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Hypothermia But Not the *N*-Methyl-D-Aspartate Antagonist, MK-801, Attenuates Neuronal Damage in Gerbils Subjected to Transient Global Ischemia

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Several laboratories have reported a significant reduction of ischemia-induced injury to hippocampal neurons in rodents treated with competitive and noncompetitive *N*-methyl-D-aspartate (NMDA) receptor-channel antagonists. This study examined the effects of the noncompetitive antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) in Mongolian gerbils subjected to 5 min of bilateral carotid artery occlusion. In adult female gerbils, single doses of MK-801 injected 1 hr prior to ischemia significantly ($p < 0.01$) reduced damage to CA1 hippocampal neurons. However, the drug rendered the postischemic animals comatose and hypothermic for several hours compared with the saline-treated animals. In subsequent experiments, animals pretreated with MK-801 and maintained normothermic during and after forebrain ischemia demonstrated no amelioration of hippocampal damage. Gerbils not treated with MK-801, but kept hypothermic in the postischemic period to approximately the same degree (34.5°C) and duration (8 hr) as was induced by MK-801 therapy showed significant ($p < 0.01$) protection of CA1 neurons against ischemia. The neuroprotective activity of MK-801 against transient global ischemia appears to be largely a consequence of postischemic hypothermia rather than a direct action on NMDA receptor-channels.

Pyramidal neurons in the CA1 zone of the hippocampus are particularly vulnerable to transient ischemia and die in a delayed fashion over hours to days in the rat (Pulsinelli et al., 1982), gerbil (Kirino, 1982), and human (Petito et al., 1987). Currently, there is no satisfactory explanation for either the marked vulnerability of these neurons to ischemia or for the temporal progression of their injury. A hypothesis to explain such injury (Meldrum, 1981; Francis and Pulsinelli, 1982; Jorgensen and Diemer, 1982) is based on the neurotoxic potential of the excitatory amino acid neurotransmitters (EAANs), glutamate, and aspartate (Olney, 1978). The EAANs are released in high concentrations following ischemia (Benveniste et al., 1984; Hagberg et al., 1985), and postsynaptic receptors of the *N*-methyl-D-aspartate (NMDA) class are present in high concentrations in the CA1 zone of hippocampus (Monaghan and Cotman, 1985). It is hypothesized that CA1 pyramidal neurons survive the transient ischemic insult, only to be subjected to excessive excitation in the aftermath of ischemia, eventuating in death. The molecular mechanism of such excitotoxic cell death is thought to be mediated by increased entry of calcium ions into the cell via calcium channels regulated by the NMDA receptor.

The development of lipid soluble noncompetitive antagonists of the NMDA receptor-channel has allowed *in vivo* experiments to test the above hypothesis. The potent noncompetitive NMDA antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate (MK-801) (Kemp et al., 1986; Wong et al., 1986) is an orally active anticonvulsant that has already undergone clinical trials for the treatment of epilepsy. In Mongolian gerbils subjected to 5 min of cerebral ischemia, various doses of MK-801 given 1 hr prior to ischemia (Gill et al., 1987) or after ischemia (Gill et al., 1988) markedly reduced pyramidal cell damage in the CA1 zone of the hippocampus. Protection against ischemia in gerbil hippocampus was also reported for animals treated with the competitive NMDA receptor antagonists cis-4-(phosphonomethyl)-2-piperidine-carboxylic acid (CGS 19755), 4-(3-phosphonopropyl)-2-piperazine-carboxylic acid (CPP), or 2-amino-7-phosphonoheptanoate (APH) (Boast et al., 1987, 1988) and for the noncompetitive antagonist, ketamine (Marcoux et al., 1988). Similar results in rat hippocampus were obtained for animals treated with APH (Swan et al., 1988), MK-801 (Church et al., 1988), or phencyclidine (Sauer et al., 1988). However, other studies of global brain ischemia found no neuroprotective effect of competitive (Block and Pulsinelli, 1987; Jensen and Auer, 1989) or noncompetitive (Jensen and Auer, 1988; Wieloch et al., 1988; Buchan and Pulsinelli, 1989) NMDA antagonists in rat hippocampus following 5–30 min of ischemia.

To date, studies in animal models of focal cerebral ischemia have consistently revealed that treatment with noncompetitive NMDA antagonists reduces cerebral infarct volume (Germano et al., 1987; Ozyurt et al., 1988; Steinberg et al., 1988). The periphery or "penumbral zone" surrounding the central core of severe ischemia is characterized by only partial blood flow reduction (Strong et al., 1983a), a mild reduction of high-energy phosphates (Selman et al., 1987; Kaplan and Pulsinelli, 1989), electrophysiological silence (Astrup et al., 1977), and cells with preserved morphology (Strong et al., 1983b) during the early hours of focal ischemia. Some have suggested that the patho-

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Table 1. Numbers of animals

	Exp. 1: Temp. monit.		Exp. 2: Temp. maint.		Exp. 3: Temp./Controlled		Exp. 4 Normoth.		Hypoth.
	Saline	MK-801	Saline	MK-801	Normoth.	Hypoth.	Saline	MK-801	MK-801
Entered	14	13	12	13	15	13	8	10	10
Deaths	6	5	8	6	6	5	2	6	4
	(43%)	(38%)	(66%)	(46%)	(40%)	(38%)	(25%)	(60%)	(40%)
Survivors	8	8	4	7	9	8	6	4	6

physiological conditions that distinguish the "penumbral zone" are particularly susceptible to NMDA receptor-channel blockade, thereby accounting for the consistent neuroprotective effect of these agents in focal cerebral ischemia (Siesjo and Bengtsson, 1989; Wieloch et al., 1989). However, since these very unique conditions of partial ischemia and partial reduction of high-energy metabolites are unlikely to exist in models that accurately mimic the conditions of global brain ischemia from cardiac arrest, the uniformly protective results reported with NMDA antagonists in focal ischemia may not apply to conditions of global brain ischemia.

The purpose of this study was to replicate the experimental conditions of global brain ischemia in gerbils where MK-801 therapy was consistently reported to be neuroprotective. Our goal was to determine whether the experimental conditions and/or species differences might help explain the discrepant results in gerbils and rats. Preliminary results of this study were published in abstract form (Buchan and Pulsinelli, 1989).

Materials and Methods

Forebrain ischemia. Female Mongolian gerbils weighing 50–70 gm were maintained on a 12 hr light:12 hr dark cycle and given free access to food and water. The animals were anesthetized with a 2% halothane: 70% N_2:28% O_2 mixture. The carotid arteries were isolated through an anterior midline cervical incision and lengths of 4-O surgical silk were loosely placed around both vessels and the wound closed. The animals were fasted overnight but were allowed free access to water. On the following day, the animals were lightly reanesthetized with halothane, the wound was reopened, and atraumatic clasps were attached to occlude both carotid arteries. The clasps were removed after 5 min, and the vessels were visually inspected for the absence of blood clots and the recovery of blood flow. The animals were allowed to survive for 5 d. Body temperature was monitored in all animals with a rectal thermistor and maintained at 38.5 ± 0.5°C in all groups during ischemia. In selected groups of animals, body temperature was maintained in the postischemic period at 38.5 ± 0.5°C with the rectal thermistor connected to a heat lamp; in other groups, body temperature was monitored only during this time.

Experimental paradigms. In *experiment 1,* separate groups of randomly selected gerbils were injected (i.p.) with a dose of 1 mg/kg of MK-801 or 1.0 ml/100 gm of normal saline, 1 hr prior to ischemia. Following drug or saline treatment, the animals were observed for any "drug effects," and their temperatures were monitored prior to ischemia, during carotid artery occlusion, and at the following times postischemia: 5, 10, 15, and 30 min; then hourly for 8 hr; and finally at 24 hr. The numbers of animals assigned to each group and their outcome are presented in Table 1.

Experiment 2 followed the exact same procedure as experiment 1, except that body temperature in all animals was maintained at 38.5 ± 0.5°C throughout the first 8 hr of cerebral recirculation.

In *experiment 3,* no drugs were given. Following 5 min of ischemia, half of the animals were maintained normothermic with heating lamps as per experiment 2, and half the animals were cooled to 34.5°C by placing them in a refrigerated room maintained at 4°C. A rectal temperature probe connected to a thermistor regulated a heat lamp and cooling fan so that body temperature was easily maintained at 34.5 ± 0.5°C. A small patch of fur was shaved from the backs of the animals to facilitate rapid heat transfer. After 8 hr of cerebral reperfusion, the animals were returned to room temperature, and their body temperature was allowed to recover to normal.

In *experiment 4,* we replicated studies 1 and 2 to ascertain whether the results were reproducible. Two groups of gerbils were treated with MK-801 (1 mg/kg, i.p.) 1 hr prior to ischemia and were either maintained normothermic or allowed to become hypothermic. These animals were compared with a single group of saline-treated (1.0 ml/100 gm) gerbils that were maintained normothermic. All animals were subjected to 5 min of bilateral carotid artery occlusion and allowed to survive 5 d postischemia.

Histological analysis. Five days after the ischemic insult, the animals were anesthetized with urethane and perfused transcardially with heparinized saline and then with a mixture of formaldehyde : acetic acid : methanol (1:1:8). Brains remained *in situ* overnight at 4°C and were then processed for paraffin embedding. Several coronal sections, 7 μm thick, were cut at the level of the dorsal hippocampus and stained with hematoxylin and eosin. Irreversible damage to CA1 neurons was graded, with the observers *blind* to the treatment group, according to the following scale (Brown and Brierley, 1968): 0, normal brain; 1, <10% of neurons injured; 2, 10–50% of neurons injured; and 3, >50% of neurons injured. Ischemic damage was accepted in any neuron showing "ischemic cell change" or "homogenizing cell change" (Brierley, 1976; Brown, 1977).

Data analysis. Mean grades of histological damage for each group (with SEs for graphic purposes) were calculated and differences between groups were statistically analyzed with the nonparametric Mann-Whitney *U* test. A Bonferroni correction for multiple comparisons was used to correct the statistical analysis of hippocampal damage in experiment 4. The mean body temperature for the various groups of animals was analyzed with repeated-measures analysis of variance.

Results

The numbers of animals entered into each study, and the mortality of the 4 experiments are presented in Table 1. On average, there was a 47% mortality in the population of gerbils subjected to 5 min of ischemia and saline injection. Animals treated with MK-801 sustained, on average, a 46% mortality. MK-801 induced a behavioral response consisting of hyperactivity followed by motor retardation. Body temperature in MK-801-treated animals was approximately 1°C higher than control animals prior to the ischemic insult.

The mean grades of histological damage to CA1 neurons for each of the groups and the mean body temperatures for these groups of animals are presented in Figures 1–4. Hippocampal CA1 damage was significantly less ($p < 0.01$) in the MK-801-treated than in saline-treated animals when body temperature was allowed to fluctuate (Fig. 1). However, the animals developed a statistically significant ($p < 0.005$) degree of hypothermia (Fig. 1) during the initial 8 hr of cerebral recirculation. When body temperature for all animals was maintained within the

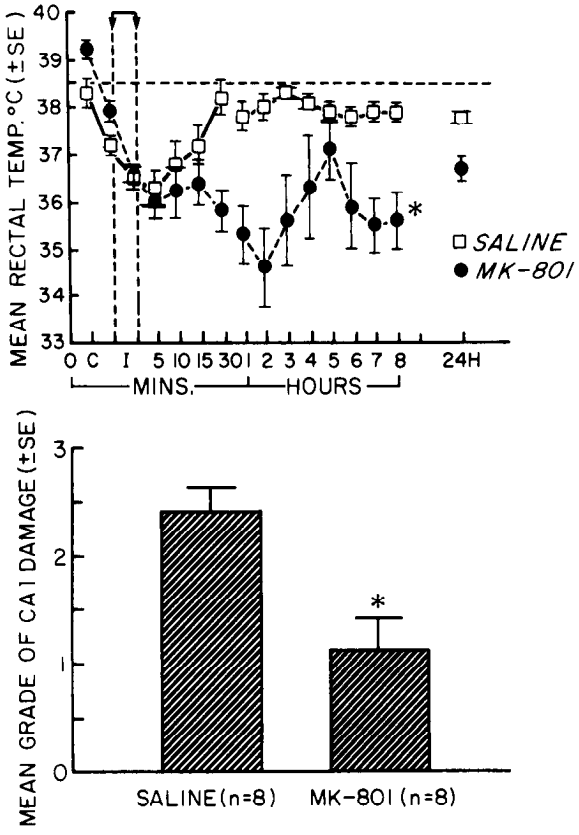

Figure 1. Mean rectal temperatures (*upper panel*) and grades of CA1 damage (*lower panel*) in experiment 1 animals. *C* signifies values 5 min prior to and *I* indicates values during bilateral carotid artery occlusion. Body temperature in the MK-801-treated group was significantly lower (*$p < 0.005$) than saline-treated animals. CA1 damage was significantly less (*$p < 0.01$) than saline-treated controls.

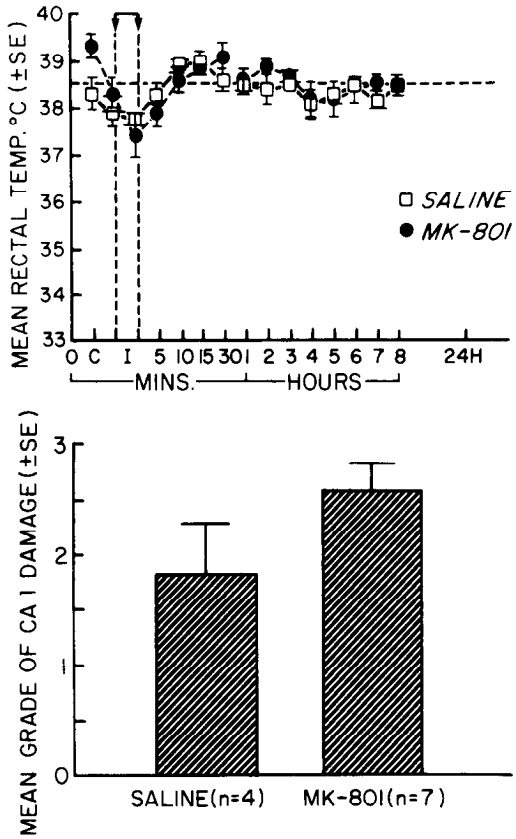

Figure 2. Mean rectal temperatures (*upper panel*) and grades of CA1 damage (*lower panel*) in experiment 2 animals. There was no difference in either body temperature or grades of injury in the MK-801 versus the saline-treated animals.

normal range, CA1 damage in MK-801-treated gerbils was slightly, but not significantly, greater than in the saline-treated group (Fig. 2).

Mean body temperature in gerbils subjected to cerebral ischemia plus MK-801 therapy fell to a nadir of 34.5°C at 2 hr postischemia, rose towards control values during the hours 4–5, and then declined once again (Figs. 1, 4). The differences in body temperature between saline- and MK-801-treated animals were approximately 2.5–3.0°C during the first 3 hr (Figs. 1, 4) and averaged approximately 2°C for the entire 8 hr of recordings. Postischemic hypothermia of the same duration (8 hr) but of slightly greater magnitude (4°C) than produced by MK-801 therapy, caused significant ($p < 0.01$) reduction of ischemic damage to CA1 neurons (Fig. 3). Experiments 1 and 2 were repeated (Fig. 4) and the same results were obtained.

Discussion

Results from this study demonstrate that systemically administered MK-801 given 1 hr prior to transient global ischemia protects the gerbil hippocampus against damage. Bilateral carotid artery occlusion in saline-treated gerbils produced transient hypothermia lasting approximately 15 min, but the combination of MK-801 plus forebrain ischemia was associated with a significant and prolonged hypothermic response lasting many hours. Prevention of the MK-801-related hypothermia resulted in a loss of the neuroprotective effect attributed to this noncompetitive NMDA antagonist. Hypothermia of the same duration but slightly greater magnitude than that produced by MK-801 treatment induced the same degree of neuroprotection in untreated animals.

These data are at variance with several studies in gerbils where therapy with competitive (Boast et al., 1987, 1988) and noncompetitive (Gill et al., 1987; Marcoux et al., 1988) NMDA receptor-channel antagonists lessened ischemic injury to hippocampal neurons. The duration of bilateral carotid artery ligation in the latter studies ranged from 5 min (Gill et al., 1987), to 10 min (Marcoux et al., 1988), to 20 min (Boast et al., 1987, 1988) compared with 5 min in our study. Mortality was reported only in the study by Marcoux et al. (1988), in which approximately 50% of the control animals died after 10 min of cerebral ischemia. The overall mortality for our saline-treated control populations was 47%. The similarity in the mortality between our study and that of Marcoux et al. (1988), together with the fact that Boast et al. (1987, 1988) used 20 min of bilateral carotid artery ligation, suggests that the degree of cerebral ischemia in our study was not extraordinarily severe and cannot explain our failure to observe neuroprotection by MK-801 therapy.

Despite the obvious importance of body and brain temperature in animal models of transient global brain ischemia, few studies testing NMDA receptor-channel antagonists in such models adequately report the conditions for maintaining body temperature. Studies which tested NMDA antagonists in gerbils subjected to bilateral carotid artery occlusion (Boast et al., 1987, 1988; Gill et al., 1987, 1988; Marcoux et al., 1988) presented

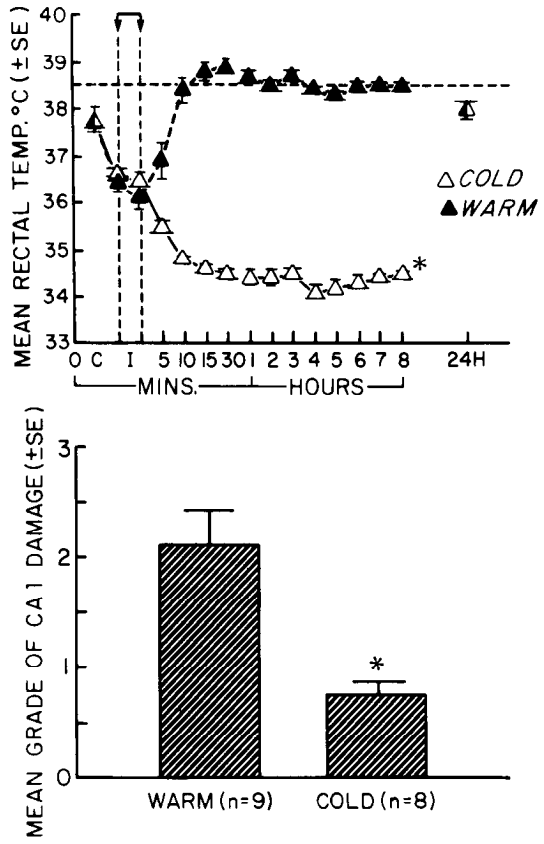

Figure 3. Mean rectal temperatures (*upper panel*) and grades of CA1 damage (*lower panel*) in experiment 3 animals. Animals placed in a 4.0°C cold room show a significant reduction of body temperature (*$p < 0.0001$) and a significant reduction of injury to CA1 hippocampus (*$p < 0.01$) compared with normothermic animals.

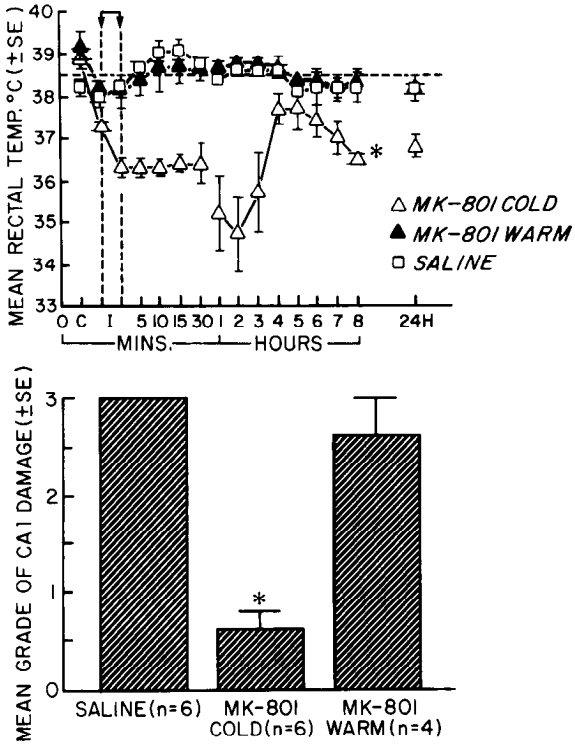

Figure 4. Mean rectal temperatures (*upper panel*) and grades of CA1 damage (*lower panel*) in experiment 4 animals. Animals treated with MK-801 and allowed to become hypothermic show a significant reduction in body temperature (*$p < 0.0001$) and a significant reduction in the mean grade of damage to CA1 neurons (*$p < 0.01$) compared with normothermic saline- and normothermic MK-801-treated animals.

no data on body temperature either during or following cerebral ischemia. While studies reporting neuroprotection against ischemia with NMDA receptor-channel antagonists in rat models of global brain ischemia (Church et al., 1988; Sauer et al., 1988; Swan et al., 1988) mention that body temperature was monitored, none clearly describe whether normothermia was maintained during ischemia and, equally as important, during the postischemic recirculation period.

Other efforts to protect the brain with NMDA receptor-channel antagonists against transient global ischemia have yielded results similar to the present study. Competitive (Block and Pulsinelli, 1987; Jensen and Auer, 1989) and noncompetitive (Jensen and Auer, 1988; Wieloch et al., 1988; Buchan and Pulsinelli, 1989) antagonism of the NMDA receptor-channel did not lessen damage to hippocampus in rats subjected to transient global ischemia. Therapy with MK-801 in cats (Fleischer et al., 1988), dogs (Michenfelder et al., 1989), and primates (Lanier et al., 1988) also failed to protect the brains of these animals against transient global ischemia. Available data either from the latter reports or from references to the animal models used in these reports indicate that normothermia was maintained during ischemia in each of these studies. With the exception of the studies performed in the rat, it is less certain whether body temperatures were maintained at a normal level after cerebral recirculation.

Bilateral carotid artery occlusion in the gerbil represents a simple, convenient model for testing pharmacologic agents in the treatment of transient global brain ischemia. However, as the results from our study show, failure to monitor physiologic variables, which have an important impact on the mechanisms of ischemic brain injury, e.g., body temperature, can lead to erroneous conclusions. It is difficult to make routine, serial measurements of important variables such as arterial pressure, oxygen, carbon dioxide, hydrogen ion, and glucose content in the gerbil because of its small body size and blood volume. Accordingly, while the gerbil may be useful for *screening* drugs, studies aimed at defining pharmacologic mechanisms of action are better served by larger models of global or focal ischemia, where appropriate physiological monitoring is both feasible and necessary.

Hypothermia has been used clinically for many years as prophylaxis against ischemic brain injury. Recent experimental studies (Busto et al., 1987) demonstrated that low *intraischemic* brain temperatures can protect brain neurons in rats subjected to transient forebrain ischemia. We show here that *postischemic* hypothermia of only a few degrees can have the same neuroprotective effect, although the mechanism is not clear. Hypothermia may alter a variety of neurochemical mechanisms, including high-energy phosphate metabolism (Welsh and Sims, 1989), pH (Norwood and Norwood, 1982), ion homeostasis (Lantos et al., 1986), neurotransmitter release (Busto et al., 1989), lipid-membrane degradation and blood–brain barrier permeability (Krantis, 1983).

Results of our study indicate that protection of CA1 hippocampal neurons by the noncompetitive NMDA antagonist, MK-

801, is related largely to the prolonged hypothermia caused by this drug in animals exposed to transient global ischemia. We do not suggest that drug-induced hypothermia is the sole explanation for the discrepant results among studies testing NMDA antagonists in global brain ischemia. One example among other potentially important factors causing such discrepancies is the experience of the investigator with reproducibly inducing cerebral ischemia below known thresholds for cell injury. Nevertheless, we conclude from this and other studies which failed to show protection of hippocampus by competitive and noncompetitive NMDA antagonists that excessive activation of the NMDA receptor is not singularly responsible for the selective vulnerability of CA1 neurons to transient global ischemia. Further studies are needed to determine whether excitation of the NMDA receptor in conjunction with other changes in synaptic neurotransmitter chemistry may still be important in selective ischemic injury to neurons.

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