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 ischemia⁴⁻⁵ and traumatic brain injury.⁴ Based on these promising preclinical studies, caffeinol was evaluated in stroke patients during a safety and feasibility study.⁵ 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.⁶⁻⁸ 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 antiexcitotoxic potential of ethanol is intriguing, our previous experience with ischemic stroke is that ethanol alone augments ischemic damage.² 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 caffeine 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).⁹

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 (36.5±0.5°C) was maintained by using a thermostatically controlled heating lamp. To produce an excitotoxic lesion, NMDA (20 nmols in 1 μL of saline) was injected under stereotactic 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+caffeine (10 mg/kg caffeine+0.32g/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 conditions offering potent anti-ischemic effect. Twenty percent of the treatment was delivered as a bolus 30 minutes before NMDA infusion, and the remaining volume was infused over 2.5 hours. MK-801 (3 mg/kg) was injected intraperitoneally 15 minutes before the onset of NMDA infusion. Forty-eight hours after the insult, rats were reanesthetized and perfused intracardially with ice-cold 2% buffered formaldehyde. The dissected brains were snap-frozen in −80°C 2-methylbutan. Hematoxilin and eosin–stained, 20-μm-thick serial coronal cryosections collected 200 μm apart were used for lesion volume determination. Lesion volume was determined morphometrically. The excitotoxic lesion volume represents the integration of all surface areas from all brain sections displaying signs of damage.

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 throughout 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 locus 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, caffeine did not affect blood pressure and other physiological variables including temperature, pH, PO2, and PCO2. After 180 minutes of ischemia, reperfusion was initiated either 15 or 30 minutes after the onset of stroke, as indicated. Unless indicated otherwise in the figure legends, 20% of each treatment was delivered as a bolus, and the remaining dose was infused over 2.5 hours.

Treatment Groups: Ischemic Stroke

Animals were divided randomly into 8 treatment groups + control: (1) saline control; (2) ethanol alone (0.32 g/kg); (3) caffeine (10 mg/kg caffeine+0.32 g/kg ethanol); (4) CNS-1102 (1.35 mg/kg); (5) CNS-1102+caffeine (1.35 to 0.12+6 mg/kg, respectively); (6) MK-801 (1 mg/kg); (7) MK-801+caffeine (1+10 mg/kg, respectively); (8) MgSO4 (67 mg/kg); and (9) control MgSO4+caffeine (67+10 mg/kg, respectively). Treatments were infused through the left femoral vein to reproduce conditions offering potent anti-ischemic effect, and all the treatments (unless indicated otherwise) were initiated either 15 or 30 minutes after the onset of stroke, as indicated. Unless indicated otherwise in the figure legends, 20% of each treatment was delivered as a bolus, and the remaining dose was infused over 2.5 hours.

Statistical Analyses

Statistical significance was determined by t test for comparison of 2 groups or ANOVA followed by the Newman–Keuls test for multiple-groups comparison.

Results

Caffeinol Reduces Cortical Damage Produced by Excitotoxic Insult

Excitotoxic cortical lesion was produced by infusing NMDA into the rat cerebral cortex as we described. The lesion volume in animals receiving NMDA alone was 29.1±7.5 mm3 (Figure 1). MK-801, a noncompetitive antagonist of the NMDA receptor, administered 15 minutes before infusion of NMDA, signif-
Caffeinol was 43% smaller (P<0.05 from saline) than the lesion volumes in the ethanol-alone and caffeine-alone groups were 25.1 and 11.7 mm³, respectively. The lesion volume in the NMDA-alone group (Figure 1) was 41% compared with a saline-treated control group (129.5 mm³). Neither ethanol alone nor caffeine alone affected lesion volume produced by NMDA; the lesion volumes in the ethanol-alone and caffeine-alone groups were 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).

**Figure 1.** Caffeinol reduces excitotoxic lesion volume in rat cerebral cortex in vivo. Cortical lesion volume (mm³) produced by 100 nM of NMDA in animals pretreated with IV administration of saline, caffeine (C/E; 0.325 g/kg ethanol and 10 mg/kg caffeine), ethanol (EtOH; 0.325 g/kg), caffeine (10 mg/kg), or MK-801 (3 mg/kg). The lesion volume was measured at 48 hours. The data are expressed as mean ± SEM. Number of animals per group is indicated above the bars. ∗P<0.05 from saline control. Figure illustrates the location of the NMDA infusion/lesion.

**Figure 2.** Infarct volume after MCA/CCA occlusion in Long–Evans rats treated with saline, CNS-1102 (CNS; 0.5 mg/kg bolus+0.345 mg/kg/h for 2.5 hours via IV infusion) or CNS-1102+caffeine (CNS+CAF; 0.5 mg/kg bolus+0.345 mg/kg/h CNS-1102+1.66 mg/kg bolus+3.33 mg/kg/h caffeine, over 2.5 hours). Treatment was started 15 minutes after onset of 180 minutes of ischemia. 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 all the other groups.

**Figure 3.** Infarct volume after MCA/CCA occlusion in Long–Evans rats treated with saline or CNS-1102 (0.5 mg/kg bolus+0.345 mg/kg/h for 2.5 hours) starting 15, 60, 90, or 120 minutes after 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.

**Figure 4.** Infarct volume as a function of time delay for CNS-1102 and caffeine in Long–Evans rats. **Note:** Data are expressed as mean ± SEM. Number of animals per group is indicated above the bars. ∗P<0.05 from saline control.
From Ischemia Has Synergistic Effect in Protecting Brain

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% (128.3/11006 9.4 versus 20.2/11006 36.6 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).

MK-801+Caffeine Is Not Sensitive to Ethanol-Induced Tolerance

Our previous studies demonstrated that chronic consumption of ethanol (but not caffeine) before ischemia significantly reduced the neuroprotective potency of caffeinol. Here, we tested whether chronic ethanol could also compromise the neuroprotective effect of MK-801+caffeine. Our study showed that ethanol pretreatment did not weaken the protective effect of MK-801+caffeine with respect to infarct volume reduction and neurological deficit score (Figure 6). In agreement with our previous study, pretreatment with ethanol significantly reduced the effect of caffeinol (Figure 6).

**Discussion**

Except for thrombolysis with tissue plasminogen activator, there is no effective, approved treatment for ischemic stroke. 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 damage2 (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 caffeinol. Specifically, we showed that caffeine, in combination with noncompetitive the NMDA antagonist CNS-1102 or MK-801, was capable of 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 ≈2 hours. In our past experience with ≥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 MgSO4, 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,19 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 MgSO4, a potential reason is that the treatment was initiated too late to offer any beneficial effect.

We previously demonstrated efficacy of MgSO4 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 MgSO4, also did not show efficacy, suggesting a short time window of opportunity for NMDA-blocking therapy. However, in contrast to MgSO4, 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 MgSO4 may not involve modulation of NMDA.

In summary, our data add evidence supporting the neuroprotective activity of caffeinol 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

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