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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-induced whole cell currents and partially blocked NMDA-induced Ca^{2+} 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 Ca^{2+} 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 therapeutics 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 NMDA[2] receptors, causing increased intracellular Ca^{2+} influx leading to the accumulation of toxic levels of intracellular calcium ions (4, 5). Elevation in intracellular Ca^{2+} concentrations activates Ca^{2+}-dependent proteases, such as calpains, which break down critical structural proteins causing neuronal death (3, 6–9).

Calpain I, activated at micromolar concentrations of Ca^{2+}, and calpain II, activated at millimolar Ca^{2+} concentrations, have similar proteolytic specificities (6, 10). For example, both calpains cleave the cytoskeletal element spectrin. Calpain-mediated proteolysis of spectrin leads to the immediate production of breakdown products (SBP) of ~150 kDa in size (6, 9–11). Previous studies have shown that a rapid (within 30 min) and sustained activation of calpain occurs following focal neocortical ischemia and global ischemia (6–10, 12–14) and following glutamate treatment of cultured hippocampal and cerebellar neurons (11). Direct inhibition of calpain reduces calpain-mediated proteolysis of spectrins and decreases brain infarction in ischemic rats and gerbils (13–17) and protects cultured hippocampal and cerebellar neurons against glutamate-induced toxicity (18–20).

Chemical compounds directly blocking glutamate toxicity to neurons may have the potential to be developed as therapeutics to stroke. But NMDA receptor blockers, such as MK-801, have failed in human stroke clinical trials due to the severe side effects possibly resulting from interference with the normal physiological functions of the NMDA receptor, despite the fact that compounds like MK-801 are very effective in preventing glutamate-mediated neuronal death in cell culture models (2). Tetracyclines are antibiotic agents with a broad spectrum of anti-microbial activities and anti-inflammation properties (21). Recent studies demonstrated that minocycline, a tetracycline derivative, has potent neuroprotective properties in animal models of various brain diseases, such as global and focal cerebral ischemia (21–23), spinal cord injury (24), retinal cell death (25), Parkinson disease (26), Huntington disease (27), multiple sclerosis (28), and amyotrophic lateral sclerosis (29). The potential mechanisms of minocycline-mediated neuroprotection are through suppression of microglial activation and inhibition of the release of apoptotic factors such as cytochrome *c* and attenuation of intracellular caspase activities (21, 27, 29). However, it is still not clear whether minocycline interferes with NMDA receptor function. Tetracyclines, in general, have been used safely as an antibiotic agent for many years in the clinic. The properties of clinical tolerance and easy penetration into the brain make some of the tetracycline derivatives potential therapeutic reagents for neuroprotection in stroke (30–33).

In the present study, we tested the protective effects of two tetracyclines against glutamate-mediated excitotoxicity and cerebral ischemia-induced brain damage. The potential molecular mechanisms of such neuroprotection were also investigated. CTC and DMC were found to be strongly neuroprotective, not through inhibition of the NMDA receptor but rather through suppression of a Ca^{2+} rise and inhibition of calpain activities.

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[2] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; SBP, spectrin breakdown product; CTC, chlortetracycline; DMC, demeclocycline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CGN, cerebellar granule neuron; CFDA, 5-(6)-carboxyfluorescein diacetate; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride; AFC, 7-amino-4-trifluoromethylcoumarin; ALLN, acetyl-Leu-Leu-norleucinal.

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 neurons prior to resuspension in medium and to remove undissociated debris prior to plating in Eagle's minimum essential medium containing 0.8 mM glutamine, 27 mM glucose, 0.01% gentamycin, 9% fetal bovine serum and supplemented with K^+ to a final concentration of 23 mM. Cells were plated onto 24-well dishes containing poly-L-lysine-coated coverslips at a density of 6×10^5 per well. After ~18 h, cytosine α-D-arabinofuranoside (AraC) was added to a final concentration of 5 μM, to prevent glial cell proliferation. 100-mm dish cultures were seeded with 21×10^6 cells in 10 ml of culture medium.

Neuronal Viability Assays

Tetracyclines were added to 8-day *in vitro* cultured CGNs at 37 °C for 15–20 min prior to treatment with 50 μM glutamate or NMDA. The plates were then incubated for 6 h at 37 °C. Untreated controls were also included. At the end of the treatment period, neuronal viability was measured using the CFDA assay as described previously (34, 35). The CFDA stock solution was diluted using Earle's balanced salts (Sigma) to a final concentration of 5 μg/ml. Cultures were incubated with 500 μl of the CFDA solution at 37 °C for 30 min. The intensities of fluorescence was quantified using a Cytofluor™ 2350 Fluorescence Measurement System (Millipore) at $\lambda_{ex} = 480$ nm and $\lambda_{em} = 530$ nm. Cellular viability was normalized against the fluorescent reading from the control cells. Neurons were also fixed in 4% formaldehyde and mounted in Dako® fluorescent mounting medium containing 5 μg/μl Hoechst 33258 to detect nuclei under a fluorescent microscope. Duplicate assessment of each treatment was made on each plate in at least three separate experiments per treatment.

Whole-cell Recording

Whole-cell recording using electrophysiological methods has been described previously (36). Briefly, CGNs, cultured on 35-mm culture dishes, were perfused continuously at 1 ml/min at 22 °C using a solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 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 resistance) 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: $B = I - I_B/I$, where I is the steady-state current evoked by NMDA, and I_B is the current evoked by NMDA in the presence of the test compound at the end of the co-application.

Intracellular Ca^{2+} 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 $\lambda_{ex} = 485$ nm and $\lambda_{em} = 530$ 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 Ca^{2+} was calculated by subtracting the initial reading from each reading divided by the initial reading.

Ratiometric Measurement of [Ca^{2+}] Using Fura-2—To quantitatively determine the effect of CTC or DMC on glutamate-induced changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) level, ratiometric measurement of $[Ca^{2+}]_i$ 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 Mg^{2+}-free buffer containing 2 mM HEPES (pH 7.2), 140 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 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 $[Ca^{2+}]_i$ concentration was measured by converting the 340/380 ratio of fura-2 florescence (after correction for background) to approximate $[Ca^{2+}]_i$ 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 (R_{min} and R_{max}) were obtained from a sample set of CGNs using 5 μM ionomycin plus 6 mM EGTA and by 10 mM $CaCl_2$, respectively. The K_d for fura-2 was assumed to be 224 nM as described by Young *et al.* (39). The basal level of $[Ca^{2+}]_i$ 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.

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 colormetric 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/Me_2SO 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 manufacture'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 Ca^{2+} 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 λ_{ex} = 400 nm and λ_{em} = 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. 1*C*). 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. 2*B*. The infarction was significantly reduced in the same areas in brains treated with CTC (Fig. 2*C*) or DMC (Fig. 2*D*).

The protective effects of these compounds were also confirmed by the improvement of the neurological behavior of the compound-treated ischemic mouse. 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. 2*E*, 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.

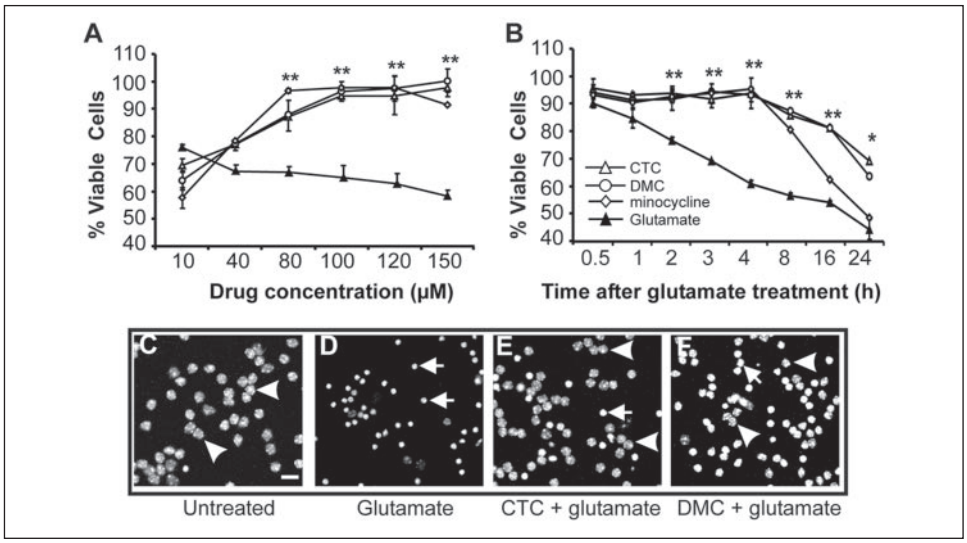

FIGURE 1. **CTC and DMC are neuroprotective *in vitro*.** *A* and *B*, cultured CGNs were treated with 50 μM glutamate with or without prior treatment with the indicated compound at the dose and duration of exposure indicated. *A* shows the dose response of compounds after 6-h incubation with glutamate, 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 lest 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.

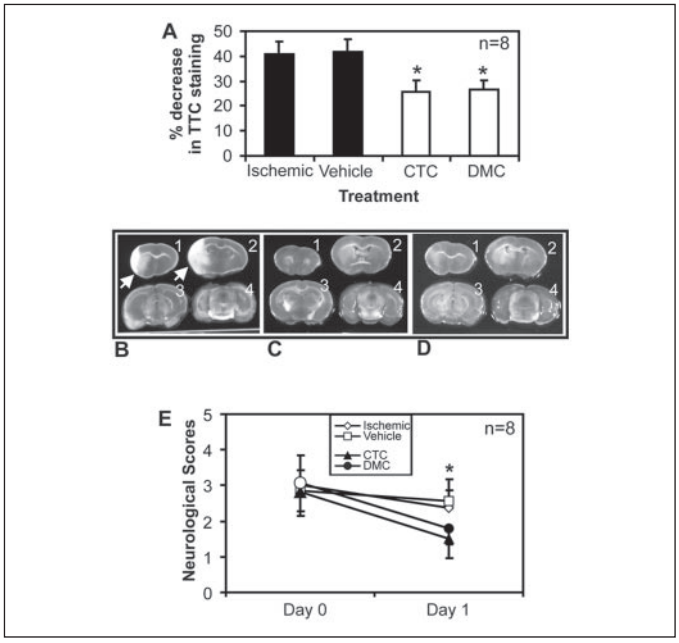

FIGURE 2. **CTC and DMC reduced cerebral infarction and improved neurological behavior in MCAO mice.** Mice at 20–23 g body weight were subjected MCAO for 1 h and reperfusion for 24 h as described in the "Experimental Procedures" section. *A*, TTC extraction and quantification were performed as described 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 Ca^{2+}—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 exitotoxicity. Both compounds at 150 μM showed weak, but rapid, antagonism to 50 μM NMDA-induced currents (Fig. 3*A*). 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 Ca^{2+} influx, we next tested whether these two compounds affect glutamate and NMDA-induced intracellular Ca^{2+} levels. As shown in Fig. 3*B*, NMDA receptor-mediated intracellular calcium influx increased immediately after the addition of glutamate. The addition of the two compounds partial blocked $[Ca^{2+}]_i$ influx, but the $[Ca^{2+}]_i$ level eventually increased to the same level as that of NMDA-treated CGNs after 40 min (Fig. 3*C*). Minocycline also exhibited a similar level of blockade of Ca^{2+} influx as compared with those from CTC and DMC at the 10- and 20-min time points (Fig. 3*C*, $p < 0.05$ compared with NMDA-mediated Ca^{2+} rise). On the other hand, MK-801, an antagonist to NMDA receptor, completely blocked glutamate and NMDA-induced Ca^{2+} 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 $[Ca^{2+}]_i$ influx during the early stages of glutamate/NMDA treatment. However, such a transient reduction in NMDA receptor current and $[Ca^{2+}]_i$ 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 Ca^{2+}-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. 4*A*, $p < 0.001$) and calpain II (Fig. 4*B*, $p < 0.001$).

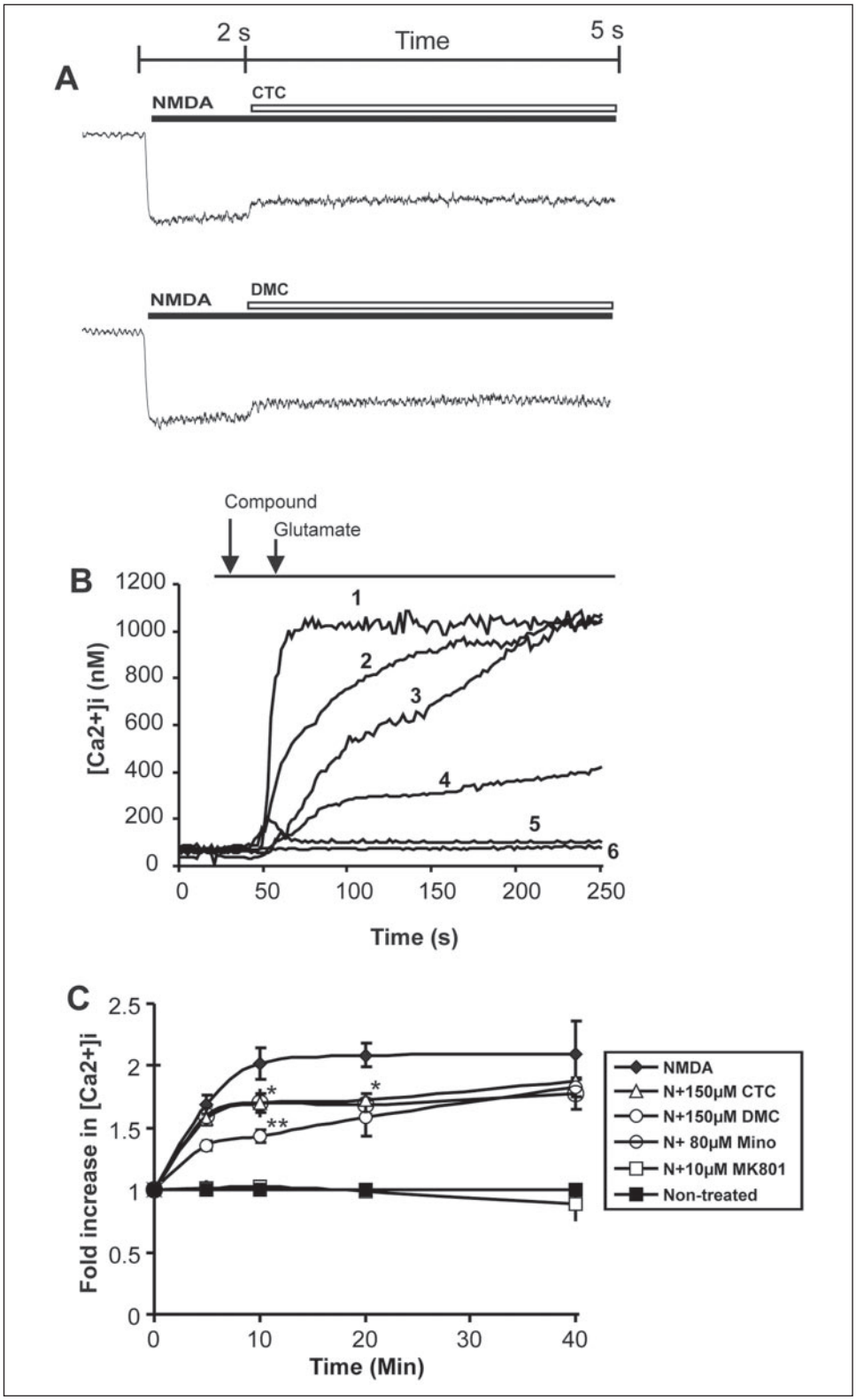

FIGURE 3. **CTC and DMC weakly antagonize NMDA receptor and calcium influx in cultured CGNs.** *A*, whole-cell membrane currents elicited by NMDA (50 μM) were recorded under voltage clamp at -60 mV. Representative recordings are presented in *A*. The average reduction in NMDA receptor activity was calculated. A 14% $\pm$ 1% ($n = 5$ cells) and 16% $\pm$ 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 florescence (after correction for background) as described under "Experimental Procedurs." 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 $\pm$ S.E.; ** indicates $p < 0.01$, and * indicates $p < 0.05$ when compared with NMDA.

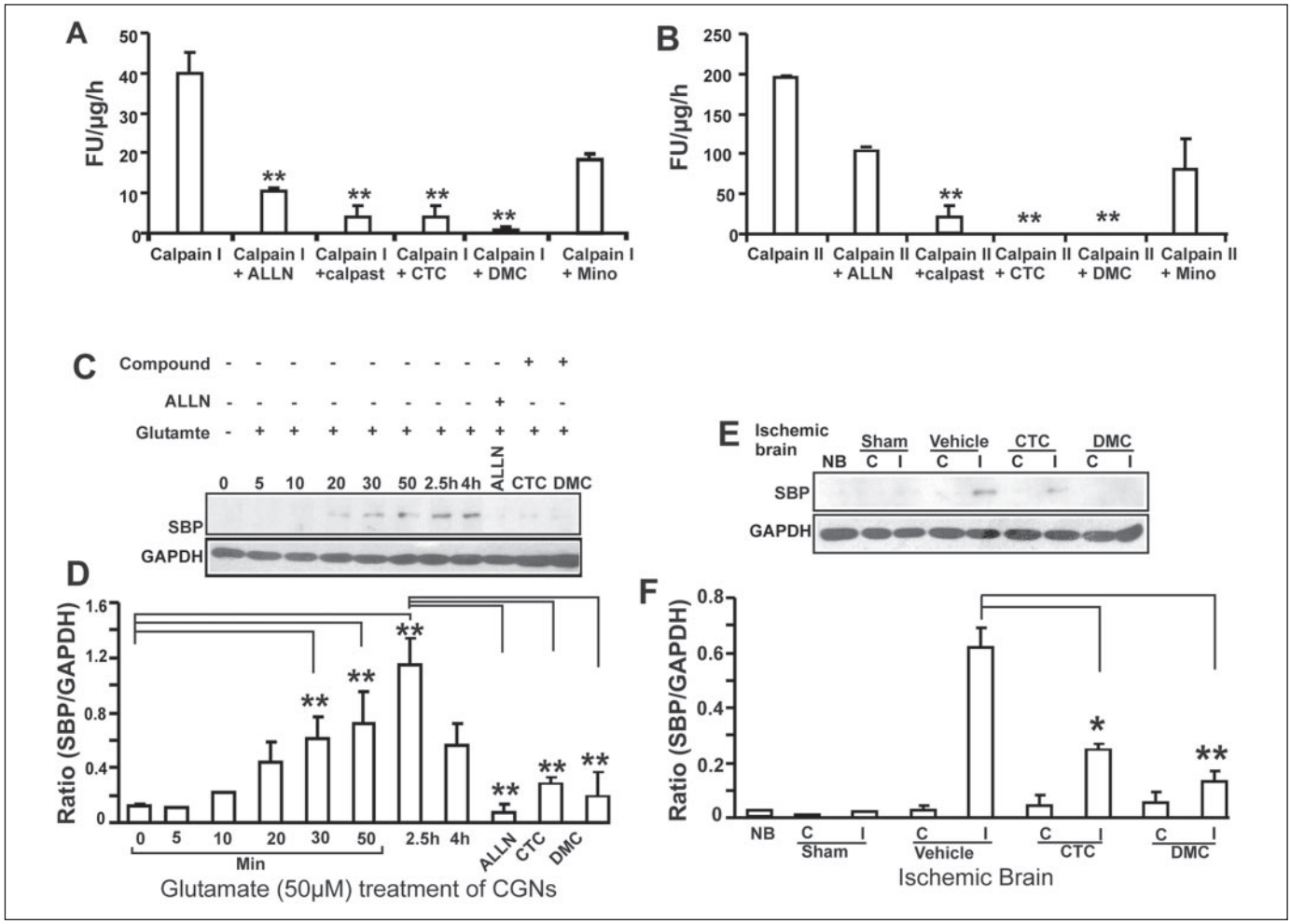

FIGURE 4. **CTC and DMC inhibit calpain activity *in vitro* (*A* and *B*) and *in vivo* (*C–F*).** Purified exogenous active calpains I and II at 0.1 unit/ml each were mixed with calpain-specific inhibitors ALLN or calpastatin at 10 μM or the compound as indicated at 150 μM concentration. After 30-min incubation with the calpain substrate, the release of fluorescent AFC was recorded using a Cytofluor. The fluorescent unit/μg of protein/h was calculated from at least three independent experiments and plotted in *A* (calpain I) and *B* (calpain II) (mean ± S.E.). ** indicates statistical significance ($p < 0.01$) by Student's *t* test. *C–F* show Western blotting and its quantification of SBP produced by the activation of calpain. Glutamate-treated CGNs in the presence or absence of calpain inhibitor or compounds were collected after the time indicated in *C*. The protein extract was subjected to Western blotting with a primary antibody against SBP. GAPDH was used as protein loading control. The production of SBP was normalized against GAPDH, and the mean ± S.E. was presented in D. Similarly, ischemic brains were collected for Western blotting to detect the production of SBP and the level of it was normalized against GAPDH (*E* and *F*). The mean ± S.E. was presented in *F*. *NB*, normal brain; C, contralateral side; *I*, ischemic side of the brain. ** indicates statistical significance ($p < 0.01$) by Student's *t* test.

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. 4*D*), and the level of SBP reached a peak after 2.5 h (Fig. 4*D*). 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. 4*D*, $p < 0.05$).

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 Ca^{2+} levels seen in the two compound-treated samples; however, it is highly unlikely that this Ca^{2+} 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 spectrin breakdown in the infarct zone (9, 20, 43). The present study, using several techniques including *in vitro* calpain activity assay and SBP quantification by Western blotting, demonstrated that calpain activity increased rapidly following treatment with glutamate in cultured CGNs or MCAO and that this induction can be ameliorated by calpain-specific inhibitors, CTC and DMC. This early induction in calpain following cerebral ischemia is consistent with previous reports that calpain-induced SBP could be detected as early as 1 min in the dendrites of pyramidal cells in the CA2/CA3 border zone and 5–10 min in CA1, cortical, and thalamic regions of the ischemic rats (9). In addition to stroke, the activities of calpain are also implicated in a wide range of pathological conditions such as traumatic brain injury, Alzheimer disease, and type 2 diabetes mellitus (44). As a result, several calpain inhibitors have been developed to target these diseases, for example, natural product-based inhibitors and synthetic inhibitors (45). However, none of them have structural similarities to tetracyclines. Thus tetracycline may inhibit calpain activation/activity in a way distinct from those described in the literature and which requires further investigation.

A compound with the potential to become a lead for development as therapeutics to stroke has to meet several requirements, such as 1) that the compound does not interfere with normal physiological functions of glutamate receptors, 2) that the compound targets intracellular death pathways to provide direct protection to neurons, and 3) that the compound should penetrate into the brain freely and have low toxicity. CTC and DMC used in the present study appear to meet all of the above requirements. CTC and DMC have been used clinically as antibiotics for many years. In addition, tetracyclines also have other beneficial activities and properties such as anti-oxidation and anti-glycation (33, 46, 47). These advantages of tetracyclines make them promising leads for drug development as stroke therapeutics.

However, further modification of CTC and DMC is required. Since these compounds are known potent antibiotics, it is desirable to use them at low doses and for a short period of time. The doses that we used in the current study were 90 mg/kg prior to MCAO and followed by 45 mg/kg with no visible toxicity present, but a very recent report suggests that low dose minocycline (at 3 mg/kg prior to cerebral ischemia and 10 mg/kg afterward intravenously) indeed conferred neuroprotection in MCAO rats (48). Current work is under way to examine the low dose effect of CTC and DMC. In addition, novel derivatives of 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. Future modification of these two compounds may lead to drugs capable of neuroprotection following cerebral ischemia.

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

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