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Antagonism of Ketamine-Induced Anesthesia by an Inhibitor of Nitric Oxide Synthesis: A Pharmacokinetic Explanation

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MUELLER, R. A. AND R. HUNT. *Antagonism of ketamine-induced anesthesia by an inhibition of nitric oxide synthesis: A pharmacokinetic explanation.* PHARMACOL BIOCHEM BEHAV **60**(1) 15–22, 1998.—Because ketamine is an antagonist of NMDA receptors, and because some NMDA receptors activate nitric oxide synthesis in brain, this study examined if nitric oxide synthase (NOS) inhibition by L-NAME altered the course of ketamine-induced behavioral impairment. Rats given progressive doses of L-NAME until NOS activity was inhibited at least 90% displayed reduced depth and duration of behavioral depression after IM ketamine. Blood and brain concentrations of ketamine, norketamine, and its dehydrogenated derivative were isolated from rats previously given saline or L-NAME as above, by ether extraction, HPLC separation, and ultraviolet quantitation. The same doses of L-NAME that altered ketamine behavior reduced blood and brain ketamine concentrations 15 min after administration to about three-fourths and one-third of control, respectively. The content of norketamine and its adventitial extraction product were similarly reduced relative to control but the ratio of metabolites to ketamine was not significantly altered ($p > 0.05$) in brain. The decreased delivery of ketamine into brain, perhaps due to L-NAME–induced alterations in blood flow, may explain the reduced behavioral response to ketamine in these rats. © 1998 Elsevier Science Inc.

Blood flow Drug metabolism Ketamine Nitric oxide Nitric oxide synthase L-NAME Norketamine Pharmacokinetics

KETAMINE is an intravenous anesthetic that produces an increase in thalamocortical seizure-like EEG activity at clinically relevant doses (29). The mechanism by which this change is produced remains unclear, but this electrical hypersynchrony is accompanied by profound analgesia and amnesia (10). Neurochemical investigation have uncovered a variety of perturbations after ketamine administration, but one of the most repeatable is ketamine's ability to act as a noncompetitive inhibitor of *n*-methyl-D-aspartic acid (NMDA) receptors (4). This action would be expected to decrease excitatory transmission at synapses dependent on these ligand gated ion channels, thus blocking Na^+ and Ca^{++} entry into the cell (27). In addition to changes in ion flux, other second messengers linked to NMDA receptor occupation may also be altered. There is now compelling evidence that some of the transmitter and toxic effects of NMDA receptor activation are linked to synthesis and release of nitric oxide (NO) (18). If those NMDA receptors blocked by ketamine are linked to NO synthesis and release, inhibition of Type 1 NO synthase (NOS) in the brain would be expected to enhance the anesthetic effect of ketamine. In addition there are also some reports that ketamine may directly inhibit NOS activity in vitro, and this could also contribute to ketamine's behavioral effects (14,15), as could additional non-NMDA modulation of NO availability (19,23,32,37).

The current studies were designed to examine if nitro^G-L-arginine methyl ester hydrochloride (L-NAME) a potent competitive inhibitor of NOS (12), would potentiate the anesthetic effect of ketamine in rats. Our results suggest that L-NAME at doses sufficient to block 90% of CNS NOS antagonizes rather than potentiates the anesthetic effect of ketamine. Further, this antagonism may be due to decreased absorption of ketamine into blood and/or decreased delivery of ketamine to the CNS, perhaps as a result of reduced peripheral and cere-

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TABLE 1
BEHAVIORAL RATING SCALE FOR KETAMINE

1. Normal (sleep, alert, eating, moving)
2. Unsteady gait
3. Immobile with abnormal posture when resting but moves with stimulus
4. Immobile, does not move more than one step with stimulus but rights self
5. No longer rights self when placed on back but moves to tail pinch
6. No movement to tail pinch
7. Respiratory problems (slow, gasping, blue color)
8. Dead

Assessments were made at 15-min intervals after time of ketamine injection, except for determination of the loss of righting reflex, which was assessed at 1-min intervals for the first 5 min, and 2-min intervals for the next 10 min.

bral blood flow. A preliminary report of these findings has appeared elsewhere (31).

METHOD

Animal Preparations

Experiments (approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill) were performed in male or female Sprague–Dawley rats from Charles River. In any single study the number of males and females was equal in control and experimental groups, and within each sex, weight matched as well. No animals were fasted before use. The behavioral scoring system used was similar to that used by Boast et al. (Table 1) (7). Animals were observed and tested for loss of righting reflex at 60-s intervals (first 5 min) or 2-min intervals (5–15 min) after drug administration to establish the onset of loss of righting ability. At subsequent 15-min intervals after drug administration a behavioral assessment was made until return to baseline activity or 2 h had elapsed. Tail pinch was delivered with a 6 inch Kocher clamp, tightened one notch, half-way from the end of the tail. Subsequent testing was done more proximal to the previous pinch (22). The skin was never broken with this technique. Behavioral scores of control and pretreated rats were compared using a Wilcoxon ranked sum test (26).

Drugs

L-NAME was obtained from Sigma Chemical Co. (St. Louis, MO). Ketamine HCl (0.1 mg benzethonium chloride/100 mg ketamine) was purchased from Parke–Davis (Ketalar), Morris Plains, NJ. Rats were given indicated doses of ketamine or L-NAME in a volume of 2 ml/kg saline; controls received saline alone. ^{3}H-arginine L-[2,3-^{3}H] 35.7 c/m mole was purchased from Dupont-NEN (Boston, MA).

Measurement of (NOS) Activity

In vitro activity was measured by the method of Bredt and Snyder (8). Animals were killed by decapitation and the brain rapidly removed and placed on crushed ice. After dissection and weighing of designated areas tissues were either frozen on dry ice and kept in a freezer (−40°C) until analysis, or were homogenized in 10 vol of 20 mM Tris pH 7.4, 2 mM EDTA with ground glass tubes with pestles. After centrifugation at 10,000 × *g* for 15 min the supernatant (50 μl) (caudate) or a one-fifth dilution of other brain areas were assayed in triplicate. After addition of 25 μl of 3 mM $CaCl_2$, the reaction was started with 50 μl of 1 mM NADPH and ^{3}H arginine (0.1 μc/tube) and incubated for 15 min at 25°C, and was stopped with 3 ml iced HEPES buffer (20 mM HEPES pH 5.5, containing 2 mM EDTA). Samples were then transferred to Na^+ Dowex columns, 2.5 cm × 6 mm. The first 3 ml of effluent and a wash of 1.0 ml of the HEPES/EDTA solution were collected in a scintillation vial and radioactivity (85% as ^{3}H citrulline by HPLC), measured in an LKB liquid scintillation counter.

Measurement of Ketamine in Blood and Brain

The method used was adapted from that of Adams et al. (3). After decapitation, trunk blood was collected in heparinized centrifuge tubes on ice, mixed, and centrifuged (2000 × *g* for 10 minutes) to remove cells. Etidocaine (1 μg) was added as an internal standard to a plasma aliquot (85–91% recovery), which was frozen for later assay. Brain areas were rapidly dissected on ice, weighed, and frozen at −40°C until later homogenization in 0.4 N $HClO_4$, removal of the 10,000 × *g*—15-min supernatant, and addition of the etidocaine internal standard. Plasma or tissue supernatants were then alkalinized with 1 N NaOH to a pH greater than 11.0, extracted with 5.0 ml diethylether, and frozen in dry ice or liquid nitrogen. The ether layers were decanted into extraction tubes containing 250 μl

TABLE 2
EFFECT OF L-NAME ON THE BEHAVIORAL EFFECTS OF KETAMINE

Ketamine Dose (mg/kg/im)	Group	Behavioral Scores After Ketamine (Minutes)							
		15	30	45	60	75	90	105	120
25	Control	2 ± 0	1.4 ± 2.5	1.2 ± 0.2	1 ± 0				
	L-NAME	2 ± 0	1.6 ± 0.25	1.4 ± 0.25	1 ± 0				
50	Control	3.0 ± 0.44	2.6 ± 0.4	1.6 ± 0.25	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	1.0	
	L-NAME	3.0 ± 0.63	1.8 ± 0.2	1.8 ± 0.2	1.6 ± 0.25	1.4 ± 0.25	1 ± 0		
75	Control	5.0 ± 0.31	3.8 ± 0.73	2.4 ± 0.67	2.4 ± 0.67	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2
	L-NAME	5.2 ± 0.18	5.0 ± 0	4.2 ± 0.52	3.4 ± 0.6	2.4 ± 0.67	2.2 ± 0.5	1.4 ± 0.25	1.4 ± 0.25

Rats were given 20 mg/2ml/kg L-NAME IV or 2 ml/kg saline IV (control) 30 min before ketamine. Behavioral scores were noted at 15-min intervals after ketamine injection. The same groups of rats received the same preketamine injection on successive days. $n = 5$ in each group.

TABLE 3
INHIBITION OF BRAIN NITRIC OXIDE SYNTHASE ACTIVITY BY L-NAME

Experiment	Treatment Group	Brain Area		
		Cerebellum	Caudate	Cortex
1	Control	0.252% ± 0.026%	0.073% ± 0.009%	0.166% ± 0.021%
	L-NAME*	0.126% ± 0.008%	0.040% ± 0.003%	0.071% ± 0.007%
	% Control	(50%)	(55%)	(42%)
2	Control	0.399% ± 0.017%	0.097% ± 0.006%	0.169% ± 0.009%
	L-NAME†	0.048% ± 0.006%	0.009% ± 0.001%	0.016% ± 0.001%
	% Control	(12%)	(10%)	(9.6%)

All values represent the percent conversion (mean ± SEM) of ^{3}H-arginine to ^{3}H-citrulline in five to seven animals. Values in brackets are the % of control values in that experiment. All values in all three brain areas in both experiments are significantly less than control values ($p < 0.01$).

*Animals received 20 mg/kg IV L-NAME, 2 days, 1 day, and 30 min before being killed.

†Animals received 50 mg/kg sq L-NAME 48 h, 24 h, and 30 min before being killed.

0.05 N H_2SO_4, mixed, frozen as above, and the ether layer discarded. The HPLC system and detector as well as assay variability used to quantitate the ketamine and metabolites in the aqueous residue was as described by Adams et al. (3).

Statistics

The mean values of NOS activity and ketamine concentration were compared with a two-tailed *t*-test (36). Significance was declared if $p < 0.05$.

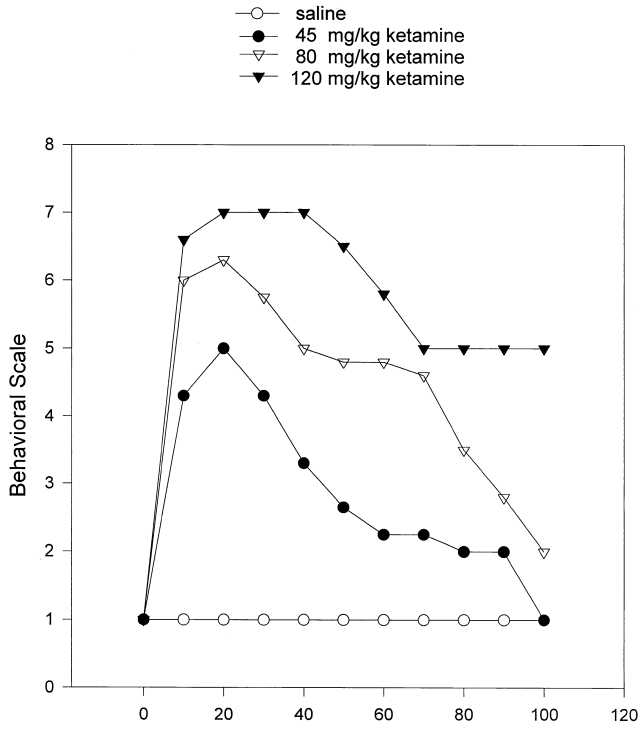

FIG. 1. Behavioral responses of rats to ketamine. Each point represents the average of six to eight rats given ketamine at zero time.

RESULTS

Inhibition of Brain NOS

Initial studies attempted to use acute intravenous administration of L-NAME to inhibit NO synthesis immediately prior to ketamine administration. Because Johns et al. (22) had previously reported that bolus doses of 30 mg/kg IV produced cardiovascular instability, we began with 20 mg/kg L-NAME IV followed 30 min later by IM ketamine 25 mg/kg. Control rats received an equivalent volume of 0.9% NaCl 30 min before ketamine. Behavioral responses (Table 2) were measured until values had returned to baseline, and the rats were allowed to recover in their home cages. The next day the same rats were given L-NAME or saline IV 30 min before 50 mg ketamine IM, and behavior assessed as above. On the third day 75 mg/kg ketamine IM was given, again 30 min after L-NAME or saline as above. These studies failed to uncover any differences between L-NAME and saline-pretreated rats in the time to development of an unsteady gait, loss of righting reflex, or in the duration of behavioral impairment produced by ketamine (Table 2). In a separate group of rats this 3 consecutive-day dosage schedule of 20 mg/kg L-NAME was successful in reducing brain NOS in vitro activity by 45 to 58% ($p < 0.01$, see Table 3). We assessed NOS activity in the cerebellum because it had previously been shown that this region of rat brain had the highest NOS activity (13). The striatum and cerebral cortex were assessed because ketamine produces cerebral cortical hypersynchrony, and ketamine-induced hallucinations in humans are antagonized by droperdol (34), a dopamine type 2 antagonist, and the striatum is richest in dopamine.

Because some NMDA receptors could be linked to the appreciable proportion of still active NOS, a schedule was developed to produce a greater inhibition of the enzyme. After examination of several dosing routes, intervals, and sizes, a final choice of 50 mg/kg of L-NAME SQ 48, 24, and 1/2 h prior to use for ketamine testing was selected. This schedule reduced regional brain NOS in vitro activity to 12% in the cerebellum and 10% in the caudate nucleus and parietal cortex, (Table 3) without any overt behavioral changes or death of animals that evidenced near-normal weight gain after L-NAME administration.

Behavioral Effect of Ketamine

The behavioral dose–response curves of large rats (310–425 g) to three doses of ketamine are shown in Fig. 1. The de-

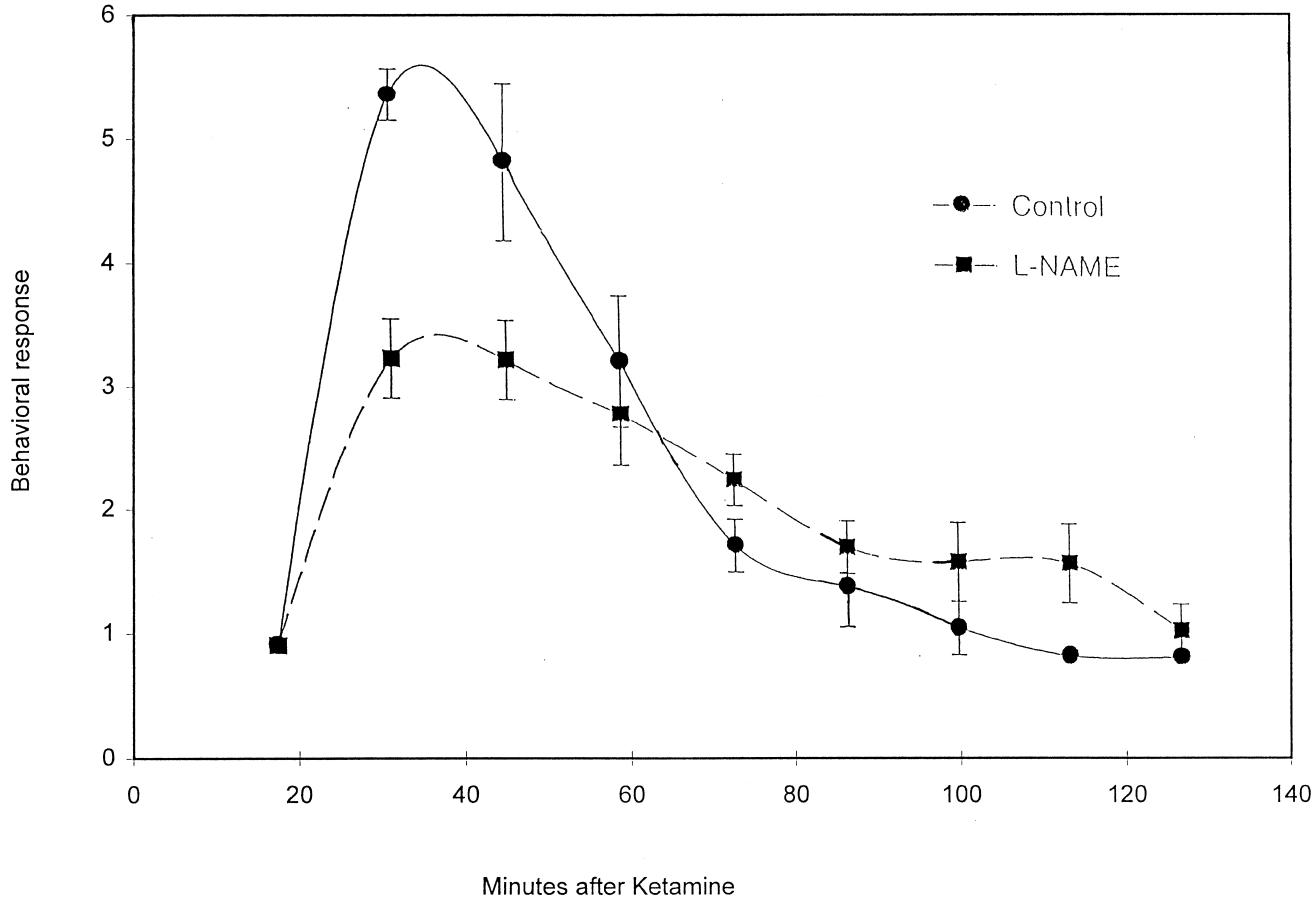

FIG. 2. Altered time course of behavioral effects of ketamine after L-NAME. Each point represents the mean ± SEM (brackets) of 6–12 rats. Experimental rats received L-NAME 48, 24, and 1/2 h before ketamine 75 mg (IM). Control rats received saline in a similar schedule before ketamine 75 mg/kg (IM). Only values at 15 and 30 min time periods in L-NAME–treated rats are significantly different ($p < 0.05$) from controls.

gree and duration of behavioral changes observed in smaller rats (160–235 g) given 75 mg/kg ketamine IM are shown in Table 2 and Fig. 2. This dose of ketamine produced a brief (15–30 min) loss of righting reflex in all saline-pretreated rats with behavioral values returning to normal by 90 min. The responses to 75 mg/kg are not maximal responses (see Fig. 1). Rats given L-NAME alone in the 3-day dosing regimen of 50 mg/kg SQ described above demonstrated no behavioral impairment. When ketamine (75 mg/kg) IM was given to rats after the above NOS inhibitor schedule, none of them ever lost their righting reflex or even became immobile. The marked differences in behavioral scores between the control and experimental groups at the 15- and 30-min evaluation points were no longer significantly different at 45 min or later time periods. Thus, the behavioral response to ketamine was antagonized rather than potentiated as hypothesized. Larger doses of ketamine (100 or 125 mg/kg IM) did produce loss of righting reflex in L-NAME–treated rats, but the behavioral scores were still significantly less than those of control rats at several later time intervals (data not shown).

Measurement of Blood and Brain Ketamine Concentrations

To examine if the antagonism of ketamine's behavioral impairment by L-NAME might be pharmacokinetic in nature, similar groups of rats were given saline or L-NAME 48, 24, and 1/2 h before ketamine 75 mg/kg IM and killed 15 min later, the time interval at which the behavioral effects were most disparate from those of saline-pretreated rats. As seen in Fig. 3, the brain concentrations of both ketamine and two of its metabolites were all significantly less in the L-NAME–treated rats. Plasma concentrations were also significantly less in L-NAME–treated rats than in control animals. Measurements of plasma and brain concentrations 1 h after ketamine, when behavioral scores were similar, were not significantly different in L-NAME-treated and saline-treated rats (data not shown). The identity of the two metabolite peaks was not verified by isolation and characterization but in the HPLC procedure used by us and by Adams et al. (3), metabolite 1 was shown to be nor-ketamine, and metabolite 2 the adventitial dehydrogenated derivative of nor-ketamine. Repeating the experiment with SQ instead of IM ketamine produced essentially similar results in cerebellum and plasma (Fig. 4).

DISCUSSION

Our original hypothesis was that since ketamine is an antagonist of CNS NMDA receptors (6,37) and some NMDA receptors appear to be linked to NO synthesis (18), an apparent potentiation of ketamine–anesthetic effects–would be

observed in rats with inhibition of NO synthesis. Using an electrochemical measure of extracellular NO concentration, Lin et al. have reported that ketamine (50 mg/kg/IP) reduces the increase in extracellular in situ NO that occurs after transient middle cerebral artery occlusion (25). Although the mechanism of inhibition of NO production by ketamine in this pathological state remains open, this effect of ketamine could take place via inhibition of NMDA-linked NOS activity, or the drug may also directly alter NOS catalytic activity in a more direct way. There are reports in the literature of a direct effect of phencyclidine, a chemical and neurochemical congener of ketamine, on NOS activity. Osawa and DaVila reported that phencyclidine can function as a suicide inhibitor of rat brain cytosol NOS in vitro, thus producing an irreversible inhibition of the enzyme (33). Very high concentrations of ketamine in vitro (0.1–1 mM) do inhibit human polymorphonuclear leukocyte NOS activity by 33 to 42%, but the inhibition was not proportional to ketamine concentration, and the effective concentrations of ketamine far exceeded anesthetic human plasma concentrations (7 μM) (15). More recently, Galley and Webster have demonstrated that ketamine at concentrations as low as 10 nM can inhibit neuronal rat NOS, but they used an in vitro assay without tetrahydrobiopterin cofactor; thus, sensitivity may be considerably different at physiologically relevant concentrations of the reduced form of this essential cofactor that may have been removed, along with arginine, from their cytosolic NOS preparations (14). In neuronal cell cultures ketamine (10–100 μM) inhibited the increase in cGMP (a possible index of NO production but also responsive to other neurochemical inputs) provoked by activation of glutamate receptors (19). An opposing conclusion was reached by Tobin et al., who found that rat cerebral cytosol NOS preparations were not inhibited by ketamine (up to 0.1 mM) (38). Bansinath et al. reported in an abstract that NOS inhibitors enhanced the behavioral depression of intravenous anesthetics, including ketamine, but the abstract was not delivered at the meeting, and we can find no subsequent publication of these observations (5).

Thus, either on the basis of NMDA receptor or NOS enzyme antagonism, we had expected the behavioral effects of ketamine to be enhanced by prior L-NAME administration. Our results, however, clearly indicate that L-NAME rather than potentiating, instead antagonized the anesthetic effects of ketamine after IM administration. Okamoto et al. recently demonstrated that the centrally initiated increase in renal sympathetic nerves of rabbits given ketamine was not increased

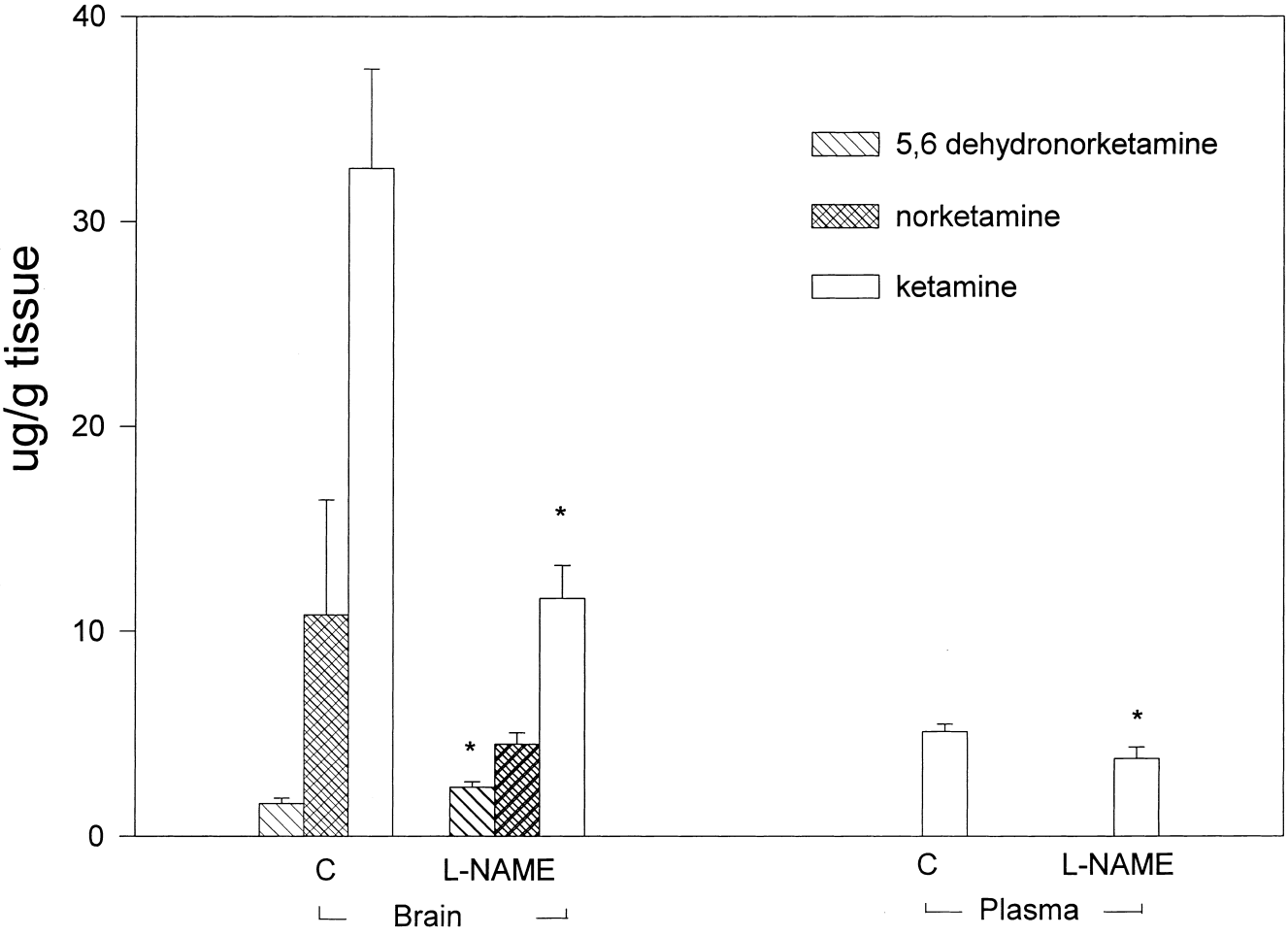

FIG. 3. Ketamine and metabolites in whole brain and plasma after intramuscular injection. Rats were given ketamine (75 mg/kg IM) 30 min after the last dose of saline (C) or L-NAME and killed 15 min later (see the Method section). Each column represents the mean ± SEM of five to eight animals. *$p < 0.05$) relative to similar measurement in control rats.

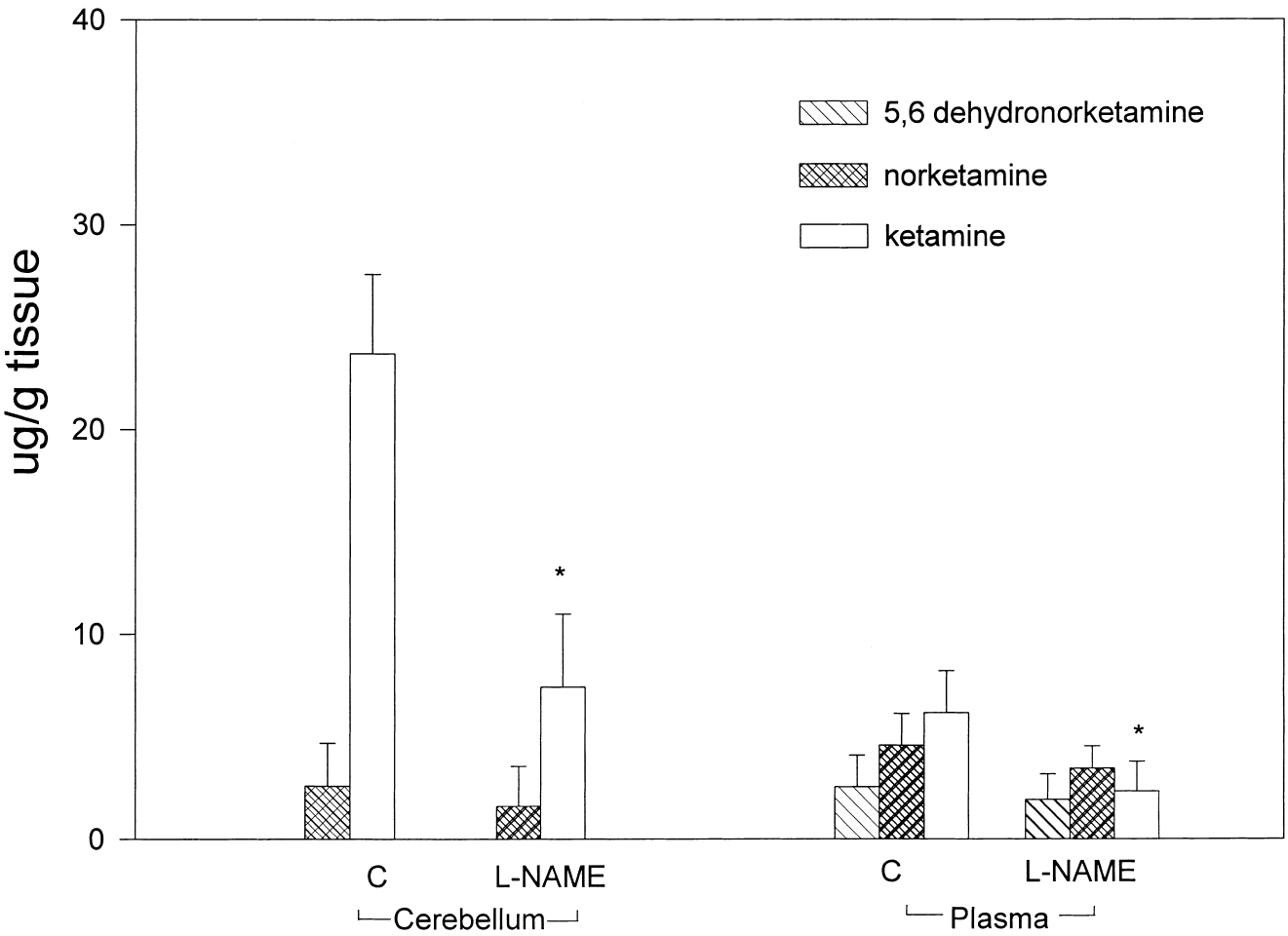

FIG. 4. Ketamine and metabolites in cerebellum and plasma after subcutaneous injection. Rats were given ketamine (75 mg/kg SQ) 30 min after the last dose of saline (C) or L-NAME and killed 15 min later (see the Method section). Each column represents the mean ± SEM of five to eight animals. *$p < 0.05$ relative to similar measurements in control rats.

further after prior exposure to L-NAME (ketamine response after L-NAME compared to ketamine response in similarly operated animals not given L-NAME) (32). In this paradigm as well, decreased delivery of ketamine by prior L-NAME administration may have prevented delivery of similar amounts of ketamine to the central site of ketamine-induced increase in sympathetic outflow (L-NAME had elevated the arterial blood pressure by the time ketamine was administered, probably reflecting altered vascular perfusion). Alternatively, the ketamine induced increase in sympathetic outflow may not be entirely due to inhibition of NOS in the brain, or other mechanisms may be involved (9).

L-NAME is a potent inhibitor of all types of NOS activity (12) and, thus, one would expect antagonism of vascular endothelial NOS as well as CNS neuronal NOS to be produced. Chronic administration of L-NAME has been shown to increase peripheral vascular resistance in a model of hypertensive disease (17,39), and acute and chronic administration can decrease blood flow in some paradigms (16,20,35). Conversely, Morikawa et al. have demonstrated that administration of arginine produced an increase in regional cerebral blood flow (30). Wei et al. have reported that administration of even small doses of L-NAME to conscious rats increased vascular resistance in all 12 measured brain areas of conscious rats by an average of over 80% (39). It is, therefore, possible that the apparent antagonism of ketamine-induced anesthesia by L-NAME may be due to reduced blood flow to both the site of administration of ketamine and to its site(s) of action in the brain.

The measurement of blood and brain contents of ketamine and its proximate metabolites support the above-proposed pharmacokinetic explanation of the antagonism of ketamine-induced behavioral impairment by L-NAME. Because blood concentrations were only 75% of control and brain concentrations 36% of control values after IM administration, it would appear that both the rate of absorption of ketamine into blood after injection, as well as the uptake of ketamine from blood into the brain are reduced by L-NAME. The single time point values of the present study after a bolus IM or SQ dose of ketamine do not permit calculations of either rate of entry into blood or brain in control or L-NAME–treated rats, however. Our study does not address if blood flow reduction is the sole cause of this reduced delivery into brain, or if uptake and/or diffusion of ketamine into brain are also impaired. Because the precise site in the CNS at which ketamine acts to produce behavioral impairment is unknown, we did not attempt to

measure brain content of ketamine in other brain regions or at larger doses of ketamine in L-NAME-treated rats.

Unfortunately, the confounding effects of L-NAME on blood flow prevent us from assessing if NO is or is not involved in communicating the consequences of NMDA blockade in brain, but with 90% inhibition of NOS it would seem unlikely. If one could measure ketamine concentrations at the NMDA receptor to assure equivalence (using a larger dose of ketamine in L-NAME–treated than in control rats) one could theoretically address the question. Finally, it would appear that the availability of vascular NO may play a significant role in the delivery of CNS active compounds, and that the use of L-NAME and perhaps other NOS inhibitors of limited specificity to discern the role of NO in physiological or pharmacological CNS investigations must be rigidly controlled to compensate for its effects on blood flow. The newer use of more selective inhibitors of neuronal NOS may help resolve these questions.

It is presently unclear if other anesthetic-induced alterations in pain perception are related to NO availability. Although Johns et al. (22) reported that intravenous L-NAME reduced the MAC of halothane to tail-pinch stimulus in rats, Adachi et al. (1) were unable to confirm this observation. Neither group measured the degree of NOS inhibition present in the CNS after single-dose acute administration of L-NAME to their animals, nor brain concentrations of anesthetic, although both did see an increase in blood pressure after IV L-NAME administration, suggesting similar effective inhibition of at least vascular NOS. In both studies each rat was given L-NAME after a control value for that rat's MAC had been measured. One would expect subsequent CNS blood flow to be reduced after L-NAME administration as reviewed above. Thus, if the same time intervals between tail clampings was allowed for "equilibration" [12–15 min in the Johns et al. study (22)] the actual CNS anesthetic partial pressure may not have decreased as far (to the new lower vaporizer setting) after L-NAME, as it did in the control pre-L–NAME MAC determinations. In the Adachi (1) study, 30 min was allowed between determinations of tail-pinch response, and this additional time may have allowed a closer approach of CNS halothane partial pressure toward the new lower vaporizer settings after L-NAME administration. Thus, the effect reported by Johns et al. may have also been due, like the effect of L-NAME on ketamine depression in the present study, to changes in CNS blood flow. Recently Ichinose et al. (21) have reported that mice congenitally deficient in neuronal NOS did not evidence an increased sensitivity to the inhalational anesthetic isoflurane, yet acute L-NAME administration did increase the sensitivity of these mice to the anesthetic effects. This latter observation may also have a pharmacokinetic basis as in the present study, or be due to other effects of L-NAME on brain function, or indicate that endothelial NOS may compensate for the loss of neuronal NOS activity.

Similar pharmacokinetic concerns may be important for injectable as well as inhalational drugs. Thus, several authors have shown that the ability of naloxone to precipitate withdrawal is reduced in morphine-tolerant rats when L-NAME has been given systemically just before or for several days before naloxone (2,24,28). If less naloxone were being absorbed and/or delivered to the CNS after L-NAME, such results would be expected. Similarly, rats given intrathecal L-NAME exhibit a prolonged analgesic effect to subsequent intrathecal morphine (11). Reduced local blood flow to the CNS by L-NAME could retard uptake into the systemic capillaries, thus increasing the residence time of morphine in the CNS. More meaningful interpretation should be possible if either changes in blood flow or actual drug levels at the site of action could complement the behavioral studies of such interactions. Notably, in none of the above studies was the degree of NOS inhibition produced by L-NAME actually measured.

We would caution others that pharmacokinetic as well as pharmacodynamic explanations for interactions between NOS inhibitors and CNS active drugs should be considered in interpretation of their data.

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