briefly, brains were dissected out and cut into four 2-mm-thick coronal slices, which were stained with 5 ml of 2% TTC for 90 min at 37 °C. Afterward, the tissue was rinsed with saline and subsequently exposed to a mixture of ethanol/dimethyl sulfoxide (1:1), which was to solubilize the formazan product. After 24-h incubation in the dark, the red solvent extracts were diluted 1:20 with fresh ethanol/Me2SO solvent in three tubes and placed in cuvettes. Absorbance was measured at 485 nm in a spectrophotometer and the values were averaged. Percentage loss in brain TTC staining in the ischemic side of the brain was compared with the contralateral side of the brain of the same animal using the following equation: % loss = (1 – (absorbance of ischemic hemisphere/absorbance of contralateral hemisphere)) × 100).

Neurological Scores

An expanded six-point scale was modified based on previous reports (40–42) and used for the present investigation. Behavioral assessments were made at 0 and 24 h after reperfusion by an individual blinded to the treatment of the mice. The neurological deficits were scored as follows: 0, normal; 1, mild turning behavior with or without inconsistent curling when picked up by tail, < 50% attempts to curl to the contralateral side; 2, mild consistent curling, > 50% attempts to curl to contralateral side; 3, strong and immediate consistent curling, mouse holds curled position for more than 1–2 s, the nose of the mouse almost reaches tail; 4, severe curling progressing into barreling, loss of walking or righting reflex; 5, comatose or moribund. At least eight mice per group were evaluated for each compound and scores were averaged for statistical analysis.

In Vitro Measurement of Calpain Activity

Calpain activity was measured using a calpain activity assay kit (Calbiochem, Mississauga, Ontario, Canada) following the manufacturer’s instructions. The assay is based on fluorometric detection of cleavage of calpain substrate Ac-Leu-Leu-Tyr-AFC using a Cytofluor™ 2350 Fluorescence Measurement System (Millipore). The cleavage resulted in the release of AFC that can be measured in a fluorometer. Briefly, constitutive calpain I or II (0.1 unit/ml) (purchased from Calbiochem) was activated by 500 μM Ca2+ and was mixed with CTC (150 μM), DMC (150 μM), minocycline (150 μM), or calpain inhibitors ALLN or calpastatin (10 μM each) and 5 μl of calpain substrate to a final volume of 100 μl. The mixture was incubated at 37 °C for 1 h in the dark. The cleavage of the substrate resulted in the release of AFC that can be detected by the Cytofluor at λex = 400 nm and λem = 505 nm.

Western Blotting

Protein at 10 μg was electrophoresed in a 7% SDS mini gel and then electroblotted onto a nitrocellulose membrane in transfer buffer (39 mM glycine, 48 mM Tris base, and 20% methanol) as described previously (34). The membrane was then probed with a polyclonal antibody selective to calpain cleaved fragment of brain spectrin at 4 °C overnight. After washing with 0.01 M phosphate-buffered saline, horseradish peroxidase-conjugated secondary antibody was applied to the membrane for 1 h at room temperature. Enhanced chemiluminescence detection of the target protein was performed using a Lumiglo substrate kit (KP Laboratories, Gaithersburg, MD) and x-ray film.

Data Analysis

Data were analyzed using Microsoft Excel and Prism. Statistical significance was determined by Student’s t test, and the significant group was determined using further post hoc Tuckey’s test. p < 0.05 was considered statistically significant.

RESULTS

CTC and DMC Protect Cultured CGNs against Glutamate Toxicity—The neuroprotective effects of CTC, DMC, and minocycline against glutamate-mediated excitotoxicity in cultured mouse primary CGNs were examined and quantified using the CFDA assay. All three compounds showed potent neuroprotection against glutamate-mediated toxicity to CGNs in a dose- and time-dependent manner (Fig. 1, A–C). More than 85% of the CGNs were protected by these two compounds at doses ranging between 80 and 150 μM. This protection lasted up to 8 h following glutamate treatment when more than 50% of the control CGNs was killed by glutamate. The appearance of dead neurons was visualized by Hoechst staining of the nuclei (Fig. 1C). CTC and DMC were not toxic to CGNs at the ranges of doses tested (data not shown).

CTC and DMC Reduce MCAO-induced Brain Damage—Since minocycline has been shown to provide neuroprotection against cerebral ischemia, we examined the neuroprotective effect of CTC and DMC in a mouse model of focal ischemia with 1 h MCAO followed by 24-h reperfusion. Each compound was administered by intraperitoneal injection 4 h prior to MCAO at 90 mg/kg and followed by two more injections (8 and 16 h following reperfusion) at 45 mg/kg. Animals were then killed to remove the brain for analysis as described under “Experimental Procedures.” Both compounds significantly reduced the infarct size in the cerebral cortex by almost 50% in comparison with the non-treated ischemic control and vehicle-treated brain (p < 0.05, Fig. 2, A and B). Coronal sections of the brain slices numbered as 1–4 were shown in Fig. 2, B–D. Most of the infarctions occurred in the first two brain slices in the cerebral cortex and striatum as indicated by the arrows in Fig. 2B. The infarction was significantly reduced in the same areas in brains treated with CTC (Fig. 2C) or DMC (Fig. 2D).

The protective effects of these compounds were also confirmed by the improvement of the neurological behavior of the compound-treated ischemic mice. Using the six point valuation system as described in the “Experimental Procedures” section, the scores of the neurological behavior of ischemic animals were compared with those of vehicle-treated or ischemic animals 0.5 h after surgery and after 24-h reperfusion. As shown in Fig. 2E, mice treated with the two compounds showed significant improvement after 24 h of reperfusion (p < 0.05) compared with the vehicle-treated or ischemic animals, demonstrating that CTC and DMC reduced MCAO-induced neurological deficits.
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FIGURE 1. CTC and DMC are neuroprotective in vitro. A and B, cultured CGNs were treated with 50 μM glutamate with or without prior treatment with the indicated compound at the dose and duration of exposure indicated. A shows the dose response of compounds after 6-h incubation with glutamate, and B shows the time course response with 150 μM CTC and DMC or 10 μM minocycline to glutamate treatment. Neuronal viability was determined by the CFDA assay as described under “Experimental Procedures.” Data represent the mean ± S.E. of at least five independent experiments. ** indicates statistical significant difference (p < 0.001). C–E, treated CGNs were also fixed with 4% formaldehyde and nuclei stained with Hoechst 33258. Representative morphologies of neurons were taken by a digital camera and presented in C–E. Arrows indicate glutamate-induced death of CGNs, while arrowheads show live CGNs. Scale bar = 80 μm.

FIGURE 2. CTC and DMC reduced cerebral infarction and improved neurological behavior in MCAO mice. Mice at 20–23 g body weight were subjected MCAO for 1 h and reperfusion for 24 h as described in the “Experimental Procedures” section. A, TTC extraction and quantification were performed as described under “Experimental Procedures,” and the percent decrease of TTC staining of at least eight groups of mice was averaged and presented in A (mean ± S.E.). B–D are repetitive images of coronal sections of brains from MCAO mouse (B), CTC-treated MCAO mouse (C), and DMC-treated MCAO mouse (D). The numbers 1–4 in B–D indicate the first to the last slice of the MCAO brain and arrows indicate ischemic infarction (white-colored region on the brain slice). E, the respective scores of neurological behavior of each mouse were plotted and presented. * indicates statistical significance (p < 0.05) by Student’s t test.

CTC and DMC Weakly Antagonize NMDA Receptor Activity and Suppress the Rise in Intracellular Ca2+—To understand the mechanisms of neuroprotection conferred by CTC and DMC, we examined whether these two compounds blocked calcium entry through the NMDA receptor, which has been implicated in mediating glutamate-induced excitotoxicity. Both compounds at 150 μM showed weak, but rapid, antagonism to 50 μM NMDA-induced currents (Fig. 3A). A 5 s co-application of NMDA plus 150 μM CTC resulted in a 14 ± 1% (n = 5) reduction in NMDA-induced current. A 5 s co-application of NMDA plus 150 μM DMC produced a 16 ± 2% (n = 5) reduction in NMDA-induced currents.

Since NMDA activation induces intracellular Ca2+ influx, we next tested whether these two compounds affect glutamate and NMDA-induced intracellular Ca2+ levels. As shown in Fig. 3B, NMDA receptor-mediated intracellular calcium influx increased immediately after the addition of glutamate. The addition of the two compounds partially blocked [Ca2+]i influx, but the [Ca2+]i level eventually increased to the same level as that of NMDA-treated CGNs after 40 min (Fig. 3C). Minocycline also exhibited a similar level of blockade of Ca2+ influx as compared with those from CTC and DMC at the 10- and 20-min time points (Fig. 3C, p < 0.05 compared with NMDA-mediated Ca2+ rise). On the other hand, MK-801, an antagonist to NMDA receptor, completely blocked glutamate and NMDA-induced Ca2+ influx (Fig. 3, B and C). Taken together, these data demonstrated that CTC and DMC are weak and transient blockers of the NMDA receptor currents and only partially block [Ca2+]i influx during the early stages of glutamate/NMDA treatment. However, such a transient reduction in NMDA receptor current and [Ca2+]i influx may not be sufficient to account for the more than 85% neuroprotection conferred by these two compounds, suggesting that these compounds may inhibit intracellular targets.

CTC and DMC Protect CGNs through Inhibition of Intracellular Calpain Activities—Calcium-activated intracellular proteases such as calpain are an important mediator of neuronal death in response to glutamate toxicity and cerebral ischemia (1). Although caspase activity may also play a role in the apoptotic component of ischemia-induced neuronal death, our previous work showed that caspase is not active in glutamate-induced neuronal death (1). To understand how CTC and DMC protect neurons in vitro and in vivo, we hypothesized that these two compounds could modulate the activities of Ca2+-activated calpains.

To do this, in vitro experiments were first performed using purified exogenous calpains. CTC and DMC significantly inhibited the activities of active calpain I (Fig. 4A, p < 0.001) and calpain II (Fig. 4B, p < 0.001).
FIGURE 3. CTC and DMC weakly antagonize NMDA receptor and calcium influx in cultured CGNs. A, whole-cell membrane currents elicited by NMDA (50 μM) were recorded under voltage clamp at −60 mV. Representative recordings are presented in A. The average reduction in NMDA receptor activity was calculated. A 14% ± 1% (n = 5 cells) and 16% ± 2% (n = 5 cells) by CTC and DMC, respectively, were recorded. B, the effect of CTC, DMC, minocycline, and MK-801 on glutamate-induced changes in [Ca^{2+}]_{i}. CGNs at 7 days in vitro were loaded with fura-2 followed by the treatment with glutamate (50 μM) in the presence or absence of the prior addition of the compound. Changes in [Ca^{2+}]_{i} was measured by converting the 340/380 ratio of fura-2 fluorescence (after correction for background) as described under “Experimental Procedures.” Data obtained from three independent experiments were averaged. Lines indicate the averaged concentrations of [Ca^{2+}]_{i} with 1 glutamate only (50 μM); 2 minocycline (150 μM) plus glutamate (50 μM); 3 DMC (150 μM) plus glutamate (50 μM); 4 CTC (150 μM) plus glutamate (50 μM); 5 MK-801 (1 μM) plus glutamate (50 μM); and 6 buffer only. C is a graph indicating that CTC and DMC partially block [Ca^{2+}]_{i} influx as measured by Fluo-4 assay. CGNs were treated with or without the compound indicated in the graph and followed by NMDA application. Intracellular calcium concentrations were measured using Fluo-4 as described under “Experimental Procedures.” The fold of increase was calculated against non-treated CGNs. At least three independent repeats were performed, and data presented are mean ± S.E.; ** indicates p < 0.01, and * indicates p < 0.05 when compared with NMDA.
Specific inhibitors to calpains (ALLN and calpastatin), which inhibited calpain activity and also prevented neuronal death, were used as positive controls for the assay. Interestingly, minocycline, a potent neuroprotectant, did not inhibit the activities of calpains (Fig. 4, A and B).

Next, we examined whether these two compounds could inhibit glutamate-induced activation of calpains in CGNs. Calpain activity was monitored by the presence and the level of the SBP on Western blot. As shown in Fig. 4, C and D, after 20-min treatment with 50 μM glutamate, the level of SBP increased significantly (p < 0.001, Fig. 4D), and the level of SBP reached a peak after 2.5 h (Fig. 4D). Calpain inhibitors, ALLN, CTC, and DMC, were applied to cultured CGNs 30 min prior to glutamate treatment. Both the calpain inhibitor, and the two compounds significantly reduced the level of SBP caused by glutamate treatment in comparison with glutamate only treated sample at 2.5 h (Fig. 4D, p < 0.05).

Furthermore, the two compounds CTC and DMC also inhibited calpain activities caused by MCAO in mice brain as shown by the reduced level of SBP on Western blot (Fig. 4, E and F). The SBP level increased sharply in the ischemic brain of vehicle-treated mouse, but the level of SBP was significantly reduced in CTC- and DMC-treated brains (Fig. 4, E and F, p < 0.05). Taken together, CTC and DMC inhibit calpains activation in response to excitotoxicity and cerebral ischemia.

**DISCUSSION**

In the present study, we report the findings that CTC and DMC are neuroprotective against glutamate toxicity in cultured mouse CGNs in vitro and focal cerebral ischemia in vivo through inhibition of calpains, a mechanism different from that of minocycline. To the best of our knowledge, the present study is the first demonstration that CTC and DMC conferred neuroprotection through inhibition of calpain activities.

The molecular targets of CTC and DMC appear to be downstream of the NMDA receptors. CTC and DMC only weakly inhibited NMDA-induced intracellular calcium influx. The 14–16% inhibition of NMDA receptors by the two compounds could contribute to the subsequent relatively slow increase in intracellular Ca²⁺ levels seen in the two compound-treated samples; however, it is highly unlikely that this Ca²⁺ entry could account for the potent neuroprotection conferred by CTC and DMC to glutamate-treated CGNs and suggests that these two com-
pounds target downstream intracellular death signal transduction pathways.

Interestingly, the molecular targets of CTC and DMC appeared to be different from those of minocycline in that CTC and DMC inhibit calpain I and II, whereas minocycline does not. Previous studies have demonstrated that minocycline provides in vivo neuroprotection by suppressing microglial activation (21, 22). Reports also showed that minocycline directly target intracellular death pathways to protect neurons through blocking cytochrome c release and the subsequent activation of caspase (23, 29). The present in vitro studies using cultured CGNs showed that neuroprotection conferred by CTC and DMC came from direct inhibition of calpain activities but not from inhibition of microglial activation, since microglial activation played no role in such acute glutamate-induced neuronal death system. In addition, caspase did not become activated in this system (1). Indeed, in vitro and in vivo experiments, as shown in Fig. 4, clearly demonstrated that CTC and DMC were potent inhibitors of calpains activated in response to both glutamate treatment and MCAO in mice brains. Calpains are major upstream proteases that are activated following ischemic injury to the brain and are responsible for the rapid and sustained induction of caspase (24). Wells, J. E., Hurlbert, R. J., Fehlings, M. G., and Yong, V. W. (2003) J. Biol. Chem. 278, 8054–8060

In our current study, we have demonstrated that CTC and DMC, which do not possess anti-microbial activities, are under development.

In summary, a battery of biochemical experiments performed in the present study demonstrated that CTC and DMC, the two clinically used antibiotics, provide neuroprotection not through blocking NMDA receptors but rather by inhibition of calpain activity. Further modification of these two compounds may lead to drugs capable of neuroprotection following cerebral ischemia.

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