Caffeinol at the Receptor Level

Anti-Ischemic Effect of N-Methyl-d-Aspartate Receptor Blockade Is Potentiated by Caffeine

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Background and Purpose—Although caffeinol (a combination of a low dose of caffeine and ethanol) was shown to robustly reduce stroke damage in experimental models and is now in clinical evaluation for treatment of ischemic stroke, little is known about the potential mechanism of its action.

Methods—We used an in vivo excitotoxicity model based on intracortical infusion of N-methyl-D-aspartate (NMDA) and a model of reversible focal ischemia to demonstrate NMDA receptor inhibition as a potential mechanism of caffeinol anti-ischemic activity.

Results—Caffeinol reduced the size of excitotoxic lesion, and substitution of ethanol in caffeinol with the NMDA antagonists CNS-1102 and MK-801 but not with MgSO4 produced treatment with strong synergistic effect that was at least as robust in reducing ischemic damage as caffeinol. This NMDA receptor antagonist and caffeine combination demonstrated a long window of opportunity, activity in spontaneously hypertensive rats, and, unlike caffeinol, was fully effective in animals chronically pretreated with ethanol.

Conclusions—Our study suggests that antiexcitotoxic properties may underlie some of the anti-ischemic effect of caffeinol. This study provides strong evidence that the anti-ischemic effect of NMDA receptor blockers in general can be dramatically augmented by caffeine, thus opening a possibility for new use of NMDA-based pharmacology in the treatment of stroke.

Key Words: neuroprotection ■ excitotoxicity ■ NMDA antagonist ■ magnesium ■ ethanol ■ caffeine

A substantial body of evidence demonstrates that low doses of ethanol+caffeine (caffeinol), can effectively reduce brain damage in rodent models of focal cerebral ischemia and traumatic brain injury. Based on these promising preclinical studies, caffeinol was evaluated in stroke patients during a safety and feasibility study. Although preclinical data indicate strong neuroprotective potential and clinical feasibility data suggest that caffeinol can be safely administered to stroke patients, little is known about its mechanism of action.

It is recognized that among myriad biological effects, ethanol can effectively inhibit the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. It is also well established that cerebral ischemia/reperfusion produces massive release of glutamate into the extracellular space, thereby causing activation of the NMDA receptor, a process believed to lead to neurotoxicity (excitotoxicity) via calcium overload. Thus, it is possible that the ability of ethanol to inhibit the NMDA receptor could represent an important component of the anti-ischemic effect of caffeinol.

Although the antixcitotoxic potential of ethanol is intriguing, our previous experience with ischemic stroke is that ethanol alone augments ischemic damage. Hence, ethanol requires caffeine for its protective effect, suggesting that caffeine not only neutralizes the deleterious aspect of ethanol, but it also interacts with ethanol in such a way that leads to a superadditive synergy. The mechanism of how caffeine modifies the effect of ethanol is also unclear. The caffeine plasma level in animals treated with caffeinol in preclinical stroke studies was ~20 μg/mL (representing ~3 to 4 cups of strong coffee), a concentration recognized to inhibit adenosine receptors (and maybe inhibition of phosphodiesterase).

In this study, we tested the hypothesis that the anti-ischemic effect of caffeinol is, at least in part, mediated through inhibition of excitotoxic damage, and that NMDA receptor inhibition by ethanol may represent an important part of caffeinol effect. Three specific questions were posed: (1) could caffeinol reduce excitotoxic damage mediated via NMDA receptor? (2) could substitution for ethanol with a pharmacological agent(s) that displays selectivity in blocking NMDA receptor? (2) could substitution for ethanol with a pharmacological agent(s) that displays selectivity in blocking NMDA receptor? (3) could this substitution approach represent new strategy for stroke treatment?
Materials and Methods
All procedures were in compliance with National Institute of Health guidelines for the humane care of animals and were approved by the institutional animal welfare committee. No deaths or seizures were observed in any of the groups analyzed.

Production and Analysis of Excitotoxic Lesions
In Vivo
The experiment was performed using a method we described previously. Briefly, male Sprague Dawley rats (250 to 300 g; Harlan Sprague Dawley) were anesthetized with chloral hydrate (0.35 g/kg). Normothermia (36.6 ± 0.5°C) was maintained by using a thermostatically controlled heating lamp. To produce an excitotoxic lesion, NMDA (20 nmol in 1 μL of saline) was injected under stereotaxic guidance over 60 minutes into the cerebral cortex. Animals were randomly divided into 5 groups: (1) NMDA alone; (2) NMDA + MK-801 (a noncompetitive NMDA receptor antagonist; used as positive control); (3) NMDA + caffeinol (10 mg/kg caffeine + 0.32 g/kg ethanol); (4) NMDA + ethanol (0.32 g/kg), and NMDA + caffeine (10 mg/kg). All the above doses were shown previously to have anti-ischemic effect. Ethanol, caffeine, and caffeinol were infused through the left femoral vein to reproduce previously to have anti-ischemic effect. Ethanol, caffeine, and caffeinol were infused through the left femoral vein to reproduce conditions offering potent anti-ischemic effect. Twenty percent of caffeinol were infused through the left femoral vein to reproduce previously to have anti-ischemic effect. Ethanol, caffeine, and caffeinol were infused through the left femoral vein to reproduce conditions offering potent anti-ischemic effect. Twenty percent of caffeinol were infused through the left femoral vein to reproduce previously to have anti-ischemic effect. Ethanol, caffeine, and caffeinol were infused through the left femoral vein to reproduce conditions offering potent anti-ischemic effect.

Ischemia Production
Focal ischemia was induced by 180 minutes of reversible left middle cerebral artery (MCA)/common carotid artery (CCA) occlusion in Long–Evans or spontaneously hypertensive rats as described previously. Briefly, animals were anesthetized with chloral hydrate (0.35 g/kg IP). The femoral vein was cannulated for drug administration. Core body temperature was maintained at 36.5 ± 0.5°C during ischemia and the first hour of reperfusion. A 0.005-inch diameter stainless-steel wire was placed underneath the MCA rostral to the rhinal fissure, proximal to the major bifurcation of the MCA, and distal to the lenticulostriate arteries. The CCA was occluded, and cerebral perfusion at the cortical surface, 3-mm distal to the lobe of the MCA occlusion, was measured using laser-Doppler flowmetry (Vesamedic). Only animals that displayed a cerebral perfusion reading <12% to 15% of the initial value were included in the study. In our original studies, we determined that under the experimental conditions used in present study, caffeinol did not affect body temperature and other physiological variables including temperature, pH, PO2, and PCO2. After 180 minutes of ischemia, reperfusion was established by reversing the occlusion procedure. At time points indicated for each study, animals were reanesthetized and perfused with 50 mL of saline intracardially. Two-mm-thick coronal brain sections were cut before staining with 2% 2,3,5-triphenyltetrazolium in PBS for 30 minutes for infarcted tissue discrimination. Because of the rapid maturation of ischemic damage in our model of ischemia, in most of the studies, infarct volume was determined 1 or 3 days after induction of ischemia. There were no differences in core body temperature and blood gases (pH, PO2, and PCO2; data not shown) between control animals and the groups receiving treatments, as determined at 60 minutes after initiation of the treatment.

Infarct Volume Analysis
Morphometric determination of indirect infarct volume based on 2,3,5-triphenyltetrazolium staining was measured using a computer-based image analyzer operated by “Brain” software (Drexel University).

Behavioral Measurements
All behavioral tests took place in a quiet dim room by an experimenter blinded with respect to the treatment groups. The footfault, forelimb placing, and postural reflex tests were performed as described previously. Neurological deficit score was measured before occlusion and 72 hours after MCA occlusion. Neurological deficit score (0 to 12) was calculated by combining the score on 3 tests.

Postural Reflex Test
The degree of abnormal posture was measured by suspending rats with their tails 10 cm above a tabletop. Intact rats extended both forelimbs toward the table surface. Rats displaying this behavior were recorded as having a score of 0. Rats that flexed only the contralateral limb toward the body were recorded as a score of 2. Rats rotating the contralateral forelimb toward the tail were graded as a 4.

Forelimb Forward Placing
Animals were held by their torsos with forelimbs hanging freely. Contralateral and ipsilateral forelimb forward-placing responses were induced by gently contacting the front side of the forelimb to the edge of a tabletop for 10 trials. The percentage of successful placing response rate was recorded as the animal successfully placed the respective forepaw on the top of the table. The neurological deficit score was calculated as the percentage of nonplacings × 4 (eg, deficit 0 = immediate and complete placing; deficit 2 = 50% placing; deficit 4 = no placing).

Footfault
Rats were placed on a grid, with openings of 2.3 cm2. As the animals traversed the grid, a footfault was scored each time the contralateral forepaw slipped through an opening in the grid. The total number of steps was also counted. The percentage of footfault was calculated as the number of footfaults/total steps × 100. A score of 0 to 4 was given to each animal according to the severity of the deficit by calculating the percentage of footfaults × 0.04.

Treatment Groups: Ischemic Stroke
Animals were divided randomly into 8 treatment groups: control: (1) saline control; (2) ethanol alone (0.32 g/kg); (3) caffeinol (10 mg/kg caffeine + 0.32 g/kg ethanol); (4) NMDA + ethanol (0.32 g/kg), and NMDA + caffeine (10 mg/kg). All the above doses were shown previously to have anti-ischemic effect, and all the treatments (unless indicated otherwise) were administered 15 minutes before infusion of NMDA, signifi-
Caffeinol was 43% smaller (P<0.05) than the lesion volume in the NMDA-alone group (Figure 1). Neither ethanol alone nor caffeine alone affected lesion volume produced by NMDA; the lesion volume in the ethanol-alone and caffeine-alone groups was 25.1±11.7 mm³ and 24.2±7.1 mm³, respectively.

**Reduction of Infarct Volume by NMDA Antagonist CNS-1102 Is Augmented by Caffeine**

Based on our previous work, we know that ethanol alone and caffeine alone has no benefit in our ischemic stroke model. However, when applied in combination, they exert robust anti-ischemic effect. Knowing that ethanol inhibits NMDA receptor/channel, we tested whether NMDA receptor antagonist (to substitute for ethanol) in combination with caffeine could also exhibit a synergistic anti-ischemic effect similar to what we found with caffeinol. Our first experiment established that in agreement with our previous data, CNS-1102, a noncompetitive NMDA receptor antagonist, reduced infarct volume by 41% compared with a saline-treated control group (127.2±14.7 versus 74.22±18.6 mm³) when administered 15 minutes after induction of stroke. Next, we established that caffeine alone has no effect on ischemic damage; however, the combination of caffeine with CNS-1102 produced far more robust reduction of infarct volume than CNS-1102 alone (Figure 2). The infarct volume was reduced by 91% (127.2±14.7 versus 10.55±4.76 mm³) in response to caffeine+CNS-1102 (Figure 2), indicating the synergistic effect.

**CNS-1102+Caffeine Has Long Window of Opportunity and Is Effective in Hypertensive Rats**

The objective of these experiments was to determine whether caffeine+CNS-1102, similar to caffeinol, has the extended window for effective treatment and if it can reduce infarct volume in a more severe model of ischemia in spontaneously hypertensive rats. By delaying the treatment, we established that the combination of caffeine and CNS-1102 is most effective if given early after the onset of the ischemia (Figure 3). However, caffeine+CNS-1102 provided 50% infarct volume reduction when treatment was delayed for up to 2 hours (Figure 3), thus being similar to the time window of opportunity demonstrated previously for caffeinol. Note that the 2-hour window of opportunity for our MCA/CCA occlusion model is the longest of any clinically relevant approaches tested in this model (data not presented). CNS-1102 alone is ineffective in reducing infarct volume when given 30 minutes after onset of MCA/CCA occlusion (127.2±14.7 mm³ for saline versus 129.5±33.7 mm³; n=5 for CNS-1102).

We also demonstrated that caffeine+CNS-1102 was effective in reducing infarct volume in spontaneously hypertensive rats (Figure 4).
MK-801, but not MgSO4, Combined With Caffeine Has Synergistic Effect in Protecting Brain From Ischemia

CNS-1102+caffeine has strong neuroprotective effect. To determine whether this neuroprotection is limited to CNS-1102 only, we tested other NMDA antagonists for their interaction with caffeine. For this study, we decided to use prototypic NMDA antagonist MK-801 and clinically relevant MgSO4, the agent proposed previously to demonstrate anti-ischemic effect through blocking NMDA receptor. Treatments were executed 30 minutes after induction of ischemia. Under these experimental conditions, the combination of caffeine and MK-801, in a similar fashion to CNS-1102, showed strong synergistic effect by reducing infarct volume by 84% (128.3±9.4 versus 20.2±9.2 mm³; Figure 5). Note that 30 minutes after treatment, MK-801 alone showed no effect on infarct volume and that the effect of MK-801+caffeine, although not statistically different, appeared to be more potent than caffeinol (Figure 5). Surprisingly, MgSO4, either alone or in combination with MK-801, had no effect on infarct volume (Figure 5).

Figure 5. Infarct volume after MCA/CCA occlusion in Long-Evans rats treated intravenously with: (1) saline; (2) ethanol (EtOH; 0.65 g/kg; groups 3 and 4) for 14 consecutive days before ischemia and then intravenously treated with: (1) saline; (2 and 3) caffeinol (0.325 g/kg ethanol and 10 mg/kg caffeine); and (4) 10 mg/kg caffeine+1 mg/kg MK-801) 30 minutes after the onset of MCA/CCA occlusion. Infarct volume was determined at 3 days. Data are expressed as mean±SEM. Number of animals per group is indicated above the bars. *P<0.05 from saline control.

Discussion

Except for thrombolysis with tissue plasminogen activator, there is no effective, approved treatment for ischemic stroke.15 We demonstrated recently that a low dose of ethanol+caffeine (caffeinol) produces remarkably potent neuroprotective effect in rodent models of ischemic stroke, and that this treatment can be administered safely to humans, including in combination with hypothermia. A randomized, placebo-controlled trial is needed to determine the efficacy effect of this combination in stroke patients.

Our present study was in part designed to investigate the mechanism of action of caffeinol with relevance to NMDA receptor inhibition. Although ethanol is known to have myriad biological effects, its role in NMDA receptor inhibition may have potential relevance to the pathophysiology of stroke. It is well established that ischemia causes a massive release of excitatory neurotransmitters and that various inhibitors of NMDA receptors are capable of reducing ischemia-induced damage in various models of ischemic stroke. The effect of ethanol on NMDA receptor inhibition appears to take place at low micromolar concentrations of ethanol and is primarily mediated via interaction with NR2B and NR2A subunits, the receptors that are abundant in stroke-affected cerebral cortex. Thus, it is intuitive to suggest that inhibition of NMDA receptor by ethanol could in part play an important role in neuroprotection.
role in the anti-ischemic effect of caffeinol. Unexpectedly, ethanol alone, instead of being neuroprotective, augmented ischemic damage\(^2\) (Figure 5), suggesting that in addition to its potential beneficial components associated with NMDA receptor inhibition, ethanol may produce some adverse effects that ultimately extinguish its beneficial effect. It could be, in part, that the role of caffeine in caffeinol is to ameliorate deleterious aspects of ethanol because caffeine alone has no beneficial role is our ischemia model.\(^2\) Ongoing studies are aimed at determining this effect of caffeine.

Nevertheless, our present experiments implicate the NMDA receptor as a component of the mode of action of caffeinol. First, we demonstrated that in analogy to what we have seen after ischemic stroke, caffeinol (but not ethanol or caffeine alone) was capable of reducing damage produced by intracortical infusion of NMDA, a well-validated model of excitotoxic damage. Although the antixcitotoxic effect of caffeinol was not as robust as its anti-ischemic effect, the resulting protection in this model was certainly substantial. Second, we demonstrated that ethanol in caffeinol could be successfully substituted by NMDA antagonist to deliver robust neuroprotection equivalent to that of caffeine. Specifically, we showed that caffeine, in combination with noncompetitive the NMDA antagonist CNS-1102 or MK-801, was capable of reducing infarct volume far more potently than the NMDA antagonist itself. We determined that the time window for effective treatment for the combination of caffeine+CNS-1102 is \(\geq 2\) hours. In our past experience with \(\geq 50\) different clinically relevant compounds, only caffeinol demonstrated a 2-hour window of opportunity for effective treatment in this model of ischemia.\(^2\) However, it was unexpected, that MgSO\(_4\), a compound with NMDA receptor blocking capacity, used at a concentration equivalent to the FAST-MAG pilot trial in humans and the range of concentration effective in experimental studies,\(^9\) alone or in combination with caffeine, did not show neuroprotective effect in our studies. Although we do not have an explanation for the lack of effect of MgSO\(_4\), a potential reason is that the treatment was initiated too late to offer any beneficial effect.

We previously demonstrated efficacy of MgSO\(_4\) in the same ischemia model with the treatment initiated 15 minutes after the MCA/CCA occlusion (Aronowski, unpublished results). Note that MK-801, under the same experimental conditions as MgSO\(_4\), also did not show efficacy, suggesting a short time window of opportunity for NMDA-blocking therapy. However, in contrast to MgSO\(_4\), which did not show effect in combination with caffeine, MK-801 in combination with caffeine reduced infarct volume by \(>80\%\) when applied under the same experimental conditions. It is possible that the lack of synergy with caffeine may suggest that the protective mechanism of action of MgSO\(_4\) may not involve modulation of NMDA.

In summary, our data add evidence supporting the neuroprotective activity of caffeine by demonstrating the effect of this combination on modulating NMDA receptor-mediated damage. Further, our data emphasize that the low dose of caffeine in the caffeinol combination plays a pivotal role in amplifying the antixcitotoxic activity of ethanol and suggests that similar doses of caffeine might be linked to other antixcitotoxic therapies to enhance their effect.

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Disclosures


References

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