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Pharmacological Induction of Ischemic Tolerance by Glutamate Transporter-1 (EAAT2) Upregulation

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Background and Purpose—Astrocytic glutamate transporter protein, GLT-1 (EAAT2), recovers extracellular glutamate and ensures that neurons are protected from excess stimulation. Recently, β-lactam antibiotics, like ceftriaxone (CTX), were reported to induce the upregulation of GLT-1. Here, we investigated ischemic tolerance induction by CTX in an experimental model of focal cerebral ischemia.

Methods—CTX (200 mg/kg per day, IP) was administered for 5 consecutive days before transient focal ischemia, which was induced by intraluminal thread occlusion of the middle cerebral artery for 90 minutes or permanently.

Results—Repeated CTX injections enhanced GLT-1 mRNA and protein expressions after 3 and 5 days of treatment, respectively. CTX-pretreated animals showed a reduction in infarct volume by 58% (reperfusion) and 39% (permanent), compared with the vehicle-pretreated animals at 24 hours postischemia ($P<0.01$). Lower doses of CTX (20 mg/kg per day and 100 mg/kg per day) reduced infarct volumes to a lesser degree. The injection of GLT-1 inhibitor (dihydrokainate) at 30 minutes before ischemia ameliorated the effect of CTX pretreatment. However, CTX administration at 30 minutes after ischemia produced no significant reduction in infarct volume. CTX reduced the levels of proinflammatory cytokines (tumor necrosis factor-α, FasL), matrix metalloproteinase (MMP)-9, and activated caspase-9 ($P<0.01$). In addition, CTX-pretreated animals showed better functional recovery at day 1 to week 5 after ischemia ($P<0.05$).

Conclusions—This study presents evidence that CTX induces ischemic tolerance in focal cerebral ischemia and that this is mediated by GLT-1 upregulation. (***Stroke***. 2007;38:177-182.)

Key Words: ceftriaxone ∎ cerebral ischemia ∎ excitotoxicity ∎ ischemic tolerance ∎ glutamate transporter-1

Cell death after cerebral ischemic insult can be induced in 3 ways: (1) by excitation (excitotoxicity), (2) by oxidative stress with free radicals, and (3) by apoptosis.[1] When the brain fails to generate sufficient ATP and this results in energy failure, ionic gradients are lost.[1] At this stage, glutamate is released, reuptake processes are impaired, and glutamate binds to its postsynaptic receptors and promotes excitotoxic processes as well as a radical-induced apoptotic course.[1] Thus, a number of NMDA receptor antagonists have been developed and used for the treatment of neurological diseases in patients, but all have failed clinical trials because of intolerable side effects or a lack of medical efficacy.[2]

Glutamate transporters play a crucial role in removing glutamate released from synaptic clefts.[3] Released glutamate is taken up by glial cells and metabolized to glutamine, which is then transported back into neurons, converted to glutamate, and sequestered into synaptic vesicles by vesicular glutamate transporters.[4] In addition, it has been demonstrated that glutamate transporter-1 (GLT-1; EAAT2) and glutamate/aspartate transporter, both glial isoforms, are the predominant means of functional glutamate transport, and that they are essential for maintaining low extracellular glutamate, preventing chronic glutamate neurotoxicity,[3,4,5] and for neuronal survival in the ischemic penumbra.[5,6] Recently and unexpectedly, it was postulated that β-lactam antibiotics are potent stimulators of GLT-1 expression.[7] Moreover, ceftriaxone (CTX), which efficiently penetrates the blood-brain barrier, was found to be neuroprotective in vitro when administered under oxygen-glucose deprived conditions, and in an in vivo mouse model of amyotropic lateral sclerosis.[7] Thus, based on the findings of this study, we investigated whether GLT-1 upregulation induced by CTX can induce ischemic tolerance

and improve functional recovery in experimental cerebral ischemia.

Materials and Methods

Animal Model and Experimental Protocol

All procedures were carried out according to an institutionally approved protocol, and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Sprague-Dawley male rats (n=103) weighing 220 to 230 g (Daehan Bio; Seoul, Korea) were used, and randomly assigned to the following experimental groups: CTX+normal control (n=18; [CTX]normal group); CTX+reperfusion ischemia (n=23; [CTX]reperfusion group); CTX+permanent ischemia (n=9; [CTX]permanent group); vehicle+reperfusion ischemia (n=23; [vehicle]reperfusion group); vehicle+permanent ischemia (n=9; [vehicle]permanent group); reperfusion ischemia+post-treatment of CTX (n=9; [CTX-post]reperfusion group). Lower dose injection (20 mg/kg per day, 100 mg/kg per day, n=9 respectively); and GLT-1 inhibitor study ([CTX]DHK group, [vehicle]DHK group, n=9 per group).

To determine the optimal time required for CTX to induce maximal GLT upregulation, in a pilot study we injected CTX (200 mg/kg, IP) for up to 7 days preoperatively (n=18). CTX dose and treatment schedules were chosen based on the findings of a previous study.[7] For GLT-1 RT-PCR and western blotting experiments, rats were euthanized at 6 hours, and at 1, 2, 3, 5, and 7 days after the first CTX injection.

Based on this pilot study, we injected CTX (200 mg/kg per day, in PBS; CJ Pharmaceuticals) or PBS (vehicle) into rats intraperitoneally for 5 days before ischemia (total 5 days). The rats for behavioral tests were also injected for additional 3 days postischemia (total 8 days). For comparison purposes, we included the [CTX-post]reperfusion group, in which daily CTX was started at 30 minutes after the ischemic insult. The lower doses of CTX (20 mg/kg per day, 100 mg/kg per day, n=9 respectively) were also tested to evaluate dosage-effects. All other injections of CTX were at 200 mg/kg per day. To verify the causal role of GLT-1 upregulation in neuroprotection, some rats were injected once with the selective GLT-1 inhibitor, dihydrokainate (DHK, 10 mg/kg, IP; Tocris), at 30 minutes before the ischemia induction ([CTX]DHK group, [vehicle]DHK group). The glial transporter GLT1 is inhibited potently by DHK.[8]

Transient focal cerebral ischemia was induced by the intraluminal occlusion of the middle cerebral artery (MCAo) using a thread for 90 minutes in both groups, as described elsewhere.[9] For comparison, permanent focal cerebral ischemia was induced by leaving the intraluminal filament in position. Physiological parameters, including mean arterial blood pressures, blood gases, and glucose concentrations, were measured throughout surgery. Rectal temperatures were maintained throughout surgery at $37\pm0.5°C$ using a thermistor-controlled heated blanket. Animals were maintained in separate cages at room temperature (25°C) with free access to food and water, under a 12-hour light-dark cycle. Modified limb placing tests (MLPT) were performed 1 day before and 1 day after ischemia, and weekly thereafter (n=6 per group) for 5 weeks, as described previously.[10,11] MLPT results were used to assess the sensorimotor integration of forelimbs and hindlimbs by verifying their responses to tactile and proprioceptive stimuli.

Infarct Volume Measurement

In order to compare infarct sizes in the ischemia-treated groups, we measured infarct volumes (at 24 hours postischemia; n=9 per group) using 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) staining, as described elsewhere.[10] Infarcted and total hemispheric areas of sections were traced from slices taken at 1-mm intervals and measured using an image analysis system (Image-Pro Plus; Media Cybernetics). Two investigators (K.C. and S.T.L.) unaware of study group identities measured infarct sizes using a computerized image analyzer (Bio-Rad). To compensate for the effect of brain edema, corrected infarct volumes were calculated as previously described using: Corrected infarct area=Measured infarct area×{1−[(Ipsilateral hemisphere area−Contralateral hemisphere area)/Contralateral hemisphere]}.[12] Infarct volumes were expressed as percentages of total hemispheric volumes.

GLT-1 Protein Expression

GLT-1 western blotting was performed as previously described,[13] using anti-GLT-1 antibody (1:200; Santa Cruz) and secondary antibody conjugated with horseradish peroxidase. Anti–β-actin antibody (Santa Cruz) was used as the control. Blots were developed by enhanced chemiluminescence (Pierce), digitally scanned (GS-700; Bio-Rad), and analyzed (Molecular Analyst; Bio-Rad). Relative optical densities were obtained by comparing measured values with the mean values of normal rats.

RT-PCR for GLT-1 and Inflammatory Mediators

Twenty-four hours after the induction of ischemia, rats were euthanized by decapitation, and brains were immediately extracted (n=4 in each group). RT-PCR for GLT-1, interleukin-6, Fas, FasL, tumor necrosis factor (TNF)-α, and MMP-2/9 were conducted using a previously described method in ischemic hemispheres (for detailed primer sets see supplemental Table I, available online at http://stroke.ahajournals.org).[11] mRNA expression levels were normalized versus GAPDH, and relative optical densities were determined by comparing mean measured values with the mean value of the [vehicle]Reperfusion group.

Measurement of Caspase Activities

Caspase activities were determined using ELISA kits (Caspase-3: Promega; Caspase-8: BD Biosciences; Caspase-9: Chemicon) in ischemic hemispheres, 24 hours after the induction of MCAo (n=4 per group), as previously described.[14] Fluorescent intensities were measured using a plate reader (FL600; Bio-Tek; excitation wavelength: 380 nm; emission wavelength: 460 nm).

Statistical Analysis

All values are expressed as mean±SD. The analysis was conducted using repeated measures of analysis of variance and the unpaired Student t test, if normally distributed (Kolmogorov–Smirov test; $P>0.05$). Alternatively, we used the Mann–Whitney U tests, or specified the test used. A 2-tailed P value of <0.05 was considered to be significant.

Results

CTX Treatment Upregulated GLT-1 Expression In Vivo

To examine GLT-1 temporal expression profiles in vivo, as a pilot study, we injected CTX daily (200 mg/kg per day, up to 7 days), and euthanized rats at various timepoints ([CTX]normal group, n=18). GLT-1 mRNA expressions in brain increased after CTX administration and peaked after 3 days (1.7-fold), whereas GLT-1 protein levels peaked after 5 days of treatment (Figure 1). Based on this result, we decided to administer CTX for 5 days before the ischemic insult.

CTX Treatment Enhanced Functional Recovery

Physiological parameters, including mean arterial blood pressure, blood gases, serum glucose, and body temperature did not differ significantly between the 2 experimental groups ([vehicle]reperfusion group and [CTX]reperfusion group) before, during, or 30 minutes after MCAo (ANOVA; supplemental Table II, available online at http://stroke.ahajournals.org). Overall mortality was 13.5% ([CTX]reperfusion group) versus 15.6% ([vehicle]reperfusion group), and this was limited to the first week postsurgery.

Whereas vehicle-treated rats ([vehicle]reperfusion group) exhibited >6 points according to MLPTs on the first day after
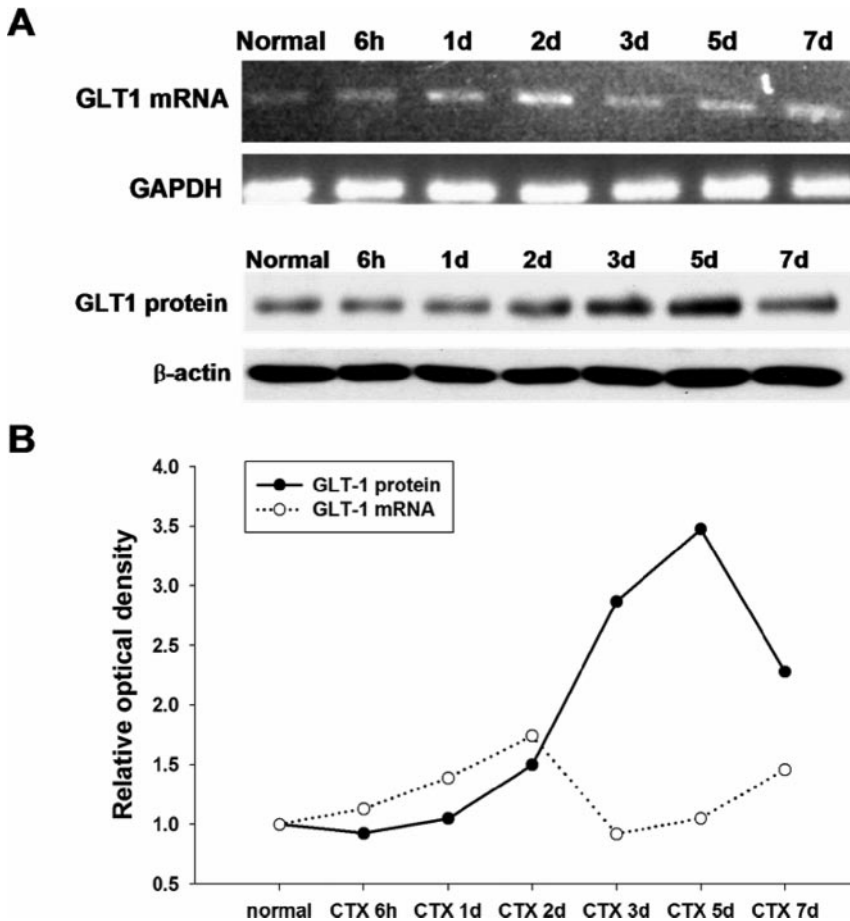
Figure 1. A, Western blotting of GLT-1 expression (upper) and RT-PCR (lower) of GLT-1 mRNA, during 7 days of CTX administration. B, The plot depicts the levels of GLT-1 protein and mRNA serially. GLT-1 protein levels peaked after 5 days of CTX administration.

MCAo, CTX-treated rats (^{CTX}reperfusion group) exhibited less profound deficits ($P<0.05$; Student t test; Figure 2a). Moreover, the ^{CTX}reperfusion group continued to recover, and MLPT differences between the 2 groups remained significant until at least 5 weeks after MCAo induction ($P<0.01$; t test). At week 5, the ^{CTX}reperfusion group had the MLPT score of <1.

CTX Pretreatment Reduced Infarct Volume

The ^{CTX}reperfusion group exhibited a reduced infarct volume (by 58%) versus the ^{vehicle}reperfusion group, at 24 hours postischemia (Figure 2b). The corrected mean infarct volume in ^{CTX}reperfusion group (CTX: 200 mg/kg per day) was 98.1 ± 32.4 mm^3 versus 235.5 ± 80.6 mm^3 in the ^{vehicle}reperfusion group ($P<0.01$). When we injected lower doses of CTX, the infarct volume of rats injected with 20 mg/kg per day of CTX was 167.1 ± 45.5 mm^3 ($P<0.05$ versus ^{vehicle}reperfusion group), and that of 100 mg/kg per day of CTX was 122.1 ± 38.8 mm^3 ($P<0.01$ versus ^{vehicle}reperfusion group). The difference between 100 mg/kg per day and 200 mg/kg per day was not significant ($P=0.226$).

When we injected dihydrokainate (a selective GLT-1 inhibitor) at 30 minutes before ischemia induction into rats pretreated with CTX (200 mg/kg) for 5 days (^{CTX}DHK group), the mean infarct volume (220.6 ± 43.6 mm^3) was found to be larger than in the ^{CTX}reperfusion ($P<0.01$) and similar to that in the ^{vehicle}reperfusion ($P=0.775$; Figure 2b). Dihydrokainate injection without CTX-pretreatment (^{vehicle}DHK group) showed no significant difference in mean infarct volume

(207.9 ± 30.5 mm^3) versus the ^{vehicle}reperfusion group. These results suggest the causal role of GLT-1 upregulation in the ischemic tolerance induced by CTX.

Mean infarct volume in the $^{CTX\text{-}post}$reperfusion group was not different from that of the ^{vehicle}reperfusion group ($P=0.87$), thus showing that post-treatment with CTX had no effect on infarct size (Figure 2c). The ^{CTX}permanent group exhibited reduced infarct volumes (by 39%) versus the ^{vehicle}permanent group, at 24 hours after ischemic insult (Figure 2d). The corrected mean infarct volume in the ^{CTX}permanent group was 165.2 ± 35.1 mm^3 versus 269.5 ± 55.3 mm^3 in the ^{vehicle}permanent group ($P<0.01$).

CTX Treatment Reduced Caspase-9 Activity and Downregulated Proinflammatory Cytokines

Caspase-9 activity in the ischemic hemisphere was significantly reduced in the ^{CTX}reperfusion group versus the ^{vehicle}reperfusion group (62% decrease; $P<0.01$; Figure 3a). However, the activities of caspases-8 and 3 were not changed by ceftriaxone treatment. We measured the mRNA expressions of cytokine-related genes, namely, TNF-α, Fas, FasL and interleukin-6, and relative optical density analysis revealed that the ^{CTX}reperfusion group showed an 86% decrease in the levels of TNF-α, and a 78% reduction in FasL mRNA levels versus the ^{vehicle}reperfusion group ($P<0.01$; Figure 3b and 3c). The levels of Fas and interleukin-6 mRNA were unchanged by CTX pretreatment. In addition, we analyzed the mRNA levels of the proteolytic enzymes (MMP-2 and MMP-9), and found that MMP-2 levels were unchanged, but that MMP-9
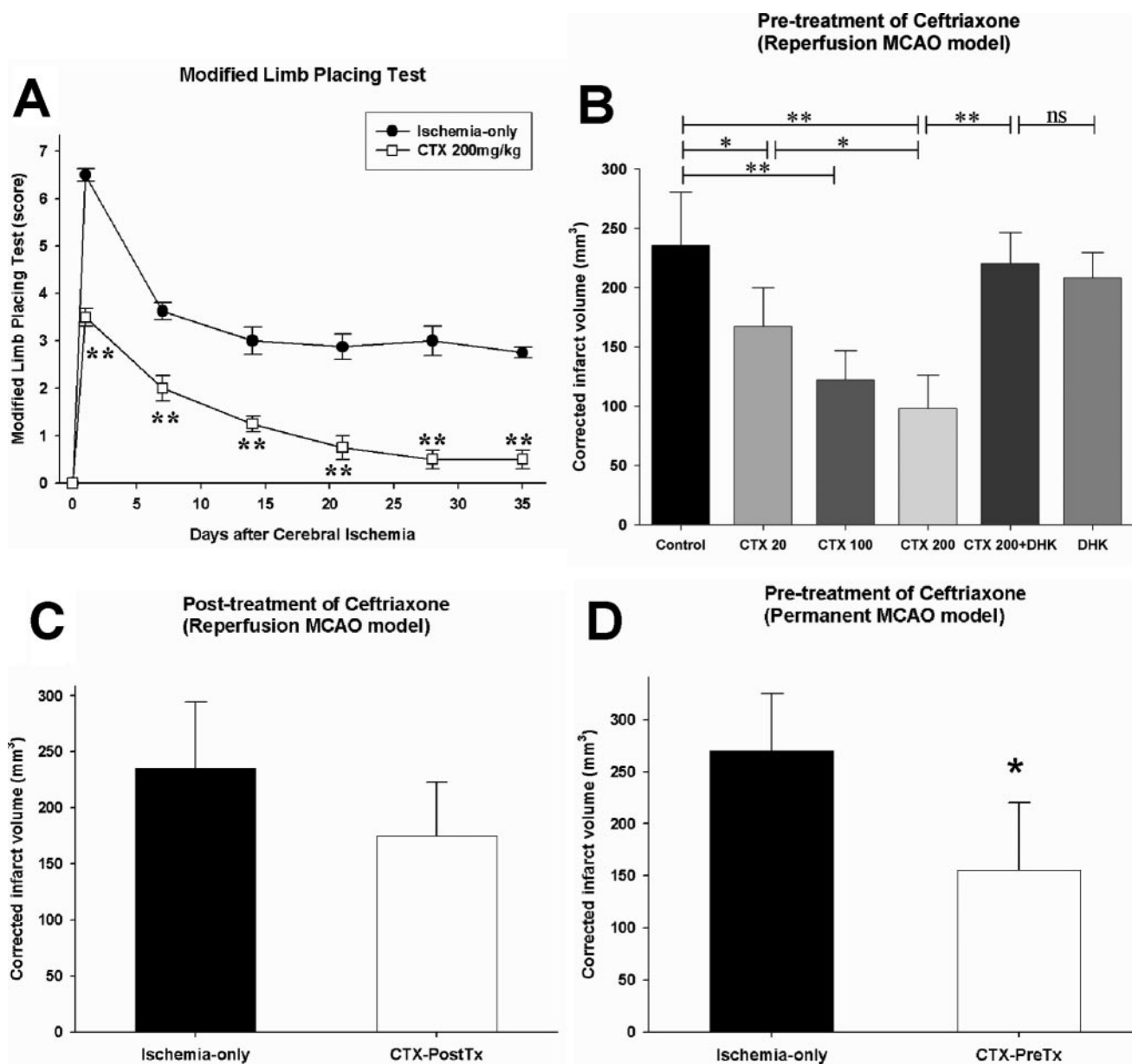
Figure 2. CTXreperfusion group showed the reduced initial neurological deficit and the enhanced functional recovery over a 5-week period (A). CTXreperfusion group also exhibited a reduced infarct volume as compared with vehiclereperfusion group (B). Lower doses of CTX (20 mg/kg per day, 100 mg/kg per day) reduced infarction volumes to a lesser degree. The selective GLT-1 inhibitor, dihydrokainate, attenuated the ischemic tolerance induced by CTX, suggesting the causal role of GLT-1 upregulation (B). CTX-postreperfusion group showed no neuroprotective effect (C). In CTXreperfusion group, infarct volume reductions were also significant but less prominent than in the reperfusion model (D). **P<0.01 versus vehiclereperfusion group in (A). *P<0.05 and **P<0.01 in (B) and (D). ns indicates statistically not significant.

mRNA levels were reduced by 95% attributable to CTX pretreatment.

Discussion

In this study, we investigated the neuroprotective effect of GLT-1 upregulation during cerebral ischemia. CTX treatment was found to upregulate GLT-1 and to reduce infarct volumes in both transient and permanent cerebral ischemic models. Moreover, the inhibition of GLT-1 ameliorated the ischemic tolerance induced by CTX, and post-treatment with CTX at 30 minutes after ischemia failed to reduce infarct volume. These results suggest that the mechanism of neuroprotection is based on GLT-1 upregulation. In addition, reduced infarct volumes corresponded with improved and sustained neurological outcomes.

Extracellular glutamate concentrations showed transient elevations immediately (within 30 minutes) after an ischemic insult.[15] In a reperfusion model, this initial peak in glutamate levels was followed by 2 secondary elevations in glutamate at 50 minutes and 90 minutes after initiating reperfusion.[15] However, although glutamate-induced excitotoxicity is a crucial component of the initial ischemic insult, all tested antiglutamatergic drugs have failed in clinical trials.[2] One of the reasons for this failure might be the rapid and early
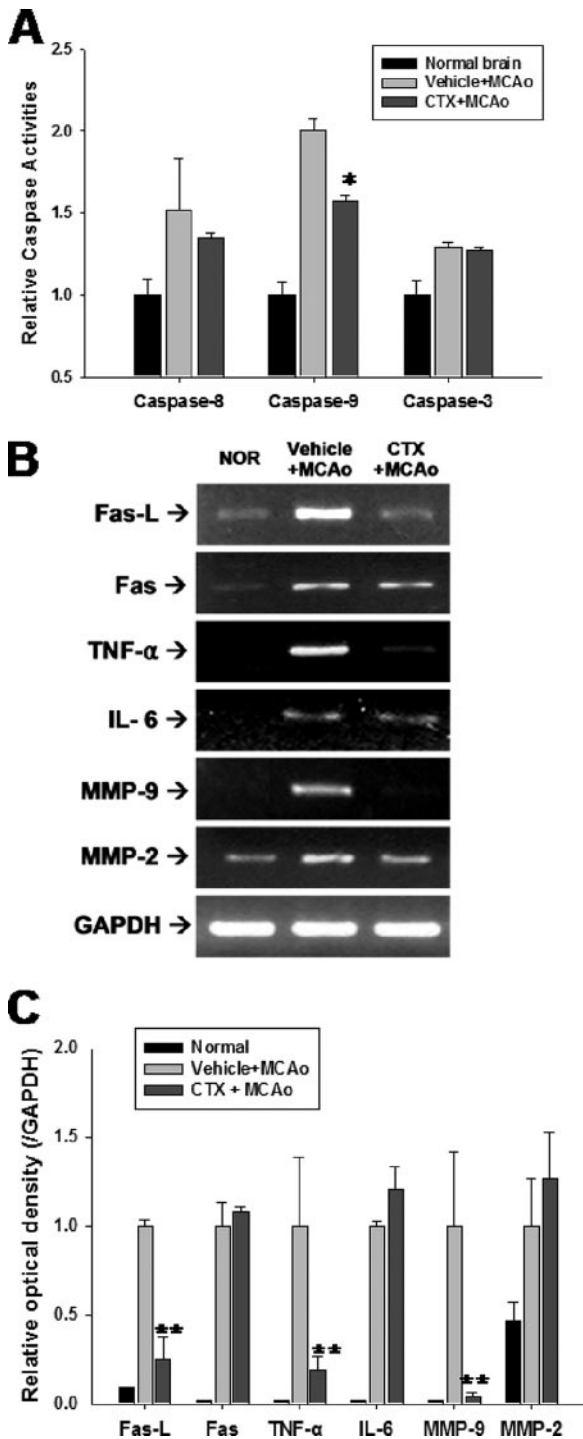
Figure 3. Caspase activities and the expression profiles of inflammatory mediators. ^{CTX}reperfusion group showed a reduction of caspase-9 activity (A). However, caspase-3 and -8 activities were unchanged. FasL, TNF-α, and MMP-9 mRNA levels were also reduced in ^{CTX}reperfusion group versus those in the ^{vehicle}reperfusion group (B and C). *$P<0.05$, **$P<0.01$ versus the ^{vehicle}reperfusion group.

increase in extracellular glutamate after ischemia, whereas drugs are administered several hours after the event. In the present study, CTX had already increased GLT-1 expression at the time of ischemia, and as such GLT-1 was available to attenuate excitotoxicity. In addition, glutamate release occurs

more than once during the early reperfusion phase.[15] Thus, increased GLT-1 levels by CTX explain the enhanced neuroprotection (reduced infarct volume) observed in the ^{CTX}reperfusion group versus the ^{CTX}permanent group.

There are at least 5 subtypes of glutamate transporters in the mammalian central nervous system.[16] GLT-1 is the predominant subtype and is responsible for the bulk of glutamate reuptake.[16] Moreover, the infusion of GLT-1 antisense led to a significant increase in infarct volumes after reperfusion in a rat ischemia model and a severe neurological deterioration.[17] On the other hand, the neuronal glutamate transporter, EAAC-1 antisense, did not influence ischemic injury.[17] Ischemic preconditioning depends on the expression of astrocytic GLT-1 and the level of its reverse operation.[18] Because glutamate transporters are dynamic proteins that can release or uptake glutamate, their function depends on their regional and cellular localizations, cerebral energy statuses, and the durations and types of neuronal insults. It is known that the reversal of GLT-1 contributes to ischemia-induced increases in excitatory amino acids in the ischemic core.[19] GLT-1 upregulation by CTX might worsen extracellular excitatory amino acids levels in situations of transporter reversal. However, excitatory amino acids release through reversed GLT-1 may not be significant in the ischemic penumbra because dihydrokainate did not reduce excitatory amino acids release in mild ischemic areas, suggesting a diminished transporter reversal role in the penumbra.[19] The normal function of GLT-1, rather than the reversed operation of GLT-1 transporter, seems to dominate in the ischemic penumbra. Our study also suggests that the enhanced expression of GLT-1 exerts a beneficial effect on the ischemic brain.

The relation between GLT-1 and inflammation is not fully understood. Reactive astrocytes express inflammatory makers, such as the inducible forms of nitric oxide synthase and cyclooxygenase, and secrete proapoptotic mediators, such as FasL, and downregulate GLT-1.[20–22] Meanwhile, TNF-α plays a facilitatory role in glutamate excitotoxicity, both directly and indirectly, by inhibiting glial glutamate transporters on astrocytes. TNF-α also has direct effects on glutamate transmission, such as by increasing the expression of AMPA receptors on synapses.[23] In addition, we previously presented evidence that NMDA blocker can reduce endogenous tissue plasminogen activator (tPA) and MMP-9 expressions.[11] In the present study, we found that GLT-1 upregulation reduced the expressions of TNF-α, FasL, and MMP-9. Accordingly, we suggest that the clearance of extracellular glutamate by upregulated GLT-1 might synergistically reduce inflammation and apoptosis. However, caspase-3 levels were not reduced by CTX in the present study, and CTX might influence other cell death pathway such as calpain, which is one of the major cell-death effecters induced by glutamate excitotoxicity in cerebral ischemia.[24] This topic requires further investigations.

The clinical implications of CTX-pretreatment require further discussion. In the amyotropic lateral sclerosis model, the chronic administration of CTX delayed neuronal loss and muscle strength, and increased mouse survival.[7] However, it needs time to upregulate GLT-1 and therefore is difficult to apply to acute neuroprotection. Thus, instead of using CTX

for acute therapy, it may be applied to induce ischemic tolerance in situations, such as carotid endarterectomy, cardiopulmonary bypass surgery, or in those with a high perioperative risk of ischemic stroke.

For bacterial infections, the usual adult daily dose of CTX is 1 to 2 g given once daily in adults, and 50 to 100 mg/kg once daily in pediatric patients. Thus, the dose used in this study (200 mg/kg per day) is relatively high as compared with clinical practice. Although ceftriaxone has a good tolerability profile, high doses of CTX can cause adverse effects, which include diarrhea, nausea, vomiting, candidiasis, and sometimes reversible biliary pseudolithiasis.[25] However, it has been shown that the EC50 required to increase GLT1 expression by ceftriaxone is 3.5 μmol/L,[7] which is comparable to the known central nervous system levels attained during therapy for meningitis (0.3 to 6 μmol/L).[26] Thus, normal antimicrobial CTX doses are likely to be sufficient to induce GLT-1 upregulation in humans.

In summary, we present evidence that ceftriaxone induces ischemic tolerance in focal cerebral ischemia, which is mediated by GLT-1 upregulation. CTX-induced GLT-1 upregulation might provide a novel antiglutaminergic approach in ischemic stroke.

Sources of Funding

Disclosures

None.

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