The Role of Inducible-Nitric Oxide in Cocaine-Induced Kindling

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Experimentally naive male Sprague Dawley rats (weighing 85–110 g) were used to examine the role of inducible nitric oxide synthase (iNOS) in cocaine-induced kindling. Repeated administration of cocaine (45 mg/kg, ip) to Sprague Dawley male rats for 7 consecutive days produced a progressive increase in the convulsive responsiveness and death. Pretreatment with iNOS inhibitors, L-N^6-(1-iminoethyl)lysine (NIL; 10 mg/kg, ip) and (−)-epigalloocatechin gallate (EGCG; 10 mg/kg, ip) 30 min before cocaine (45 mg/kg, ip) administration for 7 days attenuated the development of cocaine kindling and blocked cocaine-induced death. Results of NMDA receptor binding assay in the hippocampus showed a significant increase in the affinity without changes in the density in animals treated with cocaine, but there were no changes in these parameters in the cortex. Pretreatment with NIL or EGCG prior to cocaine administration abolished the cocaine-induced effect in the NMDA receptor affinity in the hippocampus. These results suggest that iNOS induction followed by an increase of NMDA receptor affinity in the hippocampus after repeated exposure to cocaine may participate in the process of the development of cocaine kindling.
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Key words: cocaine; kindling; NMDA receptor; seizures; iNOS; nitric oxide; hippocampus

Cocaine, a powerful psychostimulant drug of abuse, has a number of pharmacological properties, such as the development of behavioral sensitization, seizures, and lethal convulsion. In animal models, repeated administration of subthreshold doses of cocaine results in an increased sensitivity to the convulsive response of seizures (1, 2). This is referred to cocaine kindling (3). Because ischemic brain infarction, seizures, and death (4, 5) are frequently reported in humans abusing cocaine, this phenomenon is thought to be relevant to the neurotoxicity of this drug (6). The development of cocaine kindling in animal models has been used to study the neurobiological process involved in cocaine-induced neurotoxicity (7).

The underlying neurobiological mechanisms behind cocaine kindling have not been fully elucidated. Recently a number of studies have demonstrated the involvement of the N-methyl-D-aspartate (NMDA) receptor in the development of cocaine kindling. For instance, the NMDA agonist, N-methyl-D,L-aspartate (NMDLA) has been shown to produce seizures in mice (3), and the number of NMDA receptor-binding sites in the cortex was significantly increased in cocaine-kindled animals (6). Pretreatment with NMDA antagonists abolished the development of sensitization to cocaine-induced seizures (8, 9).

Nitric oxide (NO) is synthesized in the CNS by two isoforms of nitric oxide synthase (NOS), which converts L-arginine to L-citrulline: the constitutive Ca^{2+}/calmodulin-dependent neuronal NOS (nNOS) and the inducible Ca^{2+}-independent NOS (iNOS) (10). NO is a potent stimulator of guanylcyclase followed by the elevation of cyclic GMP, which is an intracellular signaling molecule (10). Thus, NO generated in brain has been reported to regulate neuronal functions in the CNS (11, 12). Several studies have recently reported the involvement of NMDA-mediated NO in the development of cocaine kindling. Pretreatment with N^g-nitro-L-arginine methyl ester (nonselective inhibitor, L-NAME) before cocaine injection completely blocked the development of cocaine kindling and protected the animals against cocaine-induced death (13, 14). Treatment with another NOS inhibitor, 7-nitroindazole (selective nNOS inhibitor, 7-NI) before cocaine administration also prevented the development of cocaine kindling (2).

However, the NOS inhibitors used previously are either nonselective NOS (L-NAME) or neuronal-selective NOS (7-NI) inhibitors. Meager data are available regarding the effect of iNOS on cocaine kindling. Recently Moore *et al.* (15) reported that L-N^6-(1-iminoethyl)lysine (NIL) is a selective iNOS inhibitor. Soliman and Mazzio (16) found an inhibitory effect of (−)-epigalloocatechin gallate (EGCG), a

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compound in green tea, on iNOS in C6 astrocyte cells. In the present study, we examined the effect of iNOS inhibitors (NIL and EGCG) on the development of cocaine kindling. In addition, because NMDA receptors have been associated with nitric oxide production and cocaine kindling, NMDA receptor affinity and density were examined in hippocampus and frontal cortex.

Materials and Methods

Materials. Experimentally naive male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), weighing 85–110 g at the start of the experiments, were used in the present study. All animals were individually housed in a standard transparent rectangular rodent cage (42 × 24 × 20 cm). They were kept under controlled environmental conditions (ambient temperature 21 ± 2°C, humidity 50–60%, 12/12-hr light/dark cycle, lights on at 7:00 AM). Feed (Harlan Teklad, Bartonville, IL) and water were allowed *ad libitum* throughout the course of the study. Experiments described beneath were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health (NIH) and adopted by Florida A&M University Animal Care and Use Committee. Before experiment began, rats were acclimatized to the housing conditions for one week and handled 1–2 min daily for 3 days prior to experiments.

Cocaine-HCl, EGCG, and NIL were purchased from Sigma Chemical Co. (St. Louis, MO). (+)-MK-801 was purchased from Research Biochemical International (Natick, MA). [^{3}H]MK-801 was purchased from New England Nuclear (Boston, MA). All the other chemicals used for binding assays were purchased from Sigma Chemical Co.

Schedule of Drug Treatment. Cocaine, EGCG, and NIL were dissolved in 0.9% saline solution. The concentrations of drugs were 45 mg/kg for cocaine, 10 mg/kg for EGCG, and NIL. Drug solutions were freshly prepared daily and administered by intraperitoneal injection (ip) for 7 days in a volume of 0.1 ml per 100 g of body weight in the animal's home cage. Pretreatment with either EGCG or NIL was injected 30 min before cocaine administration. Animal weights were monitored daily just before drug administration, and the volume of injection was adjusted accordingly. All drug administrations were performed once a day between 12:00 and 16:00 in their home cages. The control group received the same volume of 0.9% saline.

The EGCG dose was based on the previous pilot study in our lab. Doses <1 mg/kg of EGCG had no effect on cocaine-induced kindling, and the doses >50 mg/kg of EGCG resulted in a trend toward enhanced kindling. The drug concentration of NIL was based on previous studies in which dose ranged from 2.5 to 30 mg/kg (17–20). At this dose range, L-NIL almost completely blocked the *in vivo* enzymatic activity of iNOS.

Assessment of Cocaine Kindling. The effect of NIL and EGCG on the induction of sensitization to the convulsive effect of cocaine was examined immediately after cocaine injection. Because our previous pilot study showed the peak effect of cocaine was between 5 and 15 min after cocaine administration, cocaine kindling was measured for 30 min after injection and scored at 5-min intervals. The following rating scale was developed in our laboratory by modifying the Itzhak (2) and Racine (21) scales: Stage 1 (normal behavior stage)—slow moving around the cage, intermittent sniffing or asleep; Stage 2 (hyperactivity stage)—running movement characterized by rapid changes in position and sniffing; Stage 3 (stereotypy activity stage)—continuous sniffing or head nodding at the same place for several seconds, standing on the wall with continuous sniffing; Stage 4 (pre-seizure stage)—hind limbs clonus and unstable movement; Stage 5 (full motor seizure stage)—clonus of fore- and hind-limbs, flexion of head and entire body, dyskinetic movement, and loss of righting reflex. The highest stage was used for the statistical analysis during this time of period.

NMDA Receptor Binding Assay. Rats were sacrificed by decapitation 3 days after the last measurement, and their brains were removed. The hippocampus and cortex were dissected on ice, frozen on dry ice immediately, and stored at −80°C until used in the assay procedures. On the day of binding assay, the tissues were thawed and homogenized in an ice-cold 0.32 *M* sucrose using a Polytron PT 3100 membrane homogenizer (setting 7,000, 20 sec) (Littau, Switzerland). Homogenates were centrifuged at 40,000*g* for 12 min at 4°C. The pellet was resuspended and centrifuged again in binding buffer that contained 100 μ*M* glutamate, 100 μ*M* glycine, 100 μ*M* supermine, and 10 m*M* HEPES. The sample was washed one more time. The membrane pellet was then resuspended in buffer for binding assay. A small aliquot of tissue sample was used for determination of protein concentration.

[^{3}H]MK-801 binding assays were performed based on the procedure described by Diaz-Granados *et al.* (22) with a few modifications. Briefly, the assays were done in triplicate in a total volume of 300 μl, containing various concentrations of [^{3}H]MK-801, 100 μ*M* glutamate, 100 μ*M* glycine, 100 μ*M* spermidine, 10 m*M* HEPES and membrane. The density and affinity of NMDA receptor were measured by saturating the tissue with increasing concentrations of [^{3}H]MK-801 (0.312–10 n*M*). Nonspecific binding was defined by the addition of 10 μ*M* unlabeled MK-801. Homogenized tissues were incubated for 3 hr at room temperature. At the end of the incubation period, all assay mixtures were filtered by using a Brandel M-48 Cell Harvester (Gaithersburg, MD) through Whatman GF/C filters presoaked with 0.05% polyethylenimine to reduce nonspecific binding to the filters. Each filter was washed three times with ice-cold 10 m*M* Tris-HCl buffer (pH 7.4). The filters were placed into plastic vials, and 5 ml of scintillation fluid was added by using an automatic dispensing system (Brandel Inc., Gaithersburg, MD), and the vials were left standing overnight. Radioactivity was measured by a Beckman LS6500 multi-purpose scintillation counting system

(Beckman Inc., Fullerton, CA). Data were processed and analyzed using Scatchard plot analysis to determine K_d and B_{max}.

Statistical Analysis. The differences in cocaine kindling were analyzed by randomized complete block design ANOVA followed by post hoc Tukey test. The differences in NMDA receptor were analyzed by a one-way ANOVA followed by post hoc Newman–Keuls test. Significant differences were set at $P < 0.05$. All statistical analyses were performed using the SAS system.

Results

Results presented in Fig. 1 show the effect of iNOS inhibitors on cocaine kindling. Administration of cocaine (45 mg/kg, ip) for 7 consecutive days resulted in gradual increase in the convulsive response after the Day 3. Six out of seven animals developed seizures during this period including two deaths (Table I). Pretreatment with NIL and EGCG 30 min before cocaine injection attenuated the development of cocaine-induced seizures ($P < 0.05$). Statistical analysis revealed that this effect was significant ($P < 0.05$) on Day 1 and after Day 5. Two rats in the NIL group ($n = 7$) and one rat in the EGCG group ($n = 7$) exhibited seizure response during 7-day treatment. No deaths were observed in these groups. The animals pretreated with either NIL or EGCG showed generally higher activity levels than control group. They exhibited either stage 2 (hyperactivity) or stage 3 (stereotypy activity) levels of activity throughout the experiment.

In an attempt to define whether a change in NMDA receptor binding activity is associated with sensitization to

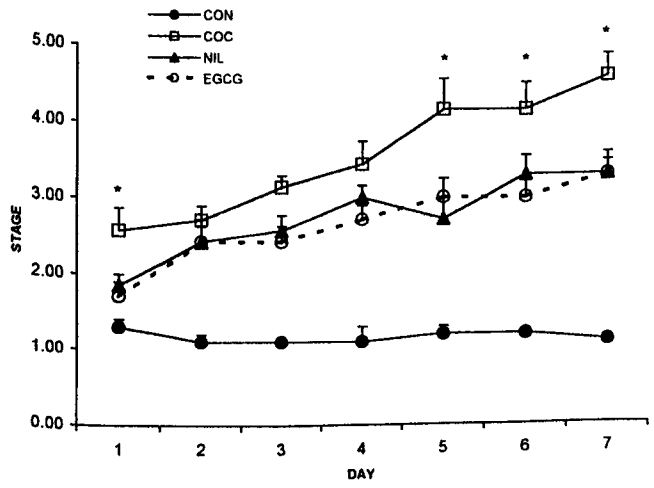

Figure 1. Effect of repeated cocaine injection on behavioral response. Male Sprague Dawley rats were administered 45 mg/kg of cocaine by ip. Pretreatment with NIL or EGCG was done 30 min before cocaine injection for 7 consecutive days. Behavioral response was observed daily for 30 min on a five-stage scale: Stage 1, normal behavior stage; Stage 2, hyperactivity stage; Stage 3, stereotypy activity stage; Stage 4, pre-seizure stage; Stage 5, full motor seizure stage. Results are shown as means ± SEM for 7 animals. *Indicates significant difference from both NIL and EGCG ($P < 0.05$). CON, control group; COC, cocaine-treated group; NIL-COC, NIL-pretreated group; EGCG-COC, EGCG-pretreated group.

Table I. Effect of iNOS Inhibitors on the Development of Seizures and Cocaine-Induced Death[a]

	Seizures ($n = 7$)	Death
COC[b]	6	2
NIL-COC	2	0
EGCG-COC	1	0

[a] Male Sprague Dawley rats were administered 45 mg/kg of cocaine by ip. Pretreatment with NIL or EGCG was done 30 min before cocaine injection for 7 consecutive days. Seizure response was observed daily for 30 min. Results show the total number of rats that developed seizures and died during 7 consecutive days of treatment.
[b] COC, cocaine-trated group; NIL-COC, NIL-pretreated group; EGCG-COC, EGCG-pretreated group.

cocaine kindling, receptor-binding assays were done in the hippocampus and cortex. As indicated in Table II, the affinity (K_d) of NMDA receptor binding site in the hippocampus was significantly increased ($P < 0.05$) in cocaine-treated group with no changes in the density (B_{max}) compared to the control group. However, there were no significant differences in either affinity or density of NMDA receptor in the cortex after cocaine treatment (Table III). Pretreatment with both NIL and EGCG did not cause a change in either affinity or density in the hippocampus and cortex compared to controls.

Discussion

Increased convulsive sensitivity after repeated administration of subthreshold doses of cocaine has previously been established (1, 3). The present study provides further evidence of cocaine effect on cocaine kindling. The novel finding of this study is that the iNOS inhibitors, NIL and EGCG, attenuated the development of sensitization to the convulsive effect of cocaine and protected the animals against cocaine-induced death. In addition, repeated administration of cocaine resulted in increased NMDA receptor affinity in the hippocampus, and the iNOS inhibitors abolished the change in cocaine-induced NMDA receptor affinity. Thus, these results demonstrate that iNOS activation in the hippocampus is crucial for cocaine kindling, and con-

Table II. Effect of iNOS Inhibitors on the Binding of [^{3}H]MK-801 to the NMDA Receptor in Hippocampus[a]

	K_d (n*M*)	B_{max} (fmol/mg protein)
CON[b]	5.65 ± 0.88	3,625 ± 339
COC	3.78 ± 0.10[c]	4,420 ± 223
NIL-COC	4.71 ± 0.19	4,250 ± 160
EGCG-COC	4.83 ± 0.18	4,236 ± 147

[a] Male Sprague Dawley rats were administered 45 mg/kg of cocaine by ip. Pretreatment with iNOS inhibitors, NIL, or EGCG was done 30 min before cocaine injection for 7 consecutive days. All animals were sacrificed 3 days after the last treatment. NMDA binding assays were done in hippocampus. Data are shown as means ± SEM for 5–7 animals.
[b] CON, control group; COC, cocaine-treated group; NIL-COC, NIL-pretreated group; EGCG-COC, EGCG-pretreated group.
[c] Significant difference from control ($P < 0.05$).

Table III. Effect of iNOS Inhibitors on the Binding of [^{3}H]MK-801 to the NMDA Receptor in Cortex

	K_d (n*M*)	B_{max} (fmol/mg protein)
CON[b]	4.56 ± 0.68	2,352 ± 141
COC	3.91 ± 0.12	2,430 ± 185
NIL-COC	4.1 ± 0.23	2,303 ± 109
EGCG-COC	4.06 ± 0.14	2,329 ± 115

[a] Male Sprague Dawley rats were administered 45 mg/kg of cocaine by ip. Pretreatment with iNOS inhibitors, NIL, or EGCG was done 30 min before cocaine injection for 7 consecutive days. All animals were sacrificed 3 days after the last treatment. NMDA binding assays were done in cortex. Data are shown as means ± SEM for 5–7 animals. [b] CON, control group; COC, cocaine-treated group; NIL-COC, NIL-pretreated grop; EGCG-COC, EGCG-pretreated group.

vulsive effect and lethality phenomenon of cocaine may be possibly through the NMDA receptor system.

The involvement of NMDA receptor in the development of cocaine kindling is implicated by the following: First, NMDA receptor is a subtype of glutamate and the role of glutamate in kindling has been well documented (23, 24). Second, repeated administration of cocaine produced a persistent increase in extracellular glutamate levels (25). Third, pretreatment with NMDA receptor antagonists attenuated cocaine-induced convulsions and mortality (6, 8, 9). Fourth, we have found change in cocaine-induced NMDA receptor system in the hippocampus. This result has corroborated previous studies in which changes in NMDA receptor system have been demonstrated either in the hippocampus or cortex (6, 26). Taken together, these findings suggest a substantial role for NMDA receptor activation, especially in the hippocampus, in the development of cocaine kindling.

In the brain, activation of the NMDA receptor induces an influx of Ca^{2+} into the cell, which binds to calmodulin. CA^{2+}-calmodulin binding consequently activates nNOS, resulting in the production of NO (10, 27). NO generated in the brain is thought to diffuse to adjacent cells and regulate neuronal functions including seizures (2, 10–12) by the elevation of cyclic GMP (27). Previously several studies have reported the effect of NO on cocaine kindling. The NOS substrate, L-arginine, produced convulsion (28) and reversed the protective effect of L-NAME in cocaine kindling (3). Exposure to LiCl and tacrine, which are associated with an increase in brain NOS activity, also produced seizures (29). Furthermore, pretreatment with nonselective NOS inhibitor, L-NAME (100 mg/kg), prior to cocaine administration completely abolished the sensitization to convulsion and lethality (13). This protective effect of NOS inhibitors was confirmed by the other studies in which both N^g-nitro-L-arginine (nonselective, 25 mg/kg) and 7-NI (selective, 25 mg/kg), prevented the development of cocaine kindling (2, 3). These results provide evidence that nNOS-induced NO is involved in the process of cocaine kindling.

Although Leib *et al.* (30) reported that pretreatment with an iNOS inhibitor, aminoguanidine, reduced incidence of seizures induced by meningitis, the effect of iNOS on cocaine kindling has not been tested. In the present study, pretreatment with NIL and EGCG 30 min before cocaine injection attenuated cocaine kindling. The selectivity of NIL and EGCG on iNOS has been previously examined in several studies. In NIL studies, NIL exhibited approximately 30 times more selectivity for iNOS than for constitutive NOS, and the IC_{50} of NIL was between 0.4 and 3.3 μM (15, 31, 32). In EGCG studies, inhibition of NO production was observed when cells were co treated with EGCG in C6 astrocyte cells (16), interferon-gamma-activated mouse peritoneal cells (33), and lipopolysaccharide-activated macrophages (34). Western blot and Northern blot analysis confirmed reduced level of iNOS and protein (33, 34), while electrophoretic mobility shift assay indicated that EGCG blocked the activation of nuclear factor-kappa B, a transcription factor necessary for iNOS induction (34). Although iNOS activity in the present study was not examined, these results provide evidence of the antagonistic effects of NIL and EGCG on iNOS.

The major cellular sources of iNOS seem to be astrocytes because they produce micromolar amounts of NO (35), and iNOS mRNAs are highly expressed in these cells (36). Furthermore, our lab has found an effect of cocaine on NO production in astrocytes (unpublished data). In addition, numerous studies have reported the expression of iNOS in the hippocampus in rats (37, 38), mice (39, 40), and human (41). The results observed in these studies may only provide a prediction about iNOS activity in the hippocampus. To better understand the mechanism(s) involved in cocaine kindling, iNOS activity in this area is under being investigated in our laboratory.

There are conflicting reports on the changes in NMDA receptor system. Treatment of cocaine (35 mg/kg) for 10 days resulted in an increase of receptor density in the hippocampus with no change in the frontal cortex when the animals were sacrificed 10 days after the last treatment (26). However, another study in which cocaine (45 mg/kg) was treated for 7 days showed an increase in density in the frontal cortex when tissues were prepared one day after the last treatment (6). Both of these studies showed no change in receptor affinity. In contrast, our study showed only a significant increase in the affinity of NMDA receptor in the hippocampus with no changes in receptor density in either area. Although the reason(s) for these different results is not clear, possible explanations for these discrepancies may include differences in dosing regimens and withdrawal period for tissue collection, species variations, and assay methods. It is interesting to note that although there was no significant difference, the receptor density in the hippocampus was also increased by 22% compared to the control group. This may explain how the change in receptor affinity only can affect cocaine kindling. That is, in the present study, a significant increase in affinity plays a major role, and the small increase in density contributes, in part, to the process of cocaine.

Although a decrease in GABAergic transmission and an increase in glutamate-mediated NMDA receptor activation are thought to be critically involved in epileptic sei-

zures (42, 43), the underlying mechanisms of cocaine kindling have not been fully elucidated. However, based on the results in the present study, it is suggested that iNOS plays a role in the process of cocaine kindling. Cocaine may induce iNOS, leading to a subsequent an excess of NO production in the hippocampus. This NO is capable of diffusing into neighboring neurons and causes them to release glutamate into synapse (44), activate NMDA receptors (45), and eventually lead to the development of seizures (6, 9). Another possibility is that iNOS may stimulate NMDA-Ca^{2+}-mediated nNOS, thus producing more NO resulting in cascade effect. Moreover, the present results do not necessarily exclude the possibility of cocaine's interaction with other neurotransmitter system such as GABAergic, muscarinic, sympathetic, and dopaminergic to elicit its convulsive effects (46–49). Further studies are required to define more exact mechanisms in the development of cocaine kindling.

In summary, the present study revealed that iNOS may be involved in the development of cocaine kindling. Since NMDA receptors are involved in the development of cocaine kindling, our data suggest a mechanism by which induction of iNOS by cocaine leads the glutamate-mediated stimulation of NMDA receptors.

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